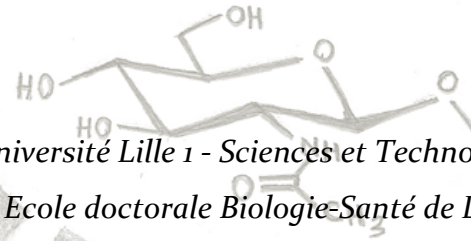

Université Lille 1 - Sciences et Technologies

Ecole doctorale Biologie-Santé de Lille

Etude de l'*O*-GlcNAcylation de la β-caténine : Des désordres métaboliques à la cancérisation

THESE DE DOCTORAT

En vue de l'obtention du titre de Docteur de l'université de Lille 1

Spécialité Biochimie et Biologie cellulaire

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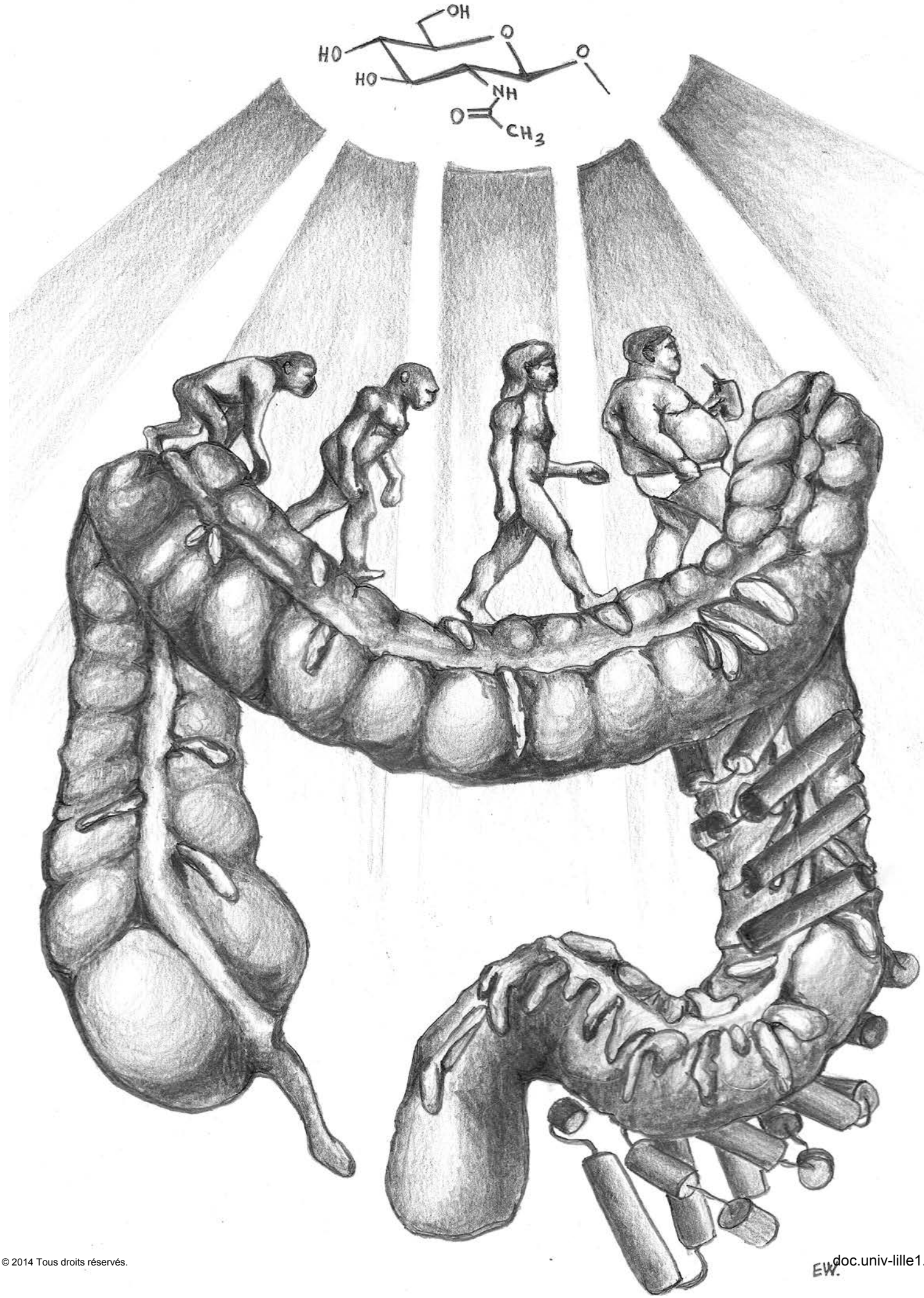

Ça y est, enfin, on y est!!! J'ai longtemps pensé au moment où j'en arriverais à rédiger cette partie et je me retrouve partagée entre deux sentiments : d'un côté très contente d'arriver à la fin de la rédaction et d'un autre côté, assez triste de sonner bientôt la fin de mes 4 ans de vie au C9.

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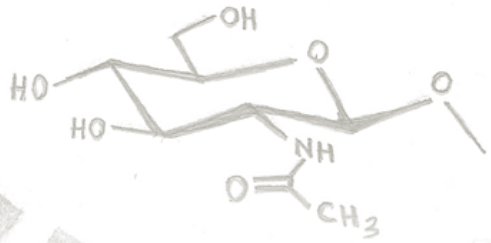
Sommaire



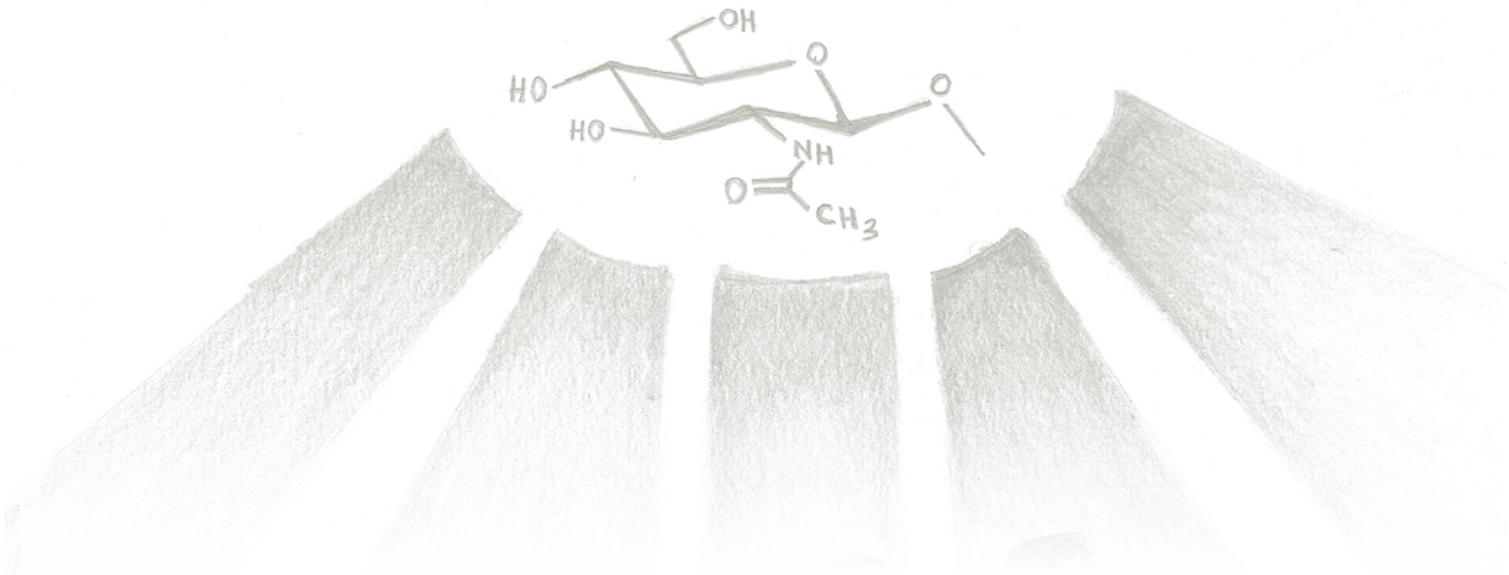
Abréviations

ADAM10: A Disintegrin And Metalloprotease 10
ADN: Acide désoxyribonucléique
AF17: ALL1-fused gene from chromosome 17
Akt/PKB: Protein Kinase B
ALP : Acide lysophosphatidique
AMPK: AMP-activated protein kinase
APC : Adenomatous Polyposis Coli
Arm.: Armadillo
ARN: Acide Ribonucléique
BAR-1: Beta-catenin/Armadillo-Related protein 1
Bcl-2–associated X protein
BCL9: B-cell CLL/lymphoma 9
BMP: Bone Morphogenic Protein
Brg1: Brahma-related gene 1
CAMKIV: Calcium/calmodulin-dependent protein Kinase IV
CBP: CREB-Binding protein
CCR : cancer colorectal
CD: Catalytic domain
CD44: Cluster de Différenciation 44
CK1 : Casein Kinase 1
CK1α : Caséine Kinase 1 α
COMPASS: COMplex Proteins ASsociated with Set1
COX2 : cyclooxygénase 2
CtBP: C-terminal Binding Protein
CTTA: C-Terminal Transcriptional Activators
D-Box : destruction-Box
DIX : Dishevelled-Axine
Dlat : dihydrolipoamide acetyltransferase
Dsh : Dishevelled
eIF: eucaryotic chain Initiation Factor
eNOS: endothelial Nitric Oxid Synthase
FAP : Familial Adenomatous Polyposis
FBPase : fructose-1,6-bisphosphatase
FCs: Facteurs de croissance
FGF: Fibroblast Growth Factor
FoxM1: Forkhead box protein M1
FoxO : Forkhead box O
FRA1: Fos-related antigen 1
Fru: Fructose
Fz: Frizzled
GFAT : Glutamine : Fructose-6-phosphate amidotransférase
Glc: Glucose
GlcNAcP Mut: N-acétylglucosamine phosphate mutase
$GlcNH_2$: Glucosamine
$GlcNH_2$6P AcT: glucosamine-6-phosphate acétyltransférase
Gln : Glutamine
Glu : glutamate

GluT: Glucose transporteur
GRB2: Growth factor receptor-bound protein 2
GRIF1: $GABA_A$ Receptor-Interacting Factor 1
GS glycogène synthétase
GSK3β: Glycogène Synthase Kinase 3β
HAT: Histone AcétylTransférase
HDAC : Histones Désacétylase
HIF1α: Hypoxia Induced Factor 1α
HK: hexokinase
HMP-2: humpback-2
ICAT: Inhibitor of β-Catenin And TCF4
ID2: Inhibitor of DNA-bindin 2
Idh3a : Isocitrate dehydrogenase
IKK : I?B kinase
Int1: Integration 1
IRS1: Insulin Receptor substrate 1
ITF2: Immunoglobulin transcription factor 2
JAs: Jonctions d'adhérence
JNK2: c-jun N-terminale kinase 2
LBH: Limb Bud and Heart
Lgs: Legless
LOH: Loss of heterozygosity
LPS: lipopolysaccharides
LRH1: Liver Receptor Homologue 1
LRP5/6: Low density Lipoprotein Receptor-related Protein 5/6
MCC: Myosin Cardiac Chain
MCM : MiniChromosome Maintenance protein
MCR : Mutation Cluster Region
MDM2: murine double minute 2
MDR1: Multidrug Resistance 1
MGEA5: Meningioma-Expressed Antigen 5
MLL: Mixed Lineage-Leukemia
MMPs : Matrix MetalloProtéinases
mOGT: mitochondrial O-GlcNAc Transferase
Msl1: male-specific lethal 1
Mtgr-1: Myeloid translocation gene related 1
MTS: Mitonchondrial Target Sequence
MYCBP: c-myc binding protein
MYPT1: Myosin phosphatase targeting subunit 1
NCOAT: Nuclear and Cytoplasmic O-GlcNAc transferase and AcetylTransferase
ncOGT: nuclear and cytoplasmic O-GlcNAc Transferase
NCoR: Nuclear receptor CoRepressor
NES : Nuclear Export Signal
NLS : Nuclear Localization Signal
NOS2: Nitrix Oxide Synthase 2
Nr-CAM: Neuronal cell adhesion molecule
Nup358: Nucléoporine 358

OGA: O-GlcNAcase
OGT: O-GlcNAc Transférase
p120: p120 caténine
PAF1: RNA Polymerase II Associated Factor 1
Parafibro.: parafibromine
PcG : Polycomb
PCP: Planar Cell Polarity
PDK1: Phosphoinositide-Dependent Kinase 1
PDX1: Pancreatic and duodenal homeobox 1
PEPCK : Phosphoenolpyruvate carboxykinase
PGC1α: PPAR gamma coactivator 1α
PGlc Is: glucose-6-phosphate isomérase
PGlcNAc Mut: N-acétylglucosamine phosphate mutase
PI3K : Phoshatidylinositol 3 kinase
PIP2: PhosphatidylInositol (4,5) bisPhosphate
PIP3: PhosphatidylInositol (3, 4, 5) triphosphate
PKA : Protéine Kinase A
PML: Promyelocytic leukemia protein
PP2A : Protein Phosphatase 2A
PP2A: Protein Phosphatase 2A
PPARδ: Peroxisome proliferator-activated receptor δ
PPO: PIP-Binding activity of OGT
pRb : protéine du Rétinoblastome
PTEN: Phosphatase and TENsin homolog
PTP1B : Protein Tyrosine Phosphatase 1B
Re.: Récépteur.
SAMPs : Sérine Alanine Méthionine Proline
SERCA: Sarco/Endoplasmic Reticulum Calcium ATPase
SERCA2a: SarcoEndoplasmic Reticulum Ca^{2+}-ATPase 2a
sFRPs: secreted Frizzled-Related Proteins
SMRT: Silencing Mediator for Retinoid and Thyroid hormone receptor
sOGA: Short O-GlcNAcase
sOG: Short O-GlcNAc Transferase
SOS: Son of sevenless
SREBP2: Sterol Regulatory Element Binding Protein 2
SWI-SNF: SWItch/Sucrose Non Fermentable
Sxc: Super sex combs
TAF110: TATA-binding-protein Associated Factor 110
TBP: TATA box binding Protein
TCF/Lef: T-Cell Factor/ Lymphoid enhancer-binding factor
TGFβ: Transforming Growth Factor β.
THAP1: Thanatos-associated domain-containing apoptosis-associated protein 1
TLE: Transducin-Like Enhancer
TPR: Tétratricopeptides repeats
Trak1: OIP106-OGT interacting Protein 106
TRRAP : Transcription/transformation domain-associated protein
TRRAP: Transformation/transcription domain-associated protein.

UDP/UMP: uridine Di/mono-phosphate.
UDP: Uridine Di-Phosphate
UDP-GlcNAc PPase: UDP-N-acétylglucosamine pyrophosphorylase
UDP-GlcNAc : Uridine Di-Phosphate-N-acétylglucosamine.
uPAR: urokinase-type Plasminogen Activator Receptor
UTP: Uridine Tri-Phosphate
VEGF: Vascular endothelial growth factor
Wg : Wingless
WISP1: WNT1 inducible signaling pathway.
WRE : Wnt response element
WRM-1: WoRM armadillo protein 1
XIC: X-inactivation center
β-TrCP : β-Transducin repeats-Containing Protein



Résumé

Une mauvaise hygiène alimentaire et certains désordres métaboliques sont décrits depuis plusieurs années comme des facteurs de risque majeurs du cancer colorectal. Ainsi, les individus diabétiques montrent une probabilité deux fois plus élevée de développer un cancer colorectal que des individus sains. Néanmoins, les mécanismes moléculaires reliant ces facteurs à la cancérisation colique et rectale restent mal compris. Comment un désordre nutritionnel ou métabolique influence-t-il l'initiation et la progression tumorale ? Pour tenter de répondre à cette question, nous nous sommes focalisés sur une voie de signalisation essentielle à l'initiation tumorale colique, la voie Wnt/β-caténine. La β-caténine, élément central de cette voie, contrôle la transcription de nombreux gènes parmi lesquels c-myc et la cycline D1. La régulation de cette activité transcriptionnelle est régie par un système de dégradation efficace. Il consiste en une séquestration de la β-caténine dans un complexe de destruction entraînant sa phosphorylation au niveau d'une séquence appelée « Destruction-box ». Cette phosphorylation est suivie de l'ubiquitinylation puis de la dégradation de la β-caténine par le protéasome. Dans un contexte de cancérisation colique et rectale, la β-caténine est stabilisée, soit par sa propre mutation, soit par des mutations du complexe de destruction, empêchant son recrutement et sa dégradation. Nous nous sommes interrogés sur le fait que des désordres métaboliques ou nutritionnels pouvaient participer à la stabilisation de la β-caténine et, de ce fait, à l'initiation tumorale.

Pour cela, nous nous sommes intéressés à une autre modification post-traductionnelle de la β-caténine susceptible d'affecter ses fonctions : la *O*-GlcNAcylation. Jusqu'alors, la modification de la β-caténine par un résidu de *N*-acétylglucosamine (GlcNAc) avait été décrite sans pour autant en comprendre la fonction. La *O*-GlcNAcylation est une modification réversible et dynamique, affectant les protéines cytosoliques, nucléaires et mitochondriales. Parce que phosphorylation et *O*-GlcNAcylation affectent les mêmes acides aminés, sérine et thréonine, il est fréquent que les deux modifications soient compétitrices l'une de l'autre. Un couple d'enzymes unique régule la *O*-GlcNAcylation des protéines : la *O*-GlcNAc transférase (OGT) et la *O*-GlcNAcase (OGA) qui catalysent respectivement l'ajout et l'hydrolyse de la GlcNAc. Ce caractère hautement dynamique en fait un élément essentiel dans la régulation de nombreux processus cellulaires physiologiques et pathologiques, au même titre que la phosphorylation. Par ailleurs, la *O*-GlcNAcylation est considérée comme un senseur nutritionnel puisque qu'elle retranscrit au sein de la cellule, le statut nutritionnel

(et notamment la concentration en glucose extracellulaire) par l'intermédiaire de la voie de biosynthèse des hexosamines. De ce fait, la *O*-GlcNAcylation reflèterait au niveau protéique le comportement alimentaire et par extension un déséquilibre métabolique se répercuterait sur le dynamisme de cette glycosylation.

Au cours de la thèse, nous avons cherché à comprendre comment les caractéristiques de la β-caténine sont affectées par des variations de *O*-GlcNAcylation. Nous avons démontré que la *O*-GlcNAcylation de cette dernière la stabilisait, notamment en réponse à une augmentation du glucose extracellulaire. Afin de mieux comprendre ce mécanisme, nous avons entrepris la cartographie des sites de *O*-GlcNAcylation. La modification de la sérine 23 et des thréonines 40 et 41 concentre la *O*-GlcNAcylation de la β-caténine dans le domaine N-terminal impliqué dans les processus de dégradation. Nous avons mis en évidence une compétition directe entre *O*-GlcNAcylation et phosphorylation sur la thréonine 41. Une élévation de *O*-GlcNAcylation diminue l'ubiquitinylation et l'interaction de la β-caténine avec le suppresseur de tumeur APC, pièce essentielle du complexe de destruction. De ce fait, nous proposons qu'en réponse à une augmentation de la concentration en glucose, la *O*-GlcNAcylation entrave le déroulement de la séquence de dégradation de la β-caténine. Nous avons également démontré que la *O*-GlcNAcylation augmente l'activité transcriptionnelle de la β-caténine, et accélère la prolifération cellulaire.

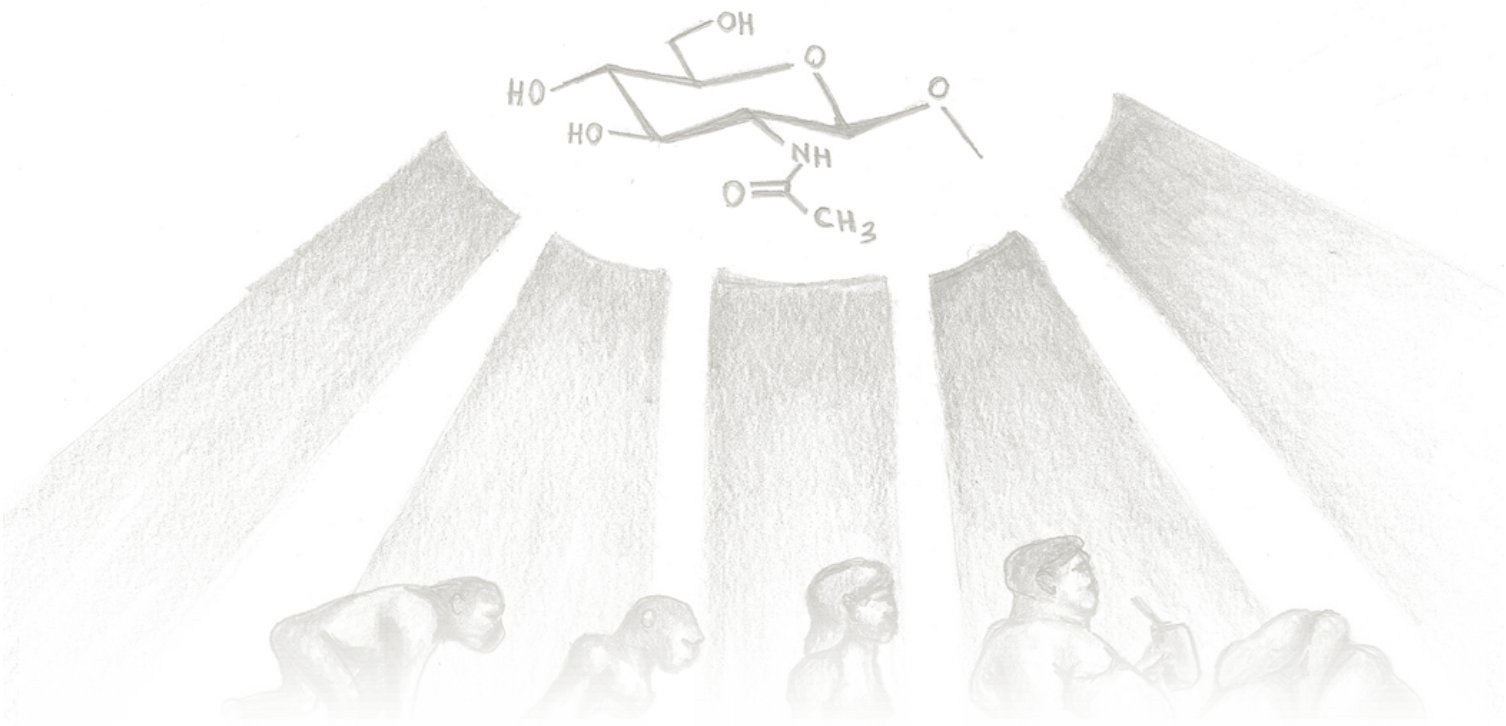
D'autre part, l'expression de β-caténine, observée au cours de la reprise du cycle cellulaire, s'accompagne d'une interaction avec l'OGT. Par ailleurs, l'expression de l'OGT est elle-même augmentée pendant la phase G1, suggérant un rôle de l'enzyme dans les étapes précoces du cycle cellulaire. Il a été décrit qu'au cours de cette phase critique, la β-caténine contrôle l'expression de la cycline D1 nécessaire à la poursuite du cycle cellulaire. A ce niveau, l'OGT jouerait un rôle essentiel puisque son inhibition bloque l'activité transcriptionnelle de la β-caténine (et la synthèse de cycline D1).

Ainsi, l'interaction de la β-caténine avec l'OGT ainsi que sa *O*-GlcNAcylation subséquente joueraient un rôle essentiel sur son activité transcriptionnelle et potentiellement sur son caractère oncogénique, notamment au cours de la cancérogénèse colique. En effet, des analyses de lignées cancéreuses et d'extraits de tumeurs humaines coliques ont montré une augmentation significative des niveaux de *O*-GlcNAcylation et de β-caténine en comparaison

respectivement à une lignée colique fœtale et aux tissus adjacents sains. De plus, la *O*-GlcNAcylation de la sérine 23 de la β-caténine a été retrouvée dans les tissus cancéreux.

L'influence de certains désordres métaboliques sur la cancérisation pourrait s'expliquer en partie par une stabilisation aberrante de la β-caténine liée à sa *O*-GlcNAcylation. Dans ce sens, nous avons montré qu'un régime alimentaire temporairement riche en glucose influence directement les niveaux de *O*-GlcNAcylation et de β-caténine dans les colons de souris C57Bl6. De même, une alimentation à long terme riche en sucres entraine le développement d'une intolérance au glucose et l'augmentation permanente de ces mêmes niveaux.

Nos travaux de thèse suggèrent qu'une stabilisation de β-caténine par *O*-GlcNAcylation en lien avec le régime alimentaire et plus largement l'hygiène de vie, impacterait sur ses propriétés et plus particulièrement son activité transcriptionnelle. Une mauvaise hygiène de vie et certains troubles métaboliques pourraient impacter la cancérisation colique et rectale par le biais d'une modification post-traductionnelle très largement exprimée, la *O*-GlcNAcylation.



Abstract

For many years, feeding and metabolic disorders are described as key risk factors for colorectal cancer emerging. As an example, diabetic patients show an increasing risk (twice-over) to develop colorectal cancer than healthy individuals. Nevertheless, molecular mechanisms connecting these factors to colorectal cancerization remain misunderstood. How nutritional or metabolic disorders affect colorectal cancer initiation? Trying to answer this question, we focused on the Wnt/β-catenin pathway, the main signaling pathway involved in this process. Inside this pathway, β-catenin plays a crucial role, shouldering transcriptional activity to stimulate cyclin D1 and c-myc expression. As a consequence, transcriptional properties control is supported by an efficient destruction complex. In a first step, β-catenin is sequestrated in a destruction complex, phosphorylated in its destruction box sequence, and then degraded through the ubiquitin/proteasome system. During the colorectal cancerization, β-catenin is stabilized, by mutations affecting either itself or its destruction complex, preventing its destruction box phosphorylation, its ubiquitinylation and finally its degradation. During our thesis, we wondered whether cell nutritional status, and particularly glucose status, affects β-catenin expression, and accordingly, whether metabolic or nutritional disorders may impact on tumoral process initiation.

Then, we studied the impact of another post-transcriptional modification which may potentially modulate β-catenin, the *O*-GlcNAcylation. For the time being, modification of β-catenin by a single *N*-acetylglucosamine (GlcNAc) moiety has been described without understanding its precise role. *O*-GlcNAcylation is a reversible and dynamic modification, affecting nuclear, cytosolic and mitochondrial proteins. Because these two modifications occur on the same amino acid residues, serine and threonine, *O*-GlcNAcylation and phosphorylation often compete in a complex interplay. A only couple of enzymes regulates the *O*-GlcNAcylation of proteins: *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) that catalyze respectively the addition and the removal of GlcNAc moieties. Like phosphorylation, the high dynamism of *O*-GlcNAcylation allows it to interfere with numerous physiological and pathological processes. Above all, *O*-GlcNAcylation is a metabolic sensor since its level tightly correlates to nutrient status (and particularly glucose rate) through the hexosamine biosynthetic pathway. By this way, *O*-GlcNAcylation reflects, at the protein level, overfeeding or metabolic disorders. On that basis, we wondered whether, and how, β-catenin is modulated by *O*-GlcNAc variations.

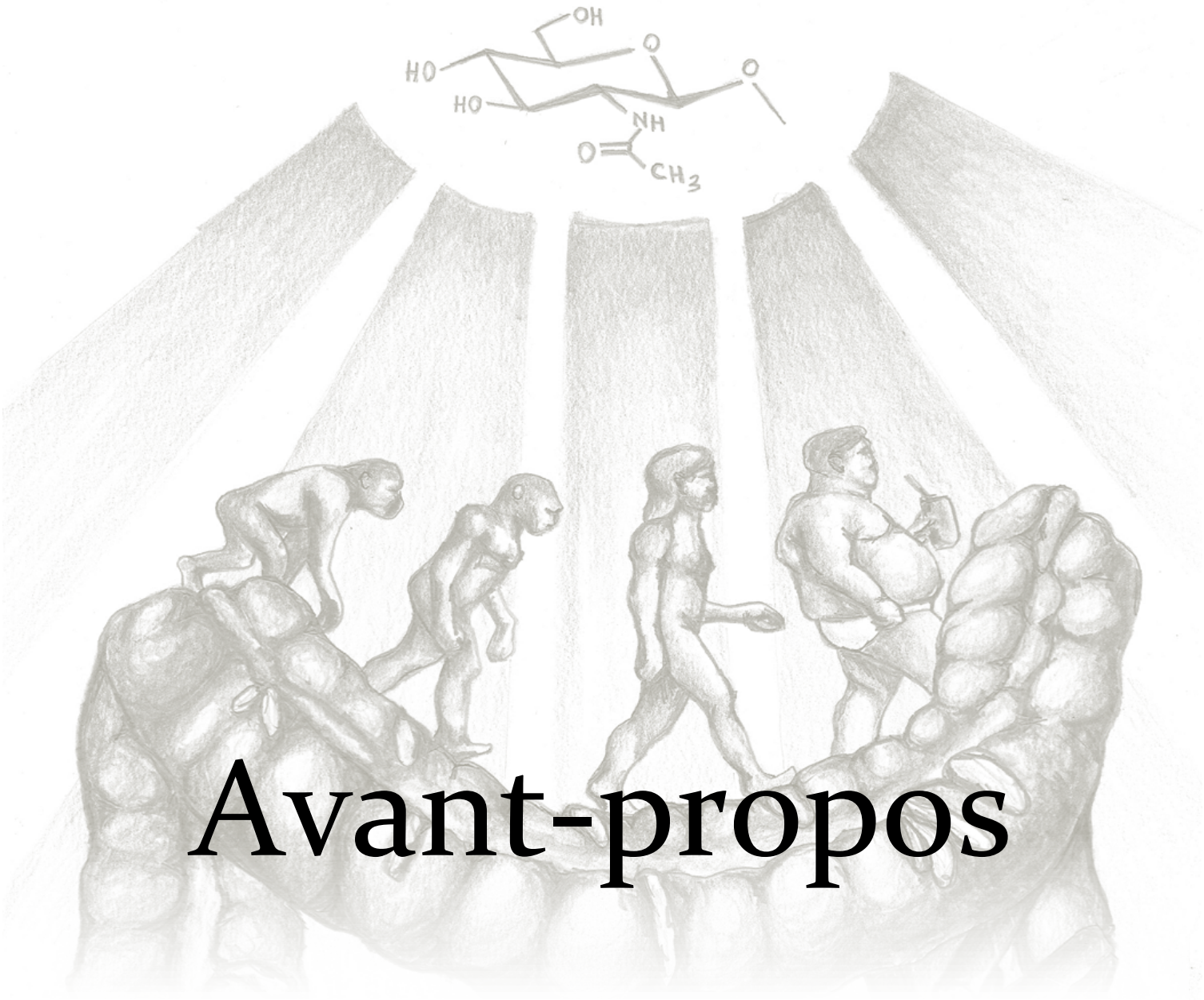
In this study, we showed that *O*-GlcNAcylation is able to stabilize β-catenin, especially in response to glucose changes. To better understand the underlying mechanism, we have undertaken the mapping of *O*-GlcNAcylation sites on β-catenin. The modification of serine 23 and threonines 40 or 41 concentrates *O*-GlcNAcylation mainly in the N-terminal domain involved in the degradation process. We also demonstrated a competition between *O*-GlcNAcylation and phosphorylation at threonine 41. In addition, increasing O-GlcNAcylation status decreases β-catenin ubiquitinylation and interaction with the tumor suppressor APC, a crucial element of the destruction complex. Accordingly we hypothesize that, in high glucose conditions, *O*-GlcNAcylation of β-catenin slows down its proteasomal degradation. Moreover, stabilization of β-catenin also increases its transcriptional activity and accelerates cell proliferation. In another part of the thesis, we have observed, especially upon cell cycle entry, an increasing interaction between β-catenin and OGT. Interestingly, during cell cycle entry, OGT expression increases, supporting the importance of this enzyme for the G0/G1 phase. Indeed, in response to growth factors, β-catenin participates in cyclin D1 synthesis, a key regulator of G1 phase, and promotes cell cycle progression. OGT may play an essential role in G1 phase since its inhibition prevents β-catenin transcriptional activity (and more particularly cyclin D1 synthesis).

Thus, interaction of β-catenin with OGT and its subsequent *O*-GlcNAcylation play a predominant role in its transcriptional activity. This event should be crucial during colorectal carcinogenesis. In this way, studies of colorectal cancer cell lines and human tumor tissues showed a significant *O*-GlcNAc and β-catenin levels increase when compared to a fetal colon cell line and adjacent healthy tissues, respectively. Moreover, O-GlcNAcylation of β-catenin at serine 23 has been found in tumor tissues.

Finally, impact of some metabolic disorders on cancerization could be explained in part by *O*-GlcNAcylation of pivotal elements like β-catenin or other proto-oncogenes. Indeed, we find that a temporary high-glucose feeding affects directly *O*-GlcNAc and β-catenin levels in C57Bl6 mice colon. Likewise, a long-term high-carbohydrate diet leads to glucose intolerance and permanent increase of these levels, getting metabolic disorders closer to colorectal cancerization.

Our work suggests that stabilization of β-catenin through *O*-GlcNAcylation linked to diet and more largely life hygiene, should impact its properties, particularly its transcriptional activity.

Metabolic disorders may favor the emergence of colorectal cancers via a widespread post-translational modification, *O*-GlcNAcylation.



Avant-propos

Ce travail de thèse a été réalisé au sein de l'Unité de Glycobiologie Structurale et Fonctionnelle (UGSF), UMR-CNRS 8576, à Villeneuve d'Ascq, FRANCE.
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L'ensemble des travaux effectués au cours de la thèse a fait l'objet des publications et des communications suivantes :

Publications:

Olivier-Van Stichelen S, Dehennaut V, Buzy A, Zachayus JL, Guinez C, Mir AM, El Yazidi-Belkoura I, Copin MC, Boureme D, Loyaux D, Ferrara P, Lefebvre T (2014) Identification of β-catenin O-GlcNAcylation sites in colon cell lines and human colorectal tumors*, FASEB J 13-243535*

Olivier-Van Stichelen S, Drougat L, Dehennaut V , El Yazidi-Belkoura I , Guinez C , Mir AM , Michalski JC , Vercoutter-Edouart AS, Lefebvre T (2012) Serum-stimulated cell cycle entry promotes ncOGT synthesis which activity is required for cyclin D expression, *Oncogenesis 1, e36*

Perez-Cervera Y, Dehennaut V, Aquino Gil M, Guedri K, Solórzano Mata CJ, **Olivier-Van Stichelen S**, Michalski JC, Foulquier F, Lefebvre T (2013) Insulin signaling controls *O*-GlcNAc transferase expression and its interaction with lipid raft, *FASEB J 27, 3478–3486*

Drougat L, **Olivier-Van Stichelen S**, Mortuaire M, Foulquier F, Lacoste AS, Michalski JC, Lefebvre T, Vercoutter-Edouart AS (2012) Characterization of O-GlcNAc Cycling and Proteomic Identification of Differentially *O*-GlcNAcylated Proteins during G1/S transition, *BBA - General Subjects 1820(12):1839-1848*

Lefebvre T, Drougat L, **Olivier-Van Stichelen S**, Michalski JC and Vercoutter-Edouard AS (2013) Antibodies and Activity Measurements for the detection of O-GlcNAc Transferase and Assay of its Substrate, UDP-GlcNAc. *Methods Mol Biol 10.1007/978-1-62703-465-4_12*

Olivier-Van Stichelen S, Guinez C, Mir A-M, Perez-Cervera Y, Liu C, Michalski J-C, Lefebvre T (2012) The hexosamine biosynthetic pathway and *O*-GlcNAcylation drive the expression of β-catenin and cell proliferation. *Am. J. Physiol. Endocrinol. Metab. 302: E417–424*

Olivier S, Mir AM, Michalski JC and Lefebvre T (2011) Signaling and metabolic predispositions linked to the colorectal cancer, *Med Sci (Paris) May;27(5):514-520*

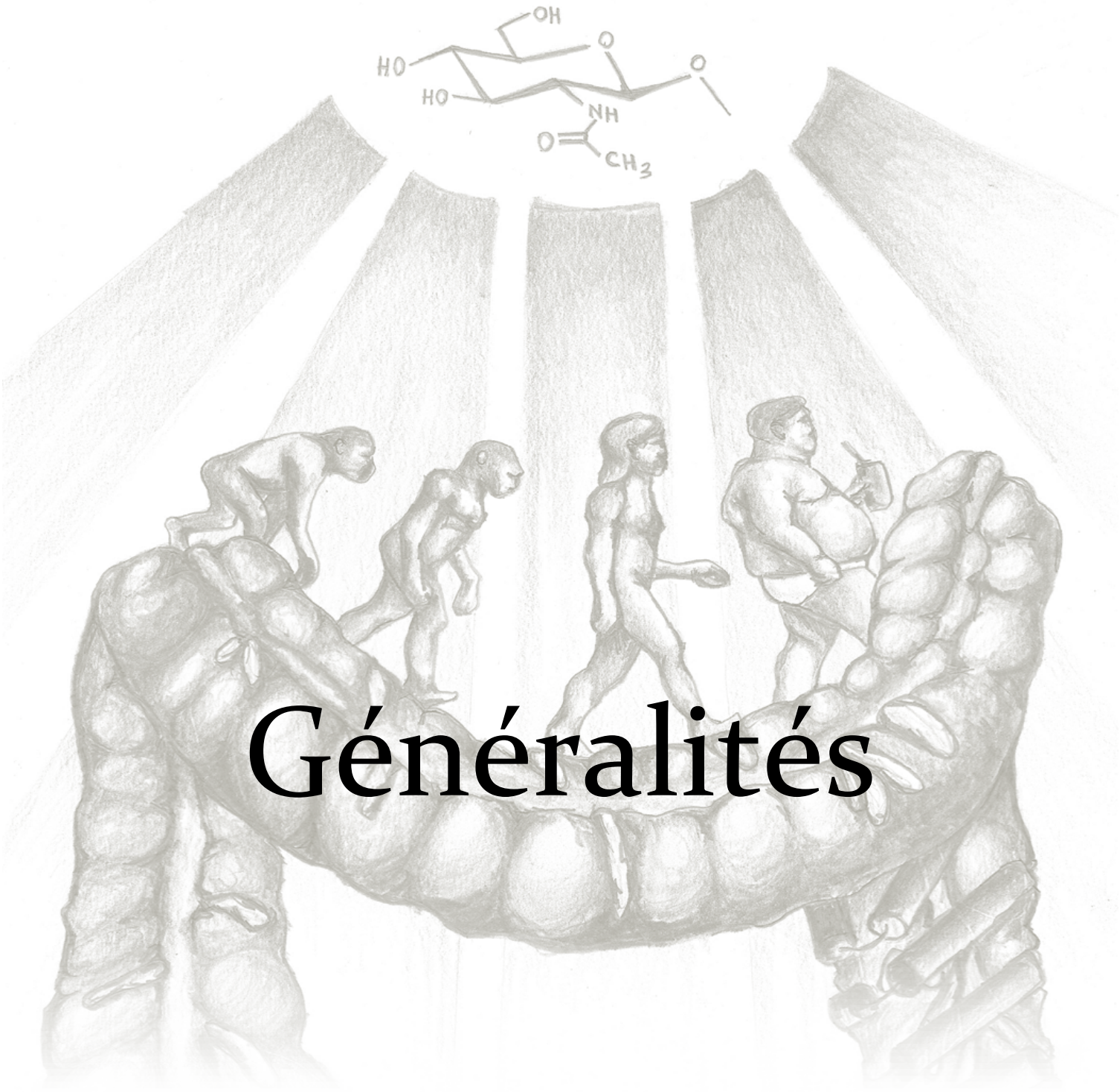
Lefebvre T, Dehennaut V, Guinez C, **Olivier S**, Drougat L, Mir AM, Mortuaire M, Vercoutter-Edouart AS and Michalski JC (2010) Dysregulation of the nutrient/stress sensor *O*-GlcNAcylation is involved in the etiology of cardiovascular disorders, type-2 diabetes and Alzheimer's disease*. Biochim. Biophys. Acta. 1800:67-79*

Communications par affiche:

Novembre 2011	22th Joint Glycobiology meeting 2011 Lille- FRANCE
Mai 2011	4ème journées scientifiques du Cancéropôle nord-ouest Deauville - FRANCE
Septembre 2010	Ecole thématique en microinformatique MIFOBIO 2010 Seignosse – FRANCE
Mars 2010	3ème journées scientifiques du Cancéropôle nord-ouest Deauville – FRANCE
Janvier 2010	Génomique du cancer : outils et état des lieux Lille – FRANCE

Communications orales:

Mai 2012	24ème congrès du Groupe Français des Glycosciences (GFG) ValJoly-FRANCE
Mars 2012	10th Jenner Glycobiology and medecine symposium La Haye-PAYS BAS
Septembre 2011	11ème journée André Verbert (EDBSL) Lille-FRANCE
Août 2011	21th International Symposium on Glycoconjugates (GlycoXXI) Vienne- AUTRICHE
Mars 2011	Université de médecine (Université autonome de Mexico (UNAM)) Mexico city – MEXICO
Mars 2011	Université autonome de Oaxaca Oaxaca – MEXICO
Novembre 2010	21th Joint Glycobiology Meeting 2010 Gent – BELGIQUE
Juin- Juillet 2010	« Early Programming of adult Modren Diseases » GDRE-691 / Summer School 2010 Villeneuve d'Ascq – FRANCE
Novembre 2009	20th Joint Glycobiology Meeting 2009 Cologne – ALLEMAGNE



Généralités

I. Avant-propos

Le cancer colorectal est le deuxième cancer le plus répandu chez la femme et le troisième chez l'homme. Il est responsable en France de près de 16.500 décès par an, sexes confondus (Dynamique d'évolution des taux de mortalité des principaux cancers en France, 2010). On note près de 1.233.600 nouveaux cas chaque année dans le monde avec parmi les pays les plus touchés, les États-Unis et la France (GLOBOCAN, 2008) (Figure 1). On note également que ces pays sont les plus affectés par les pathologies métaboliques comme par exemple le diabète de type 2 (Wild et al., 2004). Ces deux pathologies sont par conséquent des problèmes de santé publique pour les pays les plus développés et constituent un axe de recherche majeur. Des études cliniques ont permis d'établir un lien étroit entre ces deux types de pathologies. On observe par exemple une propension à développer un cancer colorectal doublée chez les diabètiques de type 2 (Khaw et al., 2004 ; Ahmed et al., 2006 ; Pais et al., 2009). Cette statistique inquiétante à amener les chercheurs à rechercher un lien moléculaire entre diabète de type 2, et plus largement désordres métaboliques, et cancer colorectal. Depuis plusieurs années, un nouveau « senseur métabolique » a fait son apparition avec la mise en évidence de la *O*-GlcNAcylation des protéines. Cette découverte a permis d'entrevoir de nouveaux modes de régulation des protéines intracellulaires, directement en relation avec les variations nutritionnelles de l'organisme. Plus précisément, la *O*-GlcNAcylation permet de retranscrire les changements de concentration en glucose extracellulaire sur l'activité cellulaire. Nous pouvons donc suggérer que ces variations affectent directement le développement du cancer par le biais de la *O*-GlcNAcylation. Cette hypothèse est par ailleurs soutenue par l'importance de l'alimentation dans les facteurs de risque du cancer colorectal (30 %) (Figure 1) (Mackay et al., 2006). Néanmoins, il nous fallait trouver une protéine cible de la *O*-GlcNAcylation permettant de relier "hygiène de vie" et cancer colorectal. La β-caténine semblait tout indiquée pour cela. Les dérégulations de cet oncogène ont été décrites dans la séquence de Fearon et Vogelstein et sont observées dans la majeure partie des cancers colorectaux (Fearon et Vogelstein, 1990). De plus, la β-caténine est impliquée dans les stades précoces de cancérisation, là où les facteurs de risque ont démontré leurs implications majeures.

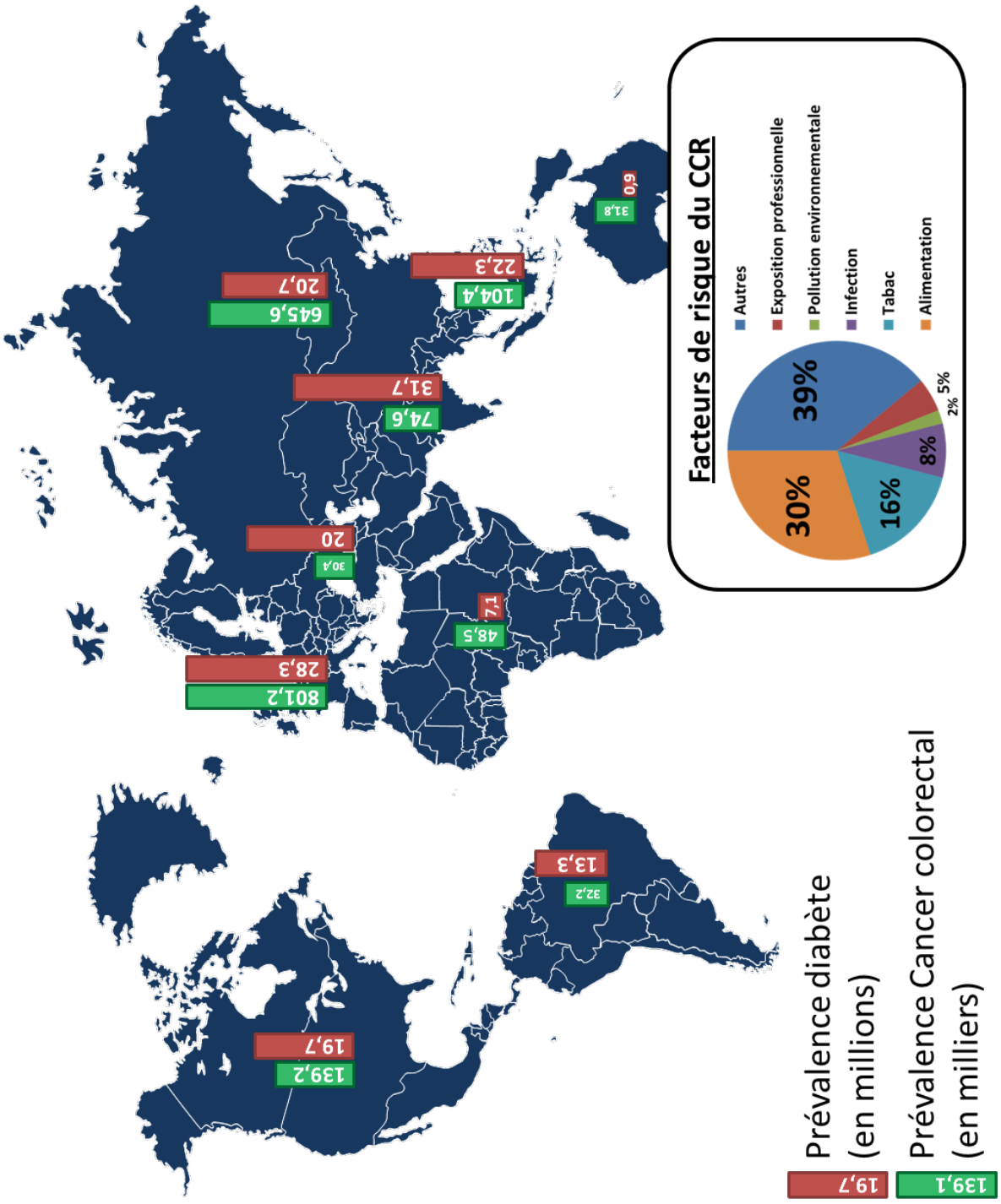


Figure 1: Prévalence du diabète de type 2 et du cancer colorectal à travers le monde et facteurs de risque du cancer colorectal. (Wild et al., 2004 ; GLOBOCAN, 2008 ; Mackay et al., 2006)

II. La β-caténine

La β-caténine est une protéine impliquée dans de nombreuses fonctions physiologiques (développement embryonnaire, renouvellement des épithéliums,…) mais elle participe également à l'emergence de certains cancers (colorectal, foie…). Cette multitude de fonctions découle en partie d'une structure bien particulière qui lui confère à la fois la possibilité d'effectuer de nombreuses interactions protéiques, d'adopter différentes localisations subcellulaires, de subir de nombreuses modifications post-traductionnelles et enfin d'adopter différents rôles au sein de la cellule.

A. Eurêka…Elle s'appellera β-caténine !

De manière indépendante au cours des années 80, deux études ont permis la découverte de la β-caténine. En 1989, la transfection de l'ovomoruline, maintenant plus connue sous le nom de E-cadhérine, a mis en lumière trois protéines inconnues de 102, 88 et 80 kDa qui seront appelées respectivement alpha- (α-), beta-(β-) et gamma-(γ-) caténine (Ozawa et al., 1989). Elles sont décrites comme des protéines « linker » permettant de lier la E-cadhérine et le cytosquelette et ainsi soutenir la structure cellulaire.

Précédemment, en 1984, l'étude de mutations affectant la segmentation de l'embryon chez la mouche du vinaigre (*Drosophila melanogaster)* a permis l'identification d'un gène responsable de cette segmentation anormale (caractérisée par la disparition de l'alternance de denticules/zones nues sur la partie ventrale de la larve) (Wieschaus et al., 1984) (Figure 2). Ce gène sera nommé *armadillo,* pour la ressemblance étrange du mutant avec l'animal, et constitue l'orthologue chez la drosophile du gène de la β-caténine humaine. Le phénotype observé chez le mutant *armadillo* est rapproché de celui du mutant nul *wingless* ce qui permettra d'inclure la β-caténine dans une voie de signalisation : la voie Wingless/Armadillo (Wnt/β-caténine) (Wieschaus et Riggleman, 1987). Des études plus approfondies permettront de montrer que la segmentation embryonnaire dépendante de la protéine Armadillo est régulée à la fois par Wingless et par un mécanisme post-traductionnel (Riggleman et al., 1990).

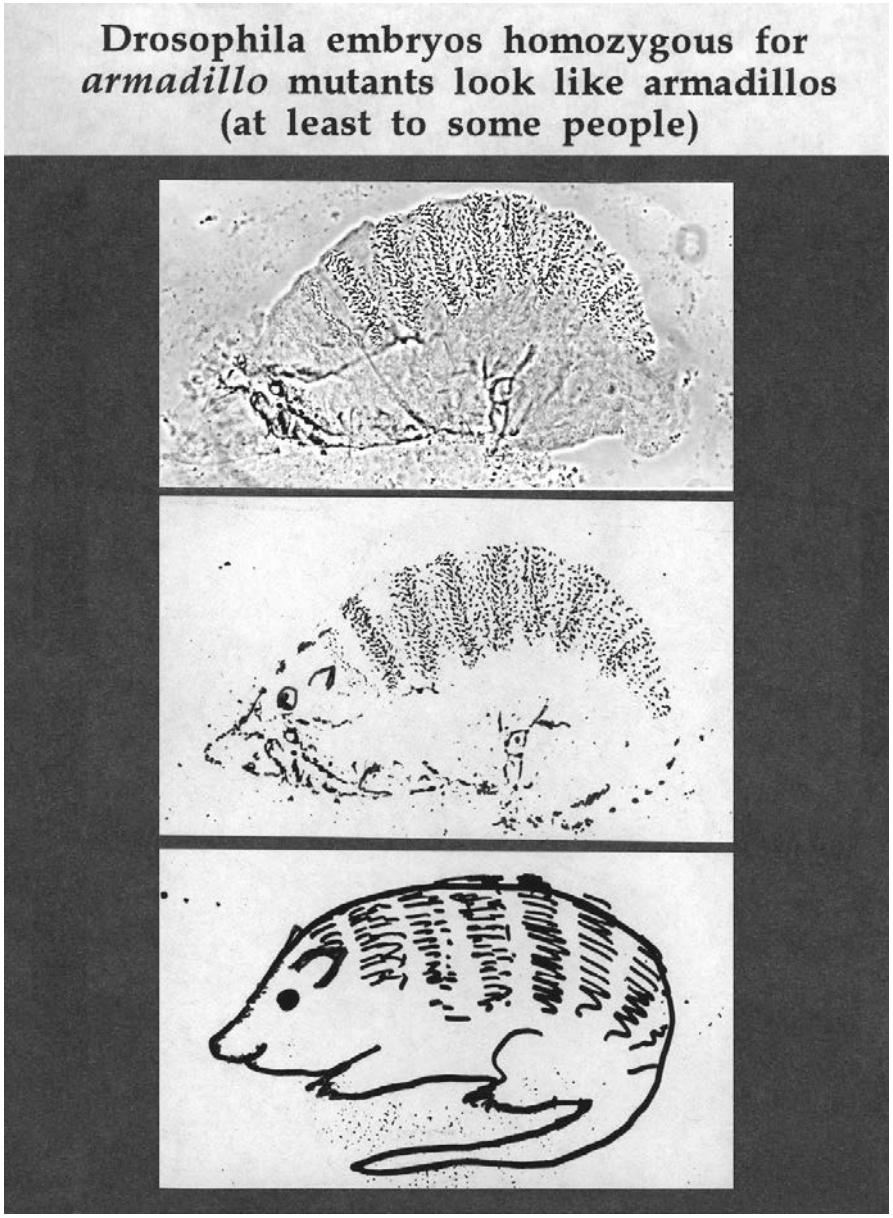


Figure 2 : Identification du gène *armadillo* chez le mutant *Drosophila melanogaster* pour sa ressemblance avec l'animal (Three decades of Wnt signaling, EMBO J, 2012).

De plus, le mutant *shaggy/zeste-white-3,* orthologue drosophilien de GSK3β (Glycogène Synthase Kinase 3β) montre des similarités avec les mutants *wingless/armadillo* démontrant ainsi l'importance des processus de phosphorylation dans la voie de signalisation (Siegfried et al., 1994 ; Steitz et al., 1998).

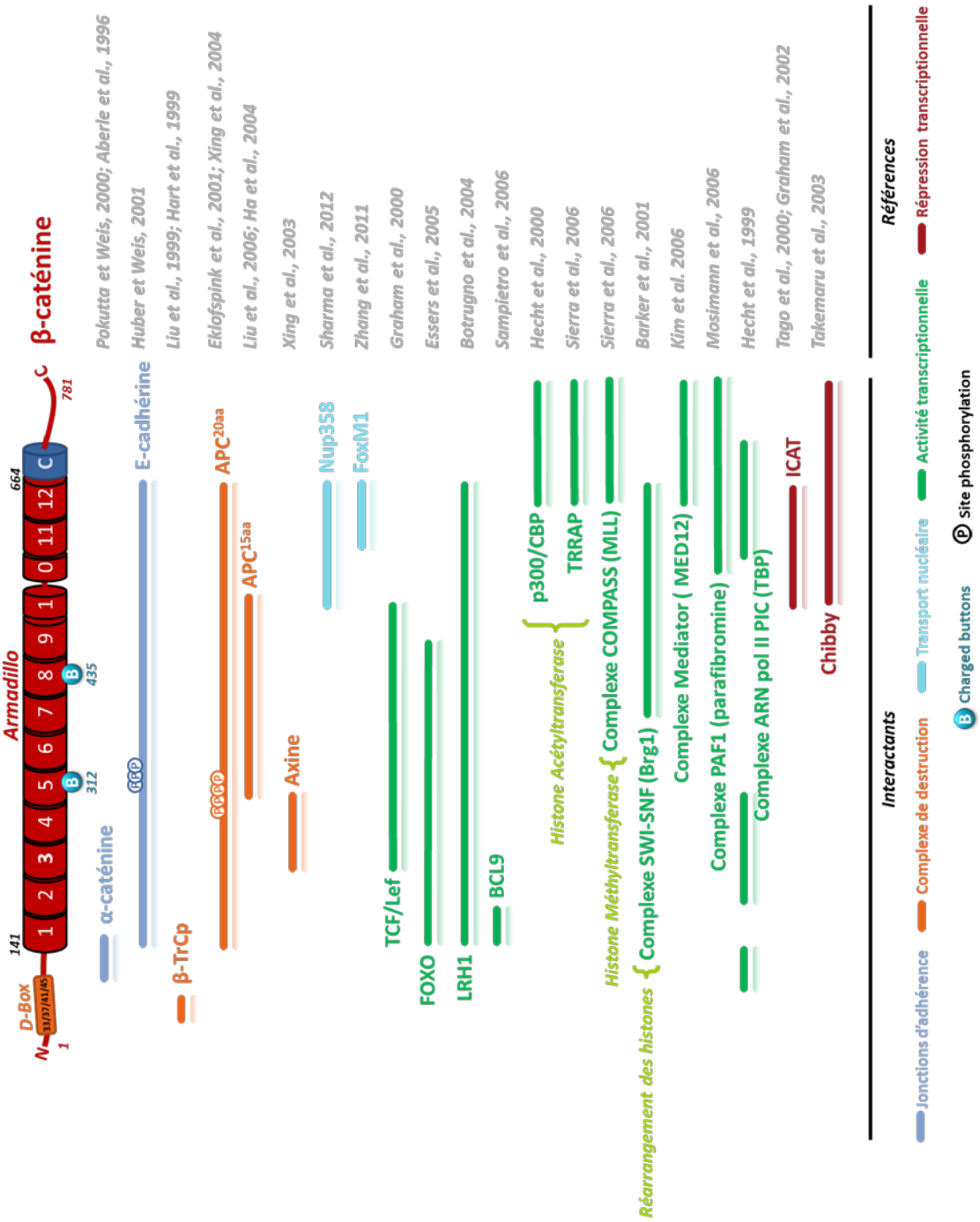
Enfin, à plusieurs reprises au cours des années 90, l'association de la β-caténine avec les facteurs de transcription de la famille T-Cell Factor (TCF)/Lymphoid enhancer-binding factor (Lef) a été décrite (Behrens et al., 1996 ; Molenaar et al., 1996 ; Van de Wetering et al., 1997). C'est à cette époque, que la qualification d'oncogène fut attribué à la β-caténine (Peifer, 1997).

Dans la ligne de ces découvertes, le rôle de la β-caténine dans la formation des jonctions d'adhérence, sa régulation par phosphorylation et son activité transcriptionnelle liée à la voie Wnt/β-caténine ont été caractérisés dans de plusieurs études qui ont fait l'objet de nombreuses publications (6665 entrées : source PUBMED).

B. Du gène à la protéine

Le gène de la β-caténine humaine, *CTNNB1*, est localisé sur le chromosome 3 au niveau de la région 3p21 (Kraus et al., 1994). Cette région chromosomique comporte 16 exons et possède une taille de 23,2 kb (Nollet et al., 1996). La région flanquante en 5' est riche en GC et ne possède pas de boite CCAAT mais on note la présence d'une boite TATA et de sites de fixation de nombreux facteurs de transcription tels qu'AP1, E2F1, NFκB, MEF1, Pax5, ISRE2, Smad3/4, GATA et ZIC (Li Q et al., 2004). Son promoteur porte également un site putatif de fixation du complexe de transcription β-caténine/TCF.

L'organisation génomique chez certains organismes est un peu différente et permet de séparer deux fonctions de la β-caténine, adhésion et transcription. Par exemple, *Caenorhabditis elegans* possède 3 gènes différents codant, d'une part une protéine HMP-2 (humpback-2) se liant aux cadhérines, et d'autre part BAR-1 (Beta-catenin/Armadillo-Related protein 1) et WRM-1 (WoRM armadillo protein 1) participant à la transcription (Natarajan et al., 2001). C'est la partie centrale contenant les armadillos qui est la plus conservée dans l'évolution, ce qui renforce l'importance de ces domaines dans les fonctions de la β-caténine.



<u>Figure 3</u> : Structure de la β-caténine et ses interactants.

APC : Adenomatous Polyposis Coli ; BCL9 : B-cell CLL/lymphoma 9 ; Brg1: brahma-related gene 1; β-TrCP : β-Transducin-repeats Containing Protein; CBP : CREB-binding protein ; COMPASS : Complex Proteins Associated with Set1; D-Box : Destruction-Box ; FoxM1 : Forkhead box protein M1 ; FOXO: Forkhead bOX O ; ICAT: Inhibitor of β-caténine and TCF4; LRH1 : liver receptor homolog-1 ; MLL: Mixed Lineage Leukemia; Nup358 : Nucléoporine 358 ; P: Phosphorylation; SWI-SNF: SWItch/Sucrose NonFermentable; TBP: TATA box binding Protein; TCF/Lef : T-Cell Factor/ Lymphoid enhancer-binding factor; TRRAP : Transformation/transcription domain-associated protein.

C. Structure de la β-caténine

Le gène *CTNNB1* encode une protéine de 781 acides aminés formée d'une région centrale rigide (141-664) et de deux extrémités flexibles appelées NTD (N-Terminal Domain) et CTD C-terminal Domain) (Figure 3).

1. Extrémités flexibles pour modifications multiples

Les extrémités flexibles de la β-caténine ont pour fonction l'amarrage de protéines régulatrices de son activité. Ces extréimtés sont également le siège modifications post-traductionnelles clés.

L'extrémité NTD constitue la plateforme d'interaction avec les kinases GSK3β et CK1α phosphorylant quatre sites de la Destruction-Box (D-Box) (S33/S37/T41/S45), centre du processus de dégradation de la protéine (cf partie II.E.).

Le domaine CTD comporte, quant à lui, une hélice très conservée appelée hélice-C (667-683) essentielle pour l'activité transcriptionnelle de la β-caténine (Xing et al., 2008). Elle est au centre du domaine CTTA (C-Terminal Transcriptional Activators) qui s'étend des derniers armadillos (Arm 10/11/12) à l'extrémité C-terminale de la β-caténine. Ce domaine permet l'interaction notamment avec de nombreux co-activateurs transcriptionnels (cf partie II.G.2) (Mosimann et al., 2009). L'extrémité CTD a également la capacité de se replier dans le sillon central et de moduler les interactions avec la E-cadhérine (Castaño et al., 2002) ou le TCF-4 (Solanas et al., 2004) par exemple. On note également la présence de quelques sites de phosphorylation en C-terminal, notamment la sérine 675 qui module l'activité transcriptionnelle de la β-caténine.

<u>Tableau 1 :</u> Les modifications post-traductionnelles de la β-caténine.

β-TrCP : β-Transducin-repeats Containing Protein; CBP : CREB-Binding Protein ; Cdk5 : Cyclin-dependent kinase 5 ; CK : Casein Kinase; GSK3β : Glycogen synthase kinase 3β; JAs : Jonctions d'adhérence ; JNK2 : c-jun N-terminal kinase 2 ; OGT : O-GlcNAc Transferase; PDK1: Pyruvate dehydrogenase kinase 1 ; PKA : Protein Kinase A ; TBP : TATA-box Binding Protein.

Modifications	Sites	Enzymes	Fonctions		Références
Phosphorylation (Ser/Thr)	S23,S29	GSK3β?, CK2?	Dégradation ?	??	Bek et Kemler, 2002; Van Noort et al., 2002
	S33,S37	GSK3β	Dégradation	Sites de reconnaissance pour β-TRcP	Liu et al., 1999; Liu et al., 2002
	T41	GSK3β	Dégradation	Site relais de phosphorylation	Wu et He, 2006; Liu et al., 2002
	S45	CK1α	Dégradation	Site amorçage pour GSK3β	Liu et al., 2002
	T112	CK2	Adhésion	Augmentation de l'affinité avec l'α-caténine	Bek et Kemler, 2002
	T120	PDK1	Localisation	Rétention golgienne du complexe E-cadhérine/β-caténine	Du et al., 2012
	S191	JNK2	Localisation	Translocation nucléaire	Wu et al., 2008
	S246	Cdk5	Dégradation	Augmentation de l'affinité avec Pin1, inhibition de l'interaction avec l'APC	Munoz et al., 2007
	T393	CK2	Dégradation	Stabilisation de la β-caténine, augmentation de l'activité transcriptionnelle	Song et al., 2003
	S552	Akt	Activité transcriptionnelle	Augmentation de l'activité transcriptionnelle	Fang et al., 2007
	S605	JNK2	Localisation	Translocation nucléaire	Wu et al., 2008
	S675	PKA	Activité transcriptionnelle	Augmentation de l'activité transcriptionnelle, interaction avec CBP	Van Veelen et al., 2011
Phosphorylation (Tyr)	Y142	Fer/Fyn; Met	Adhésion	Réduction de l'interaction avec l'α-caténine	Piedra et al., 2003 ; Brembeck et al., 2004
	Y333	Src	Activité transcriptionnelle	Augmentation de l'activité transcriptionnelle	Yang et al., 2011
	Y489	Abl	Adhésion	Rupture de l'association N-cadhérine/β-caténine	Rhee et al., 2007
	Y654	Src	Adhésion	Réduction de l'interaction avec la E-cadhérine, interaction avec TBP	Roura et al., 1999; Van Veelen et al., 2011
	Y86, Y654	Bcr-Abl	Dégradation	Stabilisation de la β-caténine	Coluccia et al., 2007
	Y654, Y670	Met	Activité transcriptionnelle	Dissociation JAs, augmentation de l'activité transcriptionnelle	zneg et al., 2006
Ubiquitinylation	K19,K49	β-TrcP	Dégradation	Dégradation protéasomale	Winer et al., 2006
Acétylation	K49	CBP	Activité transcriptionnelle	Augmentation de la transcription spécifique de c-myc	Wolf et al., 2002
	K345	p300	Activité transcriptionnelle	Augmentation de l'interaction avec TCF/Lef	Levy et al., 2004
O-GlcNAcylation	??	OGT	Localisation	Diminution de la localisation nucléaire	Sayat et al., 2008

2. Les « armadillos », siège de nombreuses interactions

La β-caténine est composée dans sa région centrale de 12 répétitions de 42 acides aminés appelées domaine Armadillo (Arm) (141-664). Chaque Armadillo est formé de trois hélices arrangées sous la forme d'un triangle; les douze domaines Arm s'enroulent et génèrent une super-hélice comportant un sillon chargé positivement servant de plateforme d'interaction (Huber et al., 1997; Xu et Kimelman, 2007). Au creux de ce sillon, les lysines 312 et 435, appelées « charged buttons », forment des ponts salins et renforcent les liaisons avec les différents partenaires. En effet, le TCF, APC (Adenomatous Polyposis Coli) ou encore la E-cadhérine interagissent avec la β-caténine au niveau de ce sillon. (Graham et al., 2000 ; Eklofspink et al., 2001; Huber et Weis, 2001). Le chevauchement des domaines d'interaction de ces différentes protéines ne permet pas leurs interactions simultanées avec la β-caténine et sépare ainsi distinctement les fonctions liées aux différents complexes, de la formation des jonctions d'adhérence à la transcription.

3. Les modifications post-traductionnelles de la β-caténine

La β-caténine possède de nombreux sites de modifications post-traductionnelles qui lui permettent de moduler ses interactions et ses fonctions biologiques. Certaines modifications, comme la *O*-GlcNAcylation, restent encore néanmoins peu renseignées (Tableau 1).

Les nombreuses intéractions et modifications post-traductionnelles répertoriées pour la β-caténine ont pour conséquence de lui conférer trois localisations et trois fonctions majeures : régulation des interactions intercellulaires à la membrane plasmique (jonctions d'adhérence), d'être sujette à la dégradation protéasomale dans le cytosol et d'acquérir son activité transcriptionnelle dans le noyau.

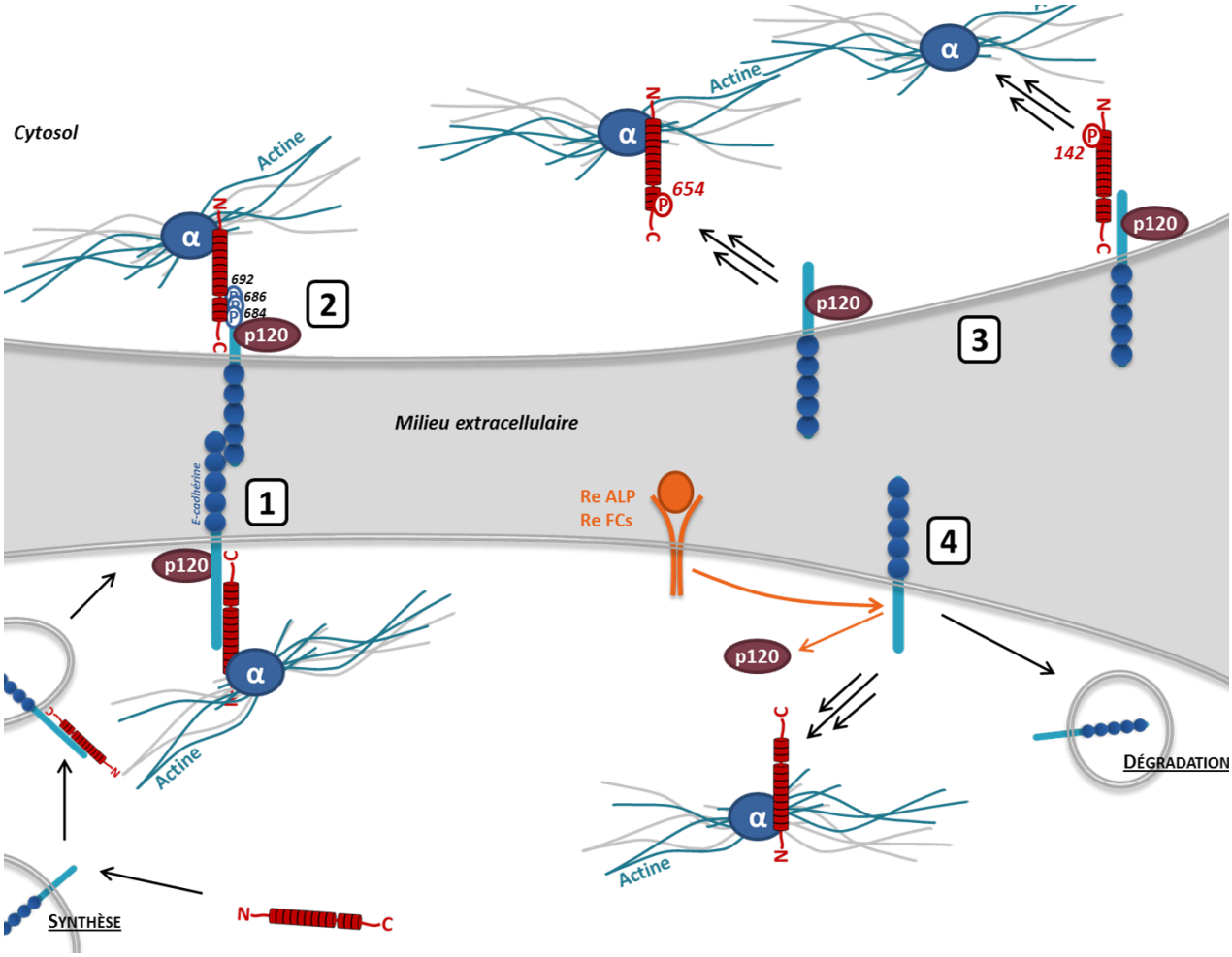


Figure 4: La β-caténine au cœur des jonctions d'adhérence : mise en place et régulations. 1) Le complexe des jonctions d'adhérence est formé de la E-cadhérine, la β-caténine et l'α-caténine et est renforcé par la présence de la p120 caténine. 2) Les phosphorylations en position 684, 686 et 692 de la E-cadhérine consolident les jonctions d'adhérence. 3) les phosphorylations 142 et 654 de la β-caténine la dissocient respectivement de l'α-caténine et de la E-cadhérine. 4) la stimulation de certains récepteurs par les facteurs de croissance ou par l'acide lysophosphatidique déstabilise le complexe notamment en dissociant la p120 caténine.

α- : α-caténine ; ALP : Acide lysophosphatidique ; FCs : Facteurs de croissance ; p120 : p120 caténine ; P : Phosphorylation ; Re : Récépteur.

D. La membrane plasmique : la β-caténine, composant essentiel des jonctions d'adhérence

1. Le complexe cadhérine/caténines (α- et β-)

En absence de stimuli externes, la β-caténine est associée en majorité (à la hauteur de 80%) aux cadhérines au niveau de la membrane plasmique formant ainsi les jonctions d'adhérence (JAs) (Figure 4).

A ce niveau, la β-caténine contribue à la formation des tissus épithéliaux polarisés et à l'intégrité de ces tissus (Meng et Takeichi, 2009). Les cadhérines appartiennent à une superfamille de glycoprotéines membranaires qui d'une part, forment des jonctions homotypiques Ca^{2+}-dépendantes grâce à leur domaine extracellulaire et d'autre part, relient le cytosquelette *via* leurs interactions avec les caténines α, β ou γ (également appelée Plakoglobine). Les cadhérines sont distinguées et nommées en fonction du tissu dans lequel elles sont exprimées majoritairement : N-cadhérine (Neurone), E-cadhérine (Epithélium), P-cadhérine (Placenta), R-… (Rétine), K-… (Rein), M-… (Myotubule), VE-… (Endothélium vasculaire)… etc… Néanmoins, elles sont co-exprimées dans de nombreux types cellulaires (Meng et Takeichi, 2009). La β-caténine et la plakoglobine sont capables d'interagir avec les différentes cadhérines et de lier l'α-caténine. Cette dernière est capable soit de s'homodimériser et de favoriser dans ce cas la polymérisation de l'actine, (Drees et al., 2005) soit de s'associer à la β-caténine et de relier les jonctions d'adhérence au cytosquelette d'actine.

Le complexe cadhérine/caténine le plus fréquemment rencontré est constitué de la E-cadhérine et de la β-caténine, c'est pourquoi nous nous focaliserons sur celui-ci. La formation de ce complexe s'effectue simultanément à la synthèse de E-cadhérine. En effet, la β-caténine s'associe à la E-cadhérine au niveau du *reticulum* endoplasmique alors que cette dernière achève sa maturation post-traductionnelle réticulaire et golgienne. Les deux protéines sont ensuite acheminées ensemble à la membrane plasmique (Hinck et al., 1994) (Figure 4-1). La β-caténine s'associe à la E-cadherine au niveau d'une séquence PEST.

Ces séquences riches en Proline proline (P), acide glutamique (E), sérine (S) et thréonine (T) sont des signaux de dégradation protéasomale pour les protéines. La β-caténine, en s'associant à ce niveau, empêche la dégradation protéasomale de la E-cadhérine en cours de maturation (Huber et al., 2001). Une autre caténine, p120, stabilise ensuite ce complexe en interagissant avec la partie juxtamembranaire de la E-cadhérine (Yap et al., 1998 ; Ohkubo et ozawa, 1999). De la même manière que pour la β-caténine, la dissociation de p120 des jonctions d'adhérence entraîne l'internalisation de la E-cadhérine et sa dégradation (Hinck et al., 1994 ; Ireton et al., 2002).

La régulation du complexe des jonctions d'adhérence est très importante, d'une part pour maintenir les jonctions cellulaires et d'autre part pour libérer la β-caténine en temps voulu pour la réalisation de ses autres fonctions.

2. Régulation du complexe des jonctions d'adhérence

La stabilisation des jonctions d'adhérence est favorisée en réponse à la phosphorylation de la E-cadhérine (S684/686/692) (Huber et Weis, 2001 ; Choi et al., 2006 ; Sampietro et al., 2006) (Figure 4-2).

Il existe malgré tout de nombreux mécanismes permettant de libérer la β-caténine des jonctions d'adhérence. La perte d'interaction avec la β-caténine dévoile les séquences PEST de la E-cadhérine et entraîne sa dégradation protéasomale, notamment *via* l'ubiquitine ligase Hakai (Fujita et al., 2002).p120 joue un rôle essentiel dans ce processus (Figure 4-4). Elle est un élément stabilisateur du complexe des jonctions d'adhérence et sa dissociation du complexe, après stimulation par exemple par l'acide lysophosphatidique, entraîne l'internalisation de la E-cadhérine et la perte des interactions cellulaires (Kam et Quaranta, 2009).

La phosphorylation de la Tyrosine 142 de la β-caténine par les kinases Fer/Fyn, membres de la famille Src, ou par le récepteur Met permet de la dissocier de l'α-caténine en réponse aux facteurs de croissance (Piedra et al., 2003 ; Brembeck et al., 2004) (Figure 4-3).

De même, la phosphorylation de la β-caténine sur la Tyrosine 654 par la kinase Src ou le récepteur Met inhibe son interaction avec la E-cadhérine et favorise son activité oncogénique (Roura et al., 1999 ; Piedra et al., 2001 ; Van Veelen et al., 2011).

Cette phosphorylation sur la Tyrosine 654 peut être contrecarrée par l'interaction avec la phosphatase PTP1B (Protein Tyrosine Phosphatase 1B), qui se fixe directement aux cadhérines, ce qui permet, par déphosphorylation de la β-caténine, de maintenir les jonctions d'adhérence (Balsamo et al., 1996). Enfin, la phosphorylation de ce résidu, en empêchant le repliement de l'extrémité CTD de la β-caténine sur les dernières répétitions Armadillo, promeut la phosphorylation de la Sérine 675 par la protéine Kinase A (PKA), qui permet à la β-caténine d'acquérir ses fonctions de facteur de transcription (Taurin et al., 2008 ; Xing et al., 2008).

En conclusion, ces mécanismes ont pour but commun d'augmenter la disponibilité de β-caténine en réponse à un stimulus prolifératif (Zeng et al., 2006) en entraînant de ce fait une déplétion de β-caténine au niveau des jonctions d'adhérence.

Afin de ne pas totalement perdre les jonctions cellulaires, la cellule met en place des systèmes de compensation. Par exemple, la plakoglobine est capable de remplacer la β-caténine au niveau des jonctions d'adhérence (Huelsken et al., 2000).

La libération d'un pool de β-caténine des jonctions d'adhérence par les différents mécanismes présentés précédemment entraine l'accumulation de la β-caténine cytosolique. Ce pool est également approvisionné par la β-caténine néo-synthétisée qui par défaut d'interaction, ne s'associe pas aux jonctions d'adhérence. La β-caténine ainsi libre dans le cytosol fait l'objet d'une dégradation protéasomale massive.

E. Là où APC passe, la β-caténine trépasse : un système de dégradation efficace

La β-caténine cytosolique libre est rapidement prise en charge par un système de dégradation très efficace, ceci dans le but de limiter son activité oncogénique.

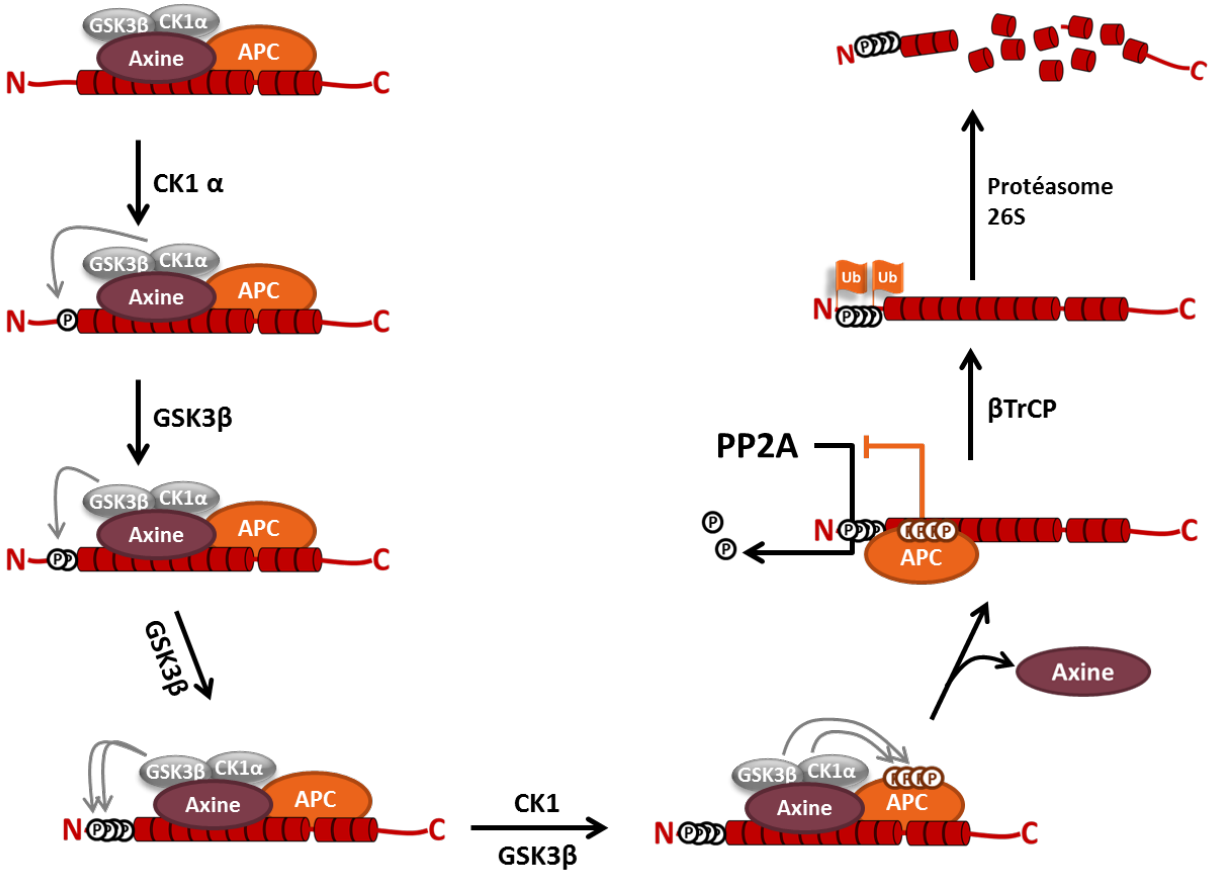


Figure 5 : Système de dégradation de la β-caténine. La séquestration de la β-caténine dans son complexe de destruction entraîne sa phosphorylation dans un premier temps par CK1α puis par GSK3β. APC est également phosphorylé au cours de ce processus permettant son rapprochement des sites phosphorylés de la β-caténine et la protection vis-à-vis de la déphosphorylation par PP2A. Enfin l'E3 ubiquitine ligase β-TrCp ubiquitinyle la β-caténine au niveau de la destruction-Box et l'adresse au protéasome.

APC : Adenomatous Polyposis Coli ; β-TrCP : β-Transducin-repeats Containing Protein ; CK1α : Casein Kinase 1α ; GSK3β : Glycogen synthase kinase 3β ; P : phosphorylation ; PP2A : Protein Phosphatase 2A ; Ub : Ubiquitine.

Pour cela, elle est reconnue par un complexe de destruction qui l'étiquette et l'adresse au protéasome, réduisant ainsi sa demi-vie, estimée à 30 minutes en moyenne (Kimelman et Xu, 2006; Papkoff et al., 1997). Dans un premier temps, la β-caténine cytosolique est reconnue par deux protéines, suppresseurs de tumeur, l'axine et l'*Adenomatous Polyposis Coli* (APC) (Roberts et al., 2011). Ce premier complexe ternaire sert de plateforme d'interaction pour les kinases GSK3β (Glycogène Synthase kinase 3 β) et CK1α (Caséine Kinase 1 α) (Dajani et al., 2003 ; Amit et al., 2002) (Figure 5).

GSK3β et CK1α permettent la phosphorylation des sérines 33, 37 et 45 et de la thréonine 41 de la Destruction-Box. Dans un premier temps, CK1α phosphoryle la sérine 45 induisant la phosphorylation par GSK3β de T41 et enfin des résidus S33 et S37 (Liu et al., 2002). Ces phosphorylations ne sont possibles qu'au sein du complexe de destruction, GSK3β étant incapable de phosphoryler la β-caténine hors de ce complexe (Hedgepeth et al., 1999). De plus, l'interaction de GSK3β avec l'axine permet de rapprocher la kinase de sa cible et augmente par conséquent considérablement son activité (Dajani et al., 2003).

Le recrutement de GSK3β dans le complexe de destruction permet également la phosphorylation d'APC qui est nécessaire pour la suite du processus de dégradation.

En effet, dans un premier temps, l'axine et APC interagissent avec la β-caténine (Eklofspink et al., 2001). APc interagit alors avec ses réépétitions de 15 acides aminés (Figure 10) que l'on nommera APC^{15aa}.

La proximité de GSK3β et de CK1α entraîne la phosphorylation des répétitions de 20 aa d'APC (APC^{20aa}), et plus particulièrement de la troisième répétition très conservée (Ha et al., 2004). Ces phosphorylations provoquent la perte d'interaction de la β-caténine avec l'axine au profit de l'APC^{20aa} phosphorylé (Xing et al., 2004 ; Ha et al., 2004 ; Liu et al., 2006). APC accompagne ainsi la β-caténine vers la dégradation protéasomale en la protégeant de la déphosphorylation par PP2A (Protein Phosphatase 2A), une phosphatase fréquemment intégrée au complexe de destruction (Su et al., 2008 ; Hsu et al., 1999). Cette phosphatase permettrait notamment de libérer la β-caténine pour qu'elle acquière sa fonction transcriptionnelle au cours de l'embryogénèse (Götz et al., 2000).

Les Sérines 33 et 37 sous leur forme phosphorylée sont ensuite reconnues par β-TrCP (β-Transducin repeats-Containing Protein) permettant le recrutement du complexe Ubiquitine Ligase $SCF^{\beta\text{-TrCP}}$ complet (Skp1/Cul1/F-$Box^{\beta\text{-TrCP}}$) (Liu et al., 1999 ; Hart et al., 1999).

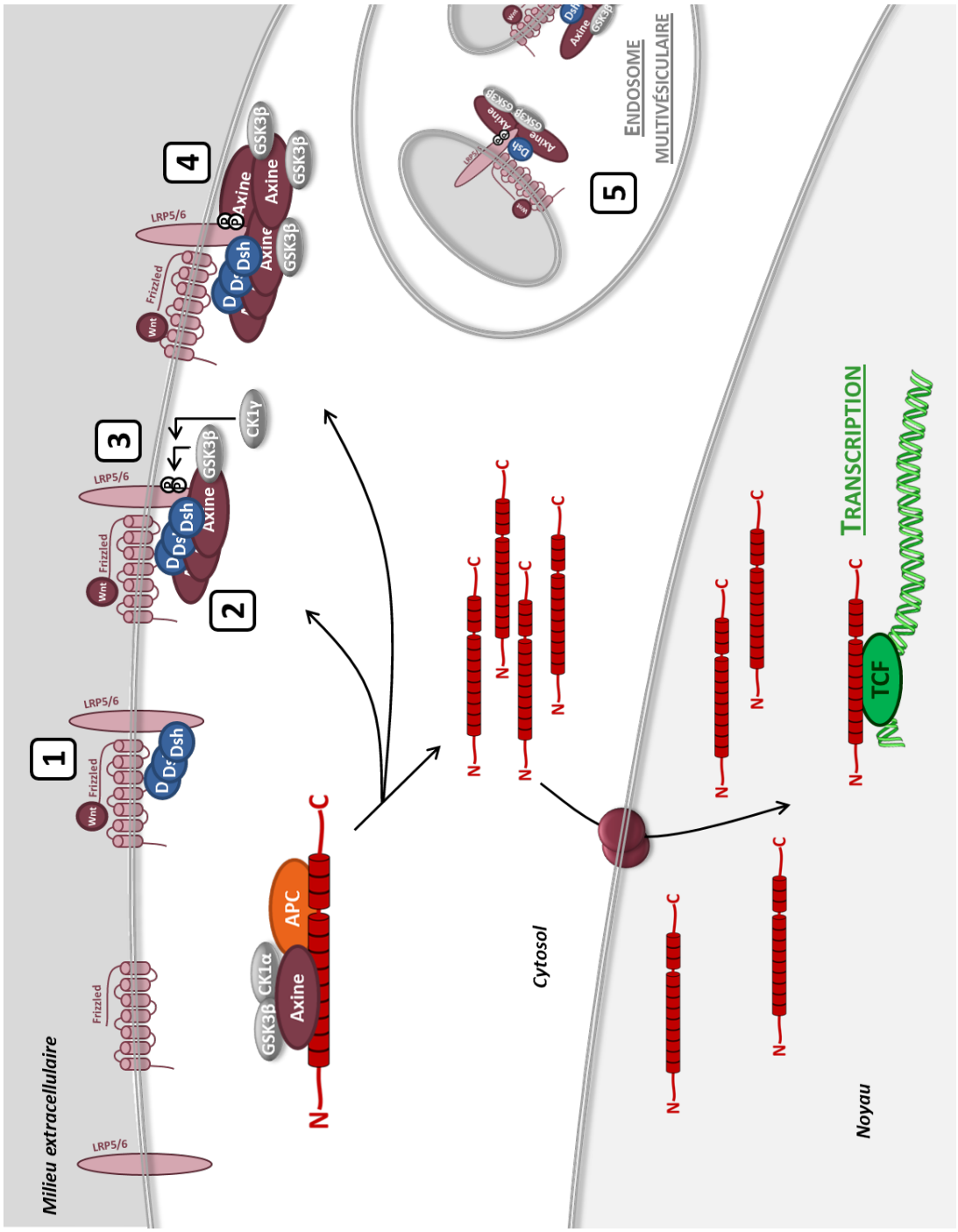


Figure 6 : La voie Wnt/β-caténine. 1) La fixation d'un facteur Wnt entraine l'association du récepteur Frizzled avec son corécepteur LRP5/6 et le recrutement de Disheveled. 2) Après sa multimérisation, Dsh recrute l'axine dissociant ainsi le complexe de destruction de la β-caténine. 3) L'axine reste notamment associée à la GSK3β. 4) Ceci entraîne la phosphorylation du corécepteur LRP5/6 et l'apparition d'un nouveau site de recrutement de l'axine. 5) Enfin, le complexe membranaire est internalisé dans des endosomes multivésiculaires afin d'empêcher la reformation du complexe de destruction.

APC : Adenomatous Polyposis Coli; CK1 : Casein Kinase 1 ; Dsh : Dishevelled ; GSK3β : Glycogen synthase kinase 3β ; P : phosphorylation ; TCF : T-Cell Factor.

La β-caténine ainsi ubiquitinylée est adressée au protéasome 26S afin d'y être dégradée. Une faible partie de la β-caténine peut également être ubiquitinylée, indépendamment de ses phosphorylations, par Jade-1. Ce mécanisme semble dépendre du suppresseur de tumeur pVHL (von Hippel-Lindau protein) impliqué notamment dans la maladie du même nom et dans la cancérogénèse rénale (Chitalia et al., 2008).

Ce système de dégradtion de la β-caténine permet le contrôle de l'activité oncogénique de la protéine. Lorsque celle-ci parvient à échapper à la dégradation, elle acquière une nouvelle fonction de facteur de transcription. Cette stabilisation est principalement décrite dans le cas de la voie Wnt/β-caténine.

F. La voie de signalisation Wnt/ β-caténine

L'appellation Wnt est un amalgame des termes Wingless et Int1 (Nusse et al., 1991). Le gène Wingless (Wg) a été découvert chez *Drosophila melanogaster* en 1973, sa mutation entraînant des phénotypes sans ailes de ces drosophiles « Wingless » (Sharma, 1973).
Int1 (Integration 1) est son homologue chez la souris et a été décrit en 1967 chez des souris présentant des malformations du cerveau antérieur (Lane, 1967). Il existe différentes voies Wnt classées selon les effecteurs qu'elles mettent en place : La voie Wnt/Ca^{2+}, la voie Wnt/PCP (Planar Cell Polarity) et la voie Wnt/β-caténine. Cette dernière possède comme effecteur central la β-caténine et est impliquée dans divers processus physiologiques du développement embryonnaire au renouvellement des épithéliums (cf partie III). La fixation d'un facteur Wnt sur son récepteur Frizzled (Fz) et co-récepteur LRP5/6 (Low density Lipoprotein Receptor-related Protein 5/6) déclenche la cascade de signalisation Wnt/β-caténine. Ils agissent de concert pour inactiver le complexe de destruction de la β-caténine, la libérer et lui permettre d'activer la transcription. On peut décrypter cette cascade en 5 étapes (Figure 6) : (1) Dishevelled (Dsh) est recruté au niveau du complexe membranaire (Wnt/Fz/LRP5/6) et se multimérise pour former le signalosome (Bilic et al., 2007). (2) Dsh interagit avec l'axine via des domaines DIX (Dishevelled-Axine). Le recrutement de l'axine au niveau de la membrane plasmique, certainement associée aux kinases du complexe de destruction, permet ainsi la dissociation de celui-ci (Schwarz-Romond et al., 2007).

(3) Enfin, le recrutement du complexe Axine-GSK3β permet la phosphorylation de LRP5/6 par GSK3β mais également par CK1γ (Zeng et al., 2005) ; (4) LRP5/6 sous sa forme phosphorylée interagit avec l'axine ancrant ainsi la protéine « scaffold » du complexe de destruction à la membrane plasmique (Mao et al., 2001). LRP5/6 phosphorylé inhibe également directement GSK3β et amplifie de ce fait la signalisation Wnt/β-caténine (Piao et al., 2008).

Par ailleurs, l'inhibition des mécanismes d'endocytoses a également montré un rôle pour ceux-ci dans la transduction du signal (Yamamoto et al., 2006). Néanmoins, ce blocage n'empêche pas la phosphorylation de LRP5/6. On aurait donc après phosphorylation de LRP5/6, une étape endocytique supplémentaire nécessaire à l'activation de la voie Wnt. Le plus probable est que le complexe ligand-récepteur serait internalisé et que ces endosomes seraient eux-mêmes séquestrés au sein d'endosomes multivésiculaires, renfermant l'axine et GSK3β et empêchant la dégradation de la β-caténine (5) (Figure 6-5) (Taelman et al., 2010).

L'accumulation cytosolique de la β-caténine suite à la stimulation de la voie Wnt/β-caténine entraine sa translocation nucléaire et l'activation de son rôle de facteur de transcription.

G. La β-caténine nucléaire : un facteur de transcription

1. Le transport nucléaire de la β-caténine

La séquence peptidique de la β-caténine ne montre pas de signal d'import nucléaire (NLS, Nuclear Localization Signal), ni d'ailleurs d'export nucléaire (NES, Nuclear Export Signal), lui permettant d'interagir avec les importines (Fagotto et al., 1998 ; Yokoya et al., 1999).

Néanmoins, la structure de la β-caténine, et notamment les répétitions armadillo 10-12, s'assimile étrangement à l'importine β et rend possible son transport nucléaire par interaction directe avec les composants du pore nucléaire tel que Nup358 (Sharma et al., 2012) (Figure 7).

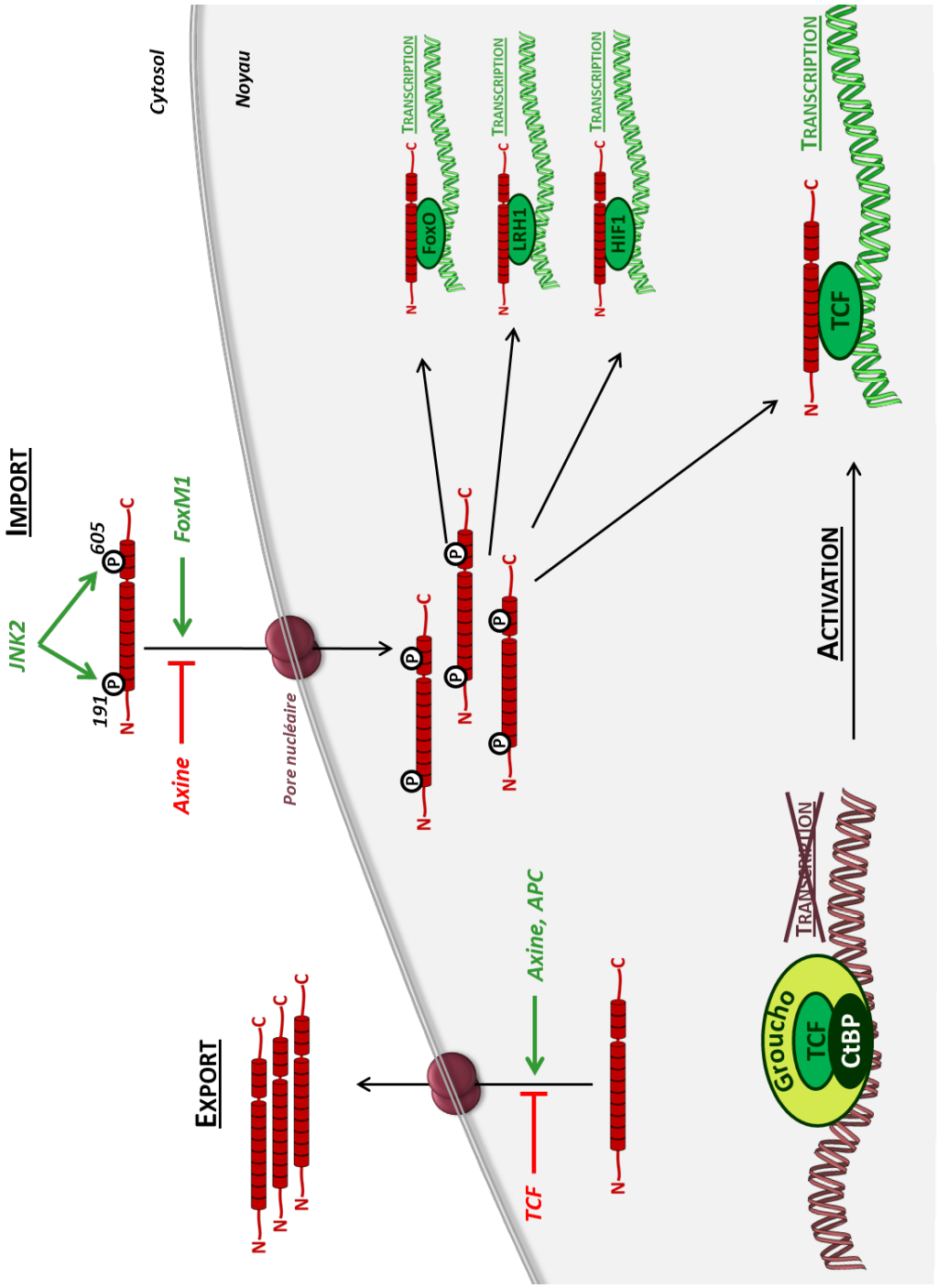


Figure 7: Import, export et activité nucléaire de la β-caténine. L'import et l'export nucléaire de la β-caténine sont régulés par différents éléments activateurs (vert) ou inhibiteurs (rouge). Au sein du noyau, la β-caténine s'associe majoritairement au TCF en déplaçant Groucho. Elle peut également s'associer à FOXO ou à LRH1 pour stimuler la transcription d'autres gènes cibles.

APC: Adenomatous Polyposis Coli; CtBP: C-terminal-binding protein ; FoxM1 : Forkhead box protein M1 ; FOXO: Forkhead bOX O ; JNK2 : c-jun N-terminale kinase 2 ; LRH1 : liver receptor homolog-1; P: Phosphorylation; TCF : T-Cell Factor.

Le transport de la β-caténine semble néanmoins sous le contrôle de certains facteurs. Tolwinski et Wieschaus ont caractérisé l'axine et le TCF, interactants de la β-caténine, comme facteurs de rétention cytosolique et nucléaire respectivement (Tolwinski et Wieschaus, 2001). Plus récemment, il a été démontré que l'interaction de la β-caténine avec FoxM1 (Forkhead box protein M1) augmentait son transport nucléaire et l'activation subséquente des gènes cibles de la voie Wnt/β-caténine (Zhang et al., 2008). FoxM1 "peut ainsi prêter sa séquence NLS" à la β-caténine afin de favoriser son transport nucléaire et l'accompagne dans sa fonction de stimulateur transcriptionnel.

Enfin, la phosphorylation des résidus Sérines 191 et 605 par JNK2 (c-Jun N-terminal kinase 2) permet de favoriser la localisation nucléaire de la β-caténine (Wu et al., 2008).

2. La β-caténine nucléaire

a) Un facteur de transcription bipartite

La β-caténine ne possède pas de domaine de liaison à l'ADN et nécessite pour son activité transcriptionnelle un partenaire capable de s'y lier.

Les protéines de la famille TCF (T-Cell Factor)/Lef (Lymphoid enhancer-binding factor) sont les partenaires les plus renseignés de la β-caténine. Il en existe 4 formes chez les vertébrés : TCF1, TCF3, TCF4 et Lef1 (Arce et al., 2006) (Figure 7).

Contrairement aux invertébrés ne possédant qu'un seul TCF, le système TCF des vertébrés permet une complémentation entre les différents membres de cette famille (Arce et al., 2006).

En dehors de son interaction avec la β-caténine, le TCF/Lef est un répresseur transcriptionnel interagissant constitutivement avec la famille de corépresseurs Groucho/TLE (Transducin-Like Enhancer) (Figure 7) (Cavallo et al., 1998). Ce complexe est renforcé par l'association de CtBP (C-terminal Binding Protein), un autre corépresseur, capable de lier des histones désacétylases (HDACs) entraînant la compaction des histones et l'inhibition de la transcription (Cuilliere-Dartigues et al., 2006).

La liaison de TCF/Lef avec la β-caténine déplace Groucho/TLE et convertit ce répresseur en activateur transcriptionnel de la voie Wnt/β-caténine (Daniels et Weis, 2005).

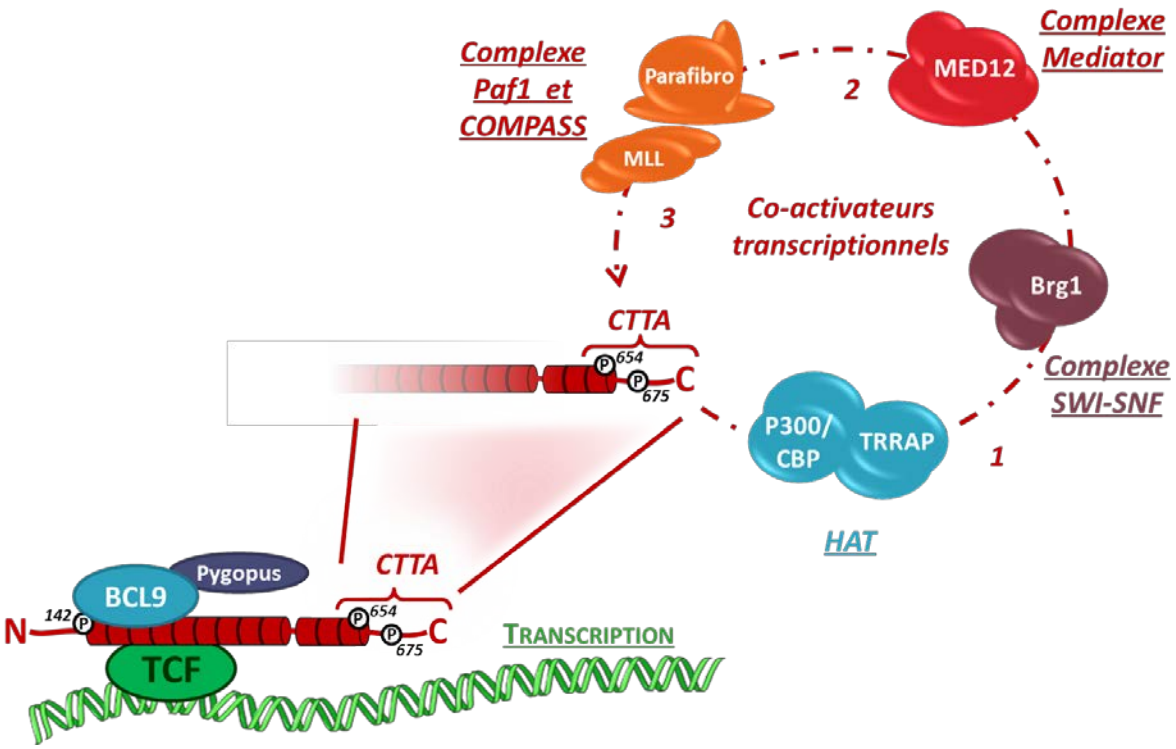


Figure 8 : Régulation de l'activité transcriptionnelle associée au complexe β-caténine/TCF. Le complexe β-caténine/TCF s'associe à différents co-activateurs transcriptionnels afin d'optimiser la transcription des gènes cibles. BCL9/Pygopus s'associe du coté N-terminal et profite notamment de la phosphorylation du résidu Y142 de la β-caténine. Le domaine CTTA, quant à lui, associe plutôt des complexes modifiant la chromatine et augmente sa capacité d'association par la phosphorylation de Y654 et S675.

BCL9 : B-cell CLL/lymphoma 9 ; Brg1: brahma-related gene 1; CBP : CREB-binding protein ; COMPASS : Complex Proteins Associated with Set1; CTTA: C-terminale Transcriptional activators; HAT: histone Acetyl Transferase; MLL: Mixed Lineage Leukemia; P: phosphorylation; Parafibro.: parafibromine ; SWI-SNF: SWItch/Sucrose Non Fermentable; TCF: T-Cell Factor; TRRAP : Transformation/transcription domain-Associated protein.

Plus rarement, la β-caténine est capable de lier d'autres facteurs de transcription comme le récepteur nucléaire LRH1 (Liver Receptor Homologue 1), HIF1α (Hypoxia Induced Factor 1α) en condition hypoxique ou encore, en réponse à un stress oxydatif, le facteur de transcription FoxO (Forkhead box O) (Botrugno et al., 2004 ; Kaidi et al., 2007 ; Almeida et al., 2007). En fonction du contexte cellulaire, la β-caténine s'associe donc à un ensemble de facteurs pour donner une réponse cellulaire appropriée.

Le contrôle de l'activité transcriptionnelle du complexe β-caténine/TCF est sous le contrôle de nombreux co-facteurs trancriptionnels décrits ci-dessous.

b) Remodelage de la chromatine et transcription

La stimulation de l'activité transcriptionnelle nécessite des changements profonds de la structure de la chromatine (Parker et al., 2008 ; Mosimann et al., 2009). De nombreux co-activateurs transcriptionnels interagissant avec la β-caténine affectent la structure de la chromatine, c'est le cas des histones acétyltransférases CBP (CREB-Binding protein)/p300 et TRRAP (Transcription/transformation domain-associated protein)) (Hecht et al., 2000; Sierra et al., 2006), et des histones méthyltransférases du système COMPASS (COMplex Proteins ASsociated with Set1) (MLL (Mixed Lineage-Leukemia)) (Sierra et al., 2006). D'autres participent au réarrangement du nucléosome, comme Brg1 (Brahma-related gene 1) du complexe SWI-SNF (SWItch/Sucrose Non Fermentable*)* (Barker et al., 2001). Le complexe PAF1 (RNA Polymerase II Associated Factor1) (Mosimann et al., 2006) ou le complexe Mediator (Kim et al., 2006b) permettent quant à eux d'associer le complexe TCF/β-caténine à l'ARN polymérase II en vue d'activer la transcription (Figure 8).

Ces différents intéractants de la β-caténine adressent les complexes nécessaires à la transcription au niveau des éléments de réponse Wnt/β-caténine (WREs).

Ces acteurs du remodelage de la chromatine s'associent à l'extrémité C-terminale de transactivation transcriptionnelle (CTTA) de la β-caténine, domaine indispensable à l'expression des gènes cibles de la voie Wnt/β-caténine (Cox et al., 1999).

Néanmoins, ces différents complexes ne peuvent se fixer à la β-caténine simultanément ce qui fait de ce domaine C-terminal une plateforme d'interaction et d'échange dynamique des co-facteurs transcriptionnels.

Voici un modèle de co-activation transcriptionnelle de la β-caténine mixant les différentes données disponibles sur ce sujet (Mosimann et al., 2006). Dans un premier temps, il est nécessaire de décompacter la chromatine. Pour cela, le complexe β-caténine/TCF recrute des histones acétyltransférases (CBP, p300, TRRAP) et le complexe SWI-SNF (Brg-1), permettant le relâchement de la chromatine et l'accès au site d'initiation de la transcription. Simultanément ou séquentiellement, le recrutement du complexe Mediator constitue la phase de pré-initiation et la mise en place de l'ARN polymérase II sur le site de transcription. Enfin, l'interaction avec certains de leurs membres (parafibromine et MLL), le complexe β-caténine/TCF recrute les complexes Paf1 et COMPASS qui recrutent la machinerie de modification post-traductionnelle des histones (ubiquitinylation, méthylation) nécessaire à l'initiation de la transcription (Mosimann et al., 2009 ; Shilatifard, 2004). L'interaction avec TBP (TATA-Box protein) s'effectue également en C-terminal (Hecht et al., 1999 ; Van Veelen et al., 2011).

c) Régulation du complexe transcriptionnel

La transcription induite par la liaison de la β-caténine au TCF est modulée, en fonction des conditions cellulaires, par l'interaction avec de nombreux co-facteurs transcriptionnels cités précédemment, permettant ainsi le renforcement ou la dissociation du complexe β-caténine/TCF (Städeli et al., 2006).

(1) Activation

BCL9 est un co-activateur transcriptionnel, qui contrairement aux autres, est spécifique de la β-caténine et s'associe à son extrémité N-terminale (Sampietro et al., 2006). Chez *Drosophilla melanogaster*, le mutant *Legless (Lgs)*, orthologue de BCL9, ou pygopus, un partenaire de Lgs, montrent de nombreuses similarités avec les mutants *armadillos*, renforçant le rôle essentiel de ces protéines (Kramps et al., 2002 ; Thompson et al., 2002).

Chez les mammifères, le système est un peu différent : il existe deux formes de BCL9 (1 et 2) et de Pygopus (Pygo1 et 2) permettant une complémentation des différents composants. Néanmoins, comme chez la drosophile, le dysfonctionnement du complexe *Lgs/Pygo* (BCL9/Pygopus) entraîne des défauts importants de stimulation de la voie Wnt/β-caténine et la mort de l'animal, faisant du complexe BCL9/Pygopus un composant essentiel de la transcription associée à cette voie de signalisation (Valenta et al., 2011 ; Schwab et al., 2007). L'extrémité C-terminale de BCL9 interagit également avec d'autres co-activateurs de la β-caténine cités précédemment (CBP/p300, TRRAP) ce qui renforce l'efficacité du complexe de transcription (Sustmann et al., 2008).

Les modifications post-traductionnelles de la β-caténine modifient également sa capacité à stimuler la transcription. En effet, la phosphorylation de la β-caténine joue un rôle très important dans son interaction avec les co-activateurs. La phosphorylation de T654, favorisant celle de S675, permet de maintenir une structure ouverte de l'hélice C et du domaine CTTA, et de lier les différents co-facteurs (Xing et al., 2008 ; Taurin et al., 2006).

A ce stade, une autre modification de la β-caténine intervient, l'acétylation. Elle est le résultat de la proximité des acétyltransférases CBP/p300 vis-à-vis de la β-caténine (cf partie II.C.3). Ainsi, CBP acétyle la β-caténine sur la lysine 49, site d'ubiquitinylation, en vue d'augmenter la transcription spécifique de c-myc (Wolf et al., 2002) tandis que p300 acétyle la lysine 345 favorisant l'interaction avec le TCF/Lef (Levy et al., 2004).

(2) Inhibition

Afin d'éviter une stimulation trop élevée ou prolongée de la voie Wnt/β-caténine, il est nécessaire de contrôler et de limiter l'activation de la transcription des gènes cibles. Pour cela, différentes possibilités s'offrent à la cellule.

L'interaction du complexe β-caténine/TCF avec des co-répresseurs transcriptionnels tels que la Reptine, Fhit, SMRT (Silencing Mediator for Retinoid and Thyroid hormone receptor) ou encore NCoR (Nuclear receptor CoRepressor) diminue l'activité transcriptionnelle, notamment en recrutant des HDACs qui compactent la chromatine (Bauer et al., 2000 ; Weiske et al., 2007 ; Song et Gelman 2008) (Figure 9).

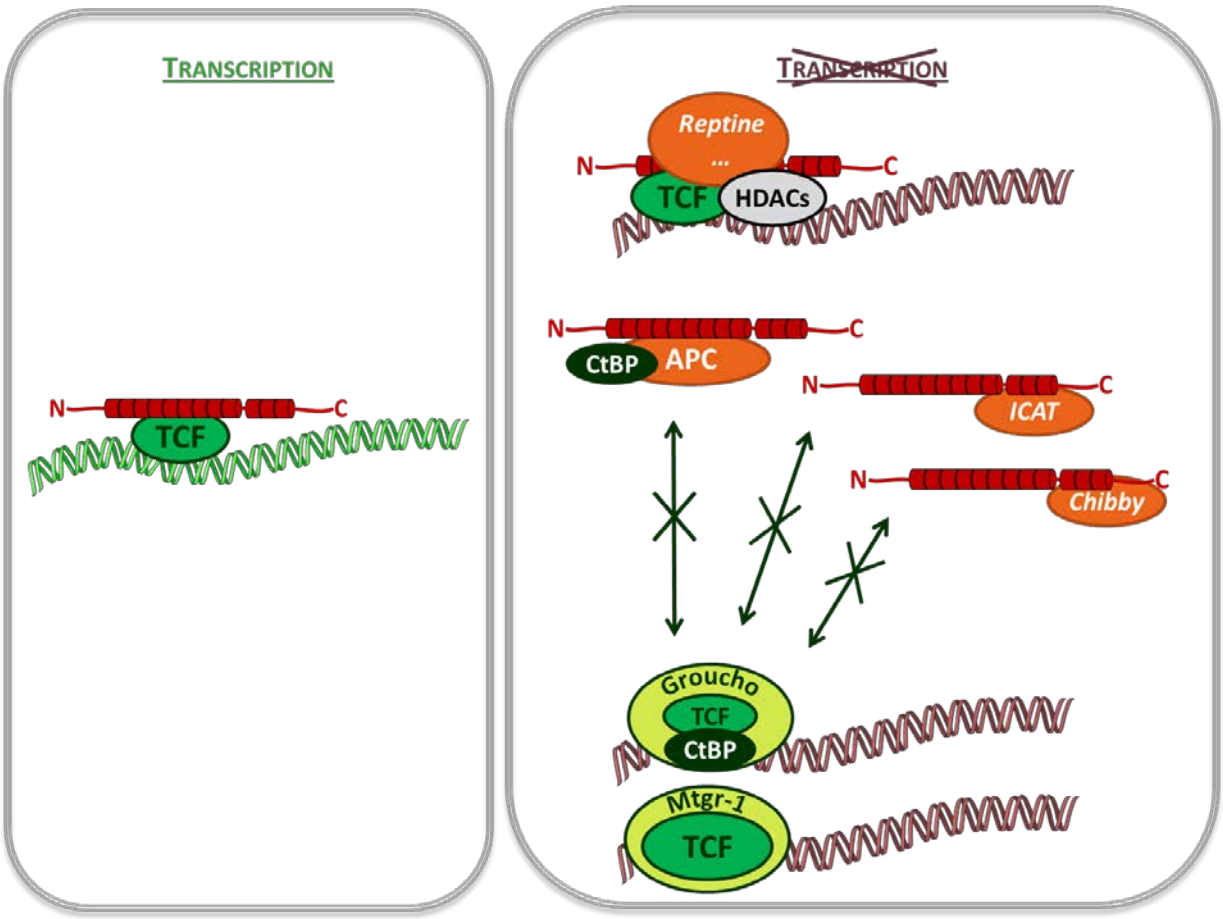


Figure 9 : Répression de la transcription associée à la β-caténine. L'inhibition de la transcription associée au complexe β-caténine/TCF apparait soit après fixation de répresseur transcriptionel du complexe comme la reptine, soit par dissociation du complexe. La compétition avec d'autres interactants de la β-caténine ou du TCF permet également l'arrêt de la transcription.

APC: Adenomatous Polyposis Coli; CtBP: C-terminal Binding Protein; HDACs: Histone Désacetylases; ICAT: Inhibitor of β-catenin and TCF4; Mtgr-1: Myeloid translocation gene related 1; TC : T-Cell Factor .

Il existe également des répresseurs transcriptionnels de ce complexe qui bloquent l'interaction entre le TCF, les co-activateurs et la β-caténine. C'est le cas de ICAT (Inhibitor of β-Catenin And TCF4) (Graham et al., 2002) ou Chibby (Takemaru et al., 2003). Ces deux protéines interagissent avec la β-caténine au niveau du domaine CTTA et entrent en compétition avec les co-activateurs transcriptionnels.

D'autre part, la réassociation du TCF avec Groucho/TLE ou encore Mtgr1 (Myeloid translocation gene related 1) lève l'interaction avec la β-caténine et limite la transcription associée à ce complexe (Cavallo et al., 1998 ; Moore et al., 2008). De plus, Groucho/TLE est capable de recruter des HDACs qui accentuent l'inhibition de la transcription (Sekiya et Zaret, 2007).

Enfin, les membres du complexe de destruction participent également à cette régulation transcriptionnelle de la β-caténine. L'APC nucléaire permettrait une séquestration de la β-caténine et en collaboration avec CtBP, promeut son export nucléaire (Hamada et Bienz, 2004). Un rôle similaire a également été décrit pour l'axine (Cong et Varmus, 2004).

3. Les gènes cibles du complexe β-caténine/ TCF

Le complexe β-caténine/TCF/LEF se fixe à l'ADN aux niveaux de régions WREs (Wnt Response Elements) et possède une liste importante de cibles transcriptionnelles dont l'expression dépend du contexte cellulaire et tissulaire (Vlad et al., 2008) (Tableau 2).

La cycline D1 est un des gènes cibles de la β-caténine les plus décrits dans la littérature du fait de son implication majeure dans le cycle cellulaire (Tetsu et McCormick 1999; Shutman et al., 1999). En effet, cette cycline participe à l'entrée des cellules en phase G1 et assure la progression dans les phases G1 et S (réplication de l'ADN) du cycle cellulaire suite à des stimuli mitogènes (Lukas et al., 1994). La cycline D1, possédant comme toutes les cyclines une demi-vie courte, est néo-synthétisée au cours de l'entrée dans le cycle cellulaire sous le contrôle, entre autres, de la β-caténine (Koepp et al., 1999 ; Blagosklonny et Pardee, 2002).

<u>Tableau 2 :</u> Les gènes cibles du complexe β-caténine/TCF.

AF17: ALL1-fused gene from chromosome 17 ; BMP4: Bone Morphogenetic Protein 4 ; CD44: Cluster de Différenciation 44; COX2: Cyclooxygenase 2; FGF: Fibroblast Growth Factor; FRA1: Fos-related antigen 1; ID2: Inhibitor of DNA-bindin 2; ITF2: Immunoglobulin transcription factor 2; LBH: Limb Bud and Heart; MDR1: Multidrug Resistance 1; MMP7: Matrix metalloproteinase 7; Msl1: male-specific lethal 1; MYCBP : c-myc binding protein; NOS2: Nitrix Oxide Synthase 2; Nr-CAM: Neuronal cell adhesion molecule; PML: Promyelocytic leukemia protein; PPARδ: Peroxisome proliferator-activated receptor δ; uPAR: urokinase-type Plasminogen Activator Receptor; VEGF: Vascular endothelial growth factor; WISP1: WNT1 inducible signaling pathway.

CATÉGORIES	GÈNES CIBLES	COMPLEXE DE TRANSCRIPTION β-CATÉNINE/...	*RÉFÉRENCES*
Prolifération cellulaire	c-myc	TCF4	He et al., 1998
	Cycline D	LEF1	Tetsu et McCormick 1999; Shtutman et al., 1999
	Gastrin	TCF4	Koh et al., 2000
	MYCBP	LEF1	Jung et Kim, 2005
Inhibition de l'apoptose	MDR1	TCF4	Yamada et al., 2000
	COX2	Lef1	Mei et al., 1999; Howe et al., 2001
	PPARδ	TCF4	He et al., 1999
	survivine	TCF4	Zhang et al., 2001
Progression tumorale	MMP7	TCF4	Brabletz et al., 1999
	uPAR	TCF/Lef??	Mann et al., 1999
	CD44	TCF4	Wielenga et al., 1999
	Laminine γ2	TCF4	Hlubeck et al., 2001
	Nr-CAM	Lef1	Conacci-Sorrell et al., 2002
	Vimentin	TCF4	Gilles et al., 2003
	LBH	TCF/Lef??	Rieger et al., 2010
	L1	TCF/Lef??	Gavert et al., 2005
Facteurs de croissance	c-met	TCF4	Boon et al., 2002
	VEGF	TCF4	Zhang et al., 2001
	WISP1	TCF/Lef??	Xu et al., 2000
	BMP4	TCF/Lef??	Kim et al., 2002
	FGF9	TCF/Lef??	Hendrix et al., 2006
	FGF18	TCF4	Shimokawa et al., 2003
	FGF20	TCF/Lef??	Chamorro et al., 2005
Facteurs de transcription	c-jun	TCF/Lef??	Mann et al., 1999
	FRA1	TCF/Lef??	Mann et al., 1999
	ITF2	TCF4	Kolligs et al., 2002
	ID2	TCF/Lef??	Rockman et al., 2001
	AF17	TCF/Lef??	Lin et al., 2001
	PML	Lef1	Shtutman et al., 2002
	Sox9	TCF4	Blache et al., 2004
Voie Wnt/ β-caténine	Axine 2	TCF4	Jho et al., 2002
	TCF1	TCF4	Roose et al., 1999
	LEF1	TCF/Lef??	Filali et al., 2002
	β-TrCP	TCF4	Spiegelman et al., 2000
	Dickkopf1	TCF4	Gonzalez-Sancho et al., 2005
Divers	Ephrine B	TCF/Lef??	*Batlle et al., 2002*
	Claudine1	TCF4	*Miwa et al., 2002*
	Endotheline 1	TCF4	*Kim et al., 2005*
	Jagged	TCF/Lef??	*Rodilla et al., 2009*
	Msl1	TCF/Lef??	*Spears et al., 2011*
	NOS 2	TCF4	*Du et al., 2006*
	Télomérase	TCF/Lef??	*Hoffmeyer et al., 2012*

Un autre gène cible de la β-caténine code l'oncogène c-myc, facteur important du cycle cellulaire : (He et al., 1998). Ce facteur de trancription permet notamment la répression transcriptionnelle de p21 (Mitchell et El-Deiry, 1999). Ce dernier fait partie de la famille des CKIs (Cyclin-dependent Kinase Inhibitors) et inhibe notamment le complexe associé à la cycline E (Cycline E/Cdk2) nécessaire à l'entrée en phase S en réduisant la synthèse de p21 mais également celle d'autres CKIs. C-myc participe de ce fait à la progression du cycle cellulaire.
En ciblant la transcription de ces deux gènes, la β-caténine participe activement à la prolifération cellulaire. En complément, on note également que le complexe β-caténine/TCF stimule l'expression de facteurs de croissance (VEGF, FGF…) et celle de facteurs d'invasion tumorale (MMPs, CD44…). Ces différentes cibles entreront notamment en jeu dans l'aspect pathologique de la β-caténine décrit plus tard (cf partie III).

Dans quelques rares cas, le complexe β-caténine/TCF réprime la transcription de gènes comme celui codant la E-cadhérine, notamment dans les kératinocytes, amplifiant ainsi la perte des jonctions d'adhérence et la libération de la β-caténine (Jamora et al., 2003).
De même, dans le foie, la β-caténine participe à la répression de différents gènes, l'impliquant dans la zonation hépatique (Benhamouche et al., 2006). Chez la drosophile, cette action répressive serait due à des éléments WREs différents de ceux utilisés pour la transcription (Blauwkamp et al., 2008).

H. Stimulation physio-pathologique de l'activité transcriptionnelle de la β-caténine

La cellule possède différents stratgèmes permettant d'augmenter la fraction cytosolique de β-caténine, disponible pour son activité transcriptionnelle. Parmi ceux-ci, on distingue la stabilisation physiologique liée à la stimulation des voies de signalisation ou à la libération de β-caténine des jonctions d'adhérence. Les nombreuses mutations décrites dans la littérature contribuent quant à elles à accumuler de manière incontrolée la β-caténine.

1. Aspects physiologiques

a) Les autres voies de signalisation

De nombreuses voies de signalisation sont capables d'affecter l'activation de la voie Wnt/β-caténine, le plus souvent en agissant directement sur la β-caténine elle-même. Par exemple, Met(HGFR), FGFR, EGFR sont capables d'induire la phosphorylation de la β-caténine sur les résidus Y142/Y654 afin de la libérer des jonctions d'adhérence augmentant ainsi le pool de β-caténine disponible pour l'activité transcriptionnelle (Brembeck et al., 2004 ; Krejci et al., 2012 ; Zeng et al., 2006). Certains d'entre eux (FGFR et EGFR) sont également capables de phosphoryler LRP5/6 et la β-caténine afin de stimuler la voie Wnt/β-caténine (Krejci et al., 2012). D'autres effecteurs sous-jacents de ces voies comme src ou Abl peuvent également avoir le même effet et favoriser la transcription associée à la β-caténine (Yang et al., 2011 ; Rhee et al., 2007 ; Roura et al., 1999 ; Van Veelen et al., 2011) (cf partie II.C.3).

Indirectement, la stimulation de nombreux récepteurs (facteurs de croissance, insuline…) activent la voie PI3K/Akt.

La cible principale de cette voie de signalisation, la protéine Kinase B (Akt/PKB), phosphoryle GSK3β sur la sérine 9 et inhibe son activité (Cross est al., 1995 ; Stambolic et al., 1994). Ce mécanisme diminue fortement la dégradation de la β-caténine. Akt est également capable d'activer l'activité transcriptionnelle de la β-caténine en phosphorylant la sérine 552 (Fang et al., 2007).

La protéine kinase A (voie cAMP-dépendante) possède la même propriété mais en ciblant la sérine 675 de la β-caténine et en favorisant son interaction avec l'histone acétyltanférase CBP (Van Veelen et al., 2011).

La stimulation des cellules par l'acide lysophosphatidique (ALP) cible p120 et dissocie le complexe des jonctions d'adhérence. Ceci entraîne une internalisation du complexe E-cadhérine/β-caténine et sa relocalisation au niveau des endosomes périnucléaires (Kam et Quaranta, 2009). A ce niveau la β-caténine est libérée du complexe et est transloquée au noyau. Ce mécanisme atypique est un nouveau mode de régulation de la voie Wnt/β-caténine.

D'autre part, un autre mécanisme d'activation de la β-caténine a également été décrit dans un contexte bien particulier d'inflammation. Au cours d'une inflammation anormale, les tissus affectés montrent une augmentation de l'expression de COX2 (cyclooxygénase 2) responsable de la synthèse des protaglandines (Vane et Botting, 1998). Particulièrement, la stimulation du récepteur par la prostaglandine PGE2 entraine la relocalisation de l'axine à la membrane plasmique par son interaction avec la sous-unité α de la protéine G couplée au récepteur (Castellone et al., 2005). Cette relocalisation entrave le bon fonctionnement du complexe de destruction et permet de stabiliser la β-caténine. Ce mécanisme entraîne également une boucle d'auto-amplification puisque COX2 est également une cible transcriptionnelle de la β-caténine (Mei et al., 1999; Howe et al., 2001).

b) La rupture des jonctions d'adhérence

La perte de E-cadhérine entraîne un relargage de la β-caténine dans le cytosol, ce qui se traduit par une stimulation de la transcription si cette dernière n'est pas dégradée par le protéasome (Zeisberg et Neilson, 2009 ; Heuberger et Birchmeier, 2010). De nombreuses protéases sont capables de cliver le domaine intracellulaire des cadhérines provoquant une diminution de l'adhésion cellulaire par rupture des jonctions d'adhérence.

Par exemple, ADAM10 (A Disintegrin And Metalloprotease 10) clive la N- ou la E-cadhérine et favorise la translocation nucléaire de la β-caténine et son activité transcriptionnelle (Reiss et al., 2005 ; Maretzky et al., 2005). De plus, ADAM10 a également été décrite comme une cible transcriptionnelle de la voie Wnt/β-caténine dans des cellules cancéreuses coliques, accentuant d'autant plus la perte des jonctions d'adhérence (Gavert et al., 2007). D'autre part, le fragment clivé de N-cadhérine joue un rôle puisqu'il est transloqué au noyau pour y stimuler la transcription du complexe β-caténine/TCF (Shoval et al., 2007).

De même, les différentes modifications post-traductionnelles décrites précédemment sont également capables de réguler la stabilité des jonctions d'adhérence et de libérer la β-caténine dans le cytosol.

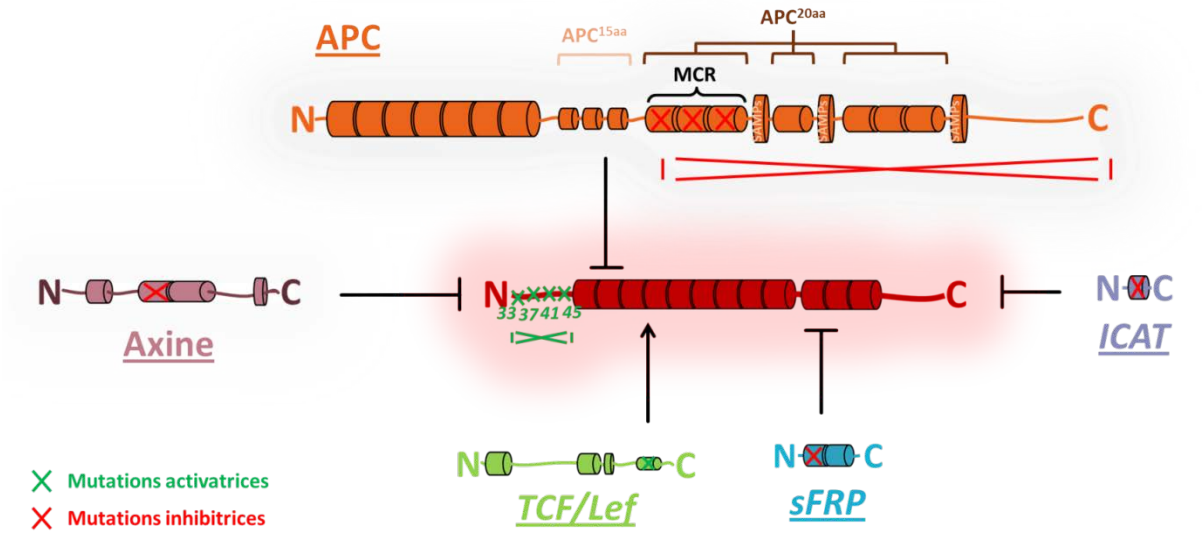


Figure 10 : L'activité transcriptionnelle de la β-caténine associée aux altérations génétiques. Les différents interactants de la β-caténine portent des mutations activatrices ou inhibitrices dans le but de stabiliser la β-caténine entrainant ainsi une augmentation de son activité oncogénique.

APC: Adenomatous Polyposis Coli; ICAT: Inhibitor of β-caténine and TCF4; MCR: Mutation Cluster Region; sFRP: secreted Frizzled Related Protein; TCF/Lef: T-Cell Factor/Lymphoid Enhancer-binding Factor.

2. Aspects pathologiques : dérégulations du complexe de destruction

La voie Wnt/β-caténine fait également l'objet de dérégulations entraînant une expression incontrôlée et prolongée des gènes cibles du complexe β-caténine/TCF. De nombreux effecteurs de cette voie sont mutés dans différents types de cancers. Le plus important est APC, un des acteurs principaux de l'initiation du cancer colorectal.

Les mutations d'APC sont décrites à la fois dans les cancers colorectaux héréditaires rassemblés sous la dénomination de FAP (Familial Adenomatous Polyposis) et dans les cancers colorectaux sporadiques (60% des mutations d'APC) (Nishisho et al., 1991 ; Powell et al., 1992). Dans le premier cas, une mutation monoallélique germinale d'APC est observée et entraîne l'apparition d'un codon stop prématuré et d'une protéine tronquée. Celle-ci perd une partie de ses répétitions 20aa et de ses domaines SAMPs (Sérine Alanine Méthionine Proline) (Figure 10) nécessaires aux interactions respectives avec la β-caténine et l'axine. Dans le deuxième cas, les mutations somatiques sont regroupées dans une zone appelée MCR (Mutation Cluster Region) au niveau de l'exon 15 entre les codons 1280 et 1500 (Miyoshi et al., 1992). Dans tous les cas, il est nécessaire d'inactiver les deux allèles de ce suppresseur de tumeur afin d'entraîner la stabilisation constitutive de la β-caténine (hypothèse de Knudson). Un des deux allèles mutés (de façon germinale ou somatique) entraîne un phénomène d'hétérozygotie. La mutation du second allèle entraîne la perte de l'hétérozygotie (LOH-Loss of heterozygosity) (Lamlum et al., 1999 ; Rowan et al., 2000) et l'incapacité totale de fixer l'axine et la β-caténine (Rubinfeld et al., 1997). Dans ce cas, la β-caténine est constitutivement stabilisée et stimule la transcription de ses gènes cibles.

Les mutations d'APC affectent les tissus coliques et rectaux, mais on observe également ces mutations dans les cancers sporadiques des poumons, des ovaires et du sein (Gershoni-Baruch et al., 2000 ; Furuuchi et al., 2000 ; Ohgaki et al., 2004).

On note également que des altérations épigénétiques, telles que la méthylation de promoteurs, modulent l'expression du gène *Apc* et affectent également la voie Wnt/β-caténine (Esteller et al., 2000).

La voie Wnt subit également des mutations inactivatrices de l'axine (Shimizu et al., 2002), d'ICAT (Reifenberger et al., 2002) ou encore des sFRPs (secreted Frizzled-Related Proteins), protéines capables de bloquer l'activation de Frizzled (Caldwell et al., 2004) (Figure 10).
D'autres composants subissent quant à eux des mutations activatrices comme la β-caténine en elle-même (Lüchtenborg et al., 2005 ; Thorstensen et al., 2005), TCF4 (Duval et al., 1999) ou encore PP2A (Arroyo et Hahn , 2005). Pour la β-caténine, la partie N-terminale est particulièrement affectée, soit par délétion de l'exon 3 ou par des mutations ponctuelles dans ce même exon au niveau des codons 33, 37, 41 ou 45 (Sparks et al., 1998, de La Coste et al., 1998). Ces modifications génétiques entraînent la perte du processus de dégradation de la β-caténine et se traduit par sa stabilisation constitutive (Morin et al., 1997). Contrairement aux suppresseurs de tumeurs, les oncogènes, comme la β-caténine ou le TCF, ne nécessitent qu'une mutation monoallélique pour entraîner le dérèglement cellulaire (Figure 10).

I. Un rôle méconnu : la stabilisation des centrosomes

La β-caténine phosphorylée par GSK3β n'est pas systématiquement dégradée. Une faible fraction est détectée par le centrosome, centre organisateur des microtubules, à partir duquel s'effectue la nucléation des microtubules. La β-caténine, au sein de ce complexe, contribue à la cohésion du centrosome et aux fonctions qui en découlent (ségrégation mitotique, polarité cellulaire…) (Huang et al., 2007 ; Chilov et al., 2011). Dans ce sens, plusieurs études ont démontré l'importance de la voie de signalisation associée à la β-caténine dans la régulation du centrosome. APC lui-même interagit avec les microtubules et permet leur stabilisation (Zumbrunn et al., 2001). La protéine Dishevelled (Dsh) intervenant en amont de la β-caténine en réponse à la stimulation de la voie Wnt/β-caténine est également impliquée dans la régulation de la stabilité des microtubules (Krylova et al., 2000).
Enfin, l'axine intervient également dans la cohésion du centrosome (Hadjihannas et al., 2010). Ces différentes études suggèrent un nouveau rôle pour la β-caténine associée à ce complexe.

III. Rôle de la β-caténine: entre physiologie et pathologie ?

A l'échelle cellulaire, la β-caténine est capable d'activer la transcription de gènes cibles. Mais, à l'échelle de l'organisme tout entier, dans quels processus physio-pathologiques s'inscrit-elle ?

A. Développement : entre prolifération et transition épithélio-mésenchymateuse

Les premiers travaux portant sur la voie Wnt ont été réalisés chez la drosophile. Des mutations de cette voie de signalisation engendrent des problèmes de développement, particulièrement au niveau des ailes, d'où le nom donné au mutant, « Wingless » (Sharma, 1973).

La β-caténine participe au développement embryonnaire. Par le biais de son activité transcriptionnelle, elle stimule la prolifération de certaines zones embryonnaires, d'une part, et permet à la cellule de migrer en amorçant la transition épithélio-mésenchymateuse, d'autre part.

Ainsi, on observe un marquage des facteurs Wnt avant la gastrulation de l'embryon dans l'endoderme viscéral ainsi que dans l'épiblaste permettant la formation du sillon primitif. On observe également un fort marquage de la β-caténine dans ce sillon primitif (Mohamed et al., 2004). La formation d'un gradient antéro-postérieur de facteurs Wnt, du à l'expression des antagonistes sFRPs, permet la mise en place et la croissance de l'endoderme viscéral (Kemp et al., 2005). On note plus particulièrement l'importance de Wnt3, essentiel à la formation du sillon primitif (Liu et al., 1999b).

Au cours du développement embryonnaire, la voie Wnt/β-caténine contrôle la formation de l'axe antéro-postérieur et la distribution neurale (Takahashi et Liu, 2006). Elle est notamment impliquée dans la prolifération des précurseurs neuronaux (Lie et al., 2005).

La β-caténine intervient également de manière précoce dans le développement hépatique chez la souris (Micsenyi et al., 2004). Elle affecte la croissance des hépatoblastes et participe, à un stade plus tardif, au développement, à la différenciation de ceux-ci en hépatocytes et en cholangiocytes (Decaens et al., 2008 ; Tan et al., 2006, Tan et al., 2008).

L'activité transcriptionnelle de la β-caténine permet également la transition épithélium-mésenchyme nécessaire à la migration cellulaire pendant le développement embryonnaire, particulièrement au cours de la gastrulation. Certaines cibles transcriptionnelles de la β-caténine sont des effecteurs de cette transition comme les répresseurs transcriptionnels de la E-cadhérine, Twist (Howe et al., 2003), Slug (Snail2) (Barallo-Cimeno et al., 2003) ou ZEB1 (Sanchez-Tillo et al., 2011).

D'autre part, une stimulation indirecte de Snail1, un autre répresseur transcriptionnel de la E-cadhérine, *via* GSK3β est également possible par la voie Wnt (Zhou et al., 2004 ; Yook et al., 2006). Celui-ci est par ailleurs capable d'interagir avec la β-caténine afin de stimuler son activité transcriptionnelle (Stemmer et al., 2008).

B. Renouvellement des tissus chez l'adulte

La voie Wnt/β-caténine joue un rôle très important dans le renouvellement des tissus chez l'adulte.

Elle participe au développement du système pileux en agissant sur le bourgeon de cellules souches épidermales, fournissant les cellules nécessaires à la pousse du poil (Van Genderen et al., 1994). La voie Wnt interviendrait non seulement dans le maintien du pool de cellules souche mais également dans la différenciation des différentes lignées du follicule pileux (Lowry et al., 2005).

Dans un contexte différent, il a été démontré que les facteurs Wnt étaient sécrétés par les cellules hématopoïétiques ainsi que par l'environnement de la moelle épinière, agissant ainsi sur la prolifération des cellules souches hématopoïétiques (Reya et al., 2003).

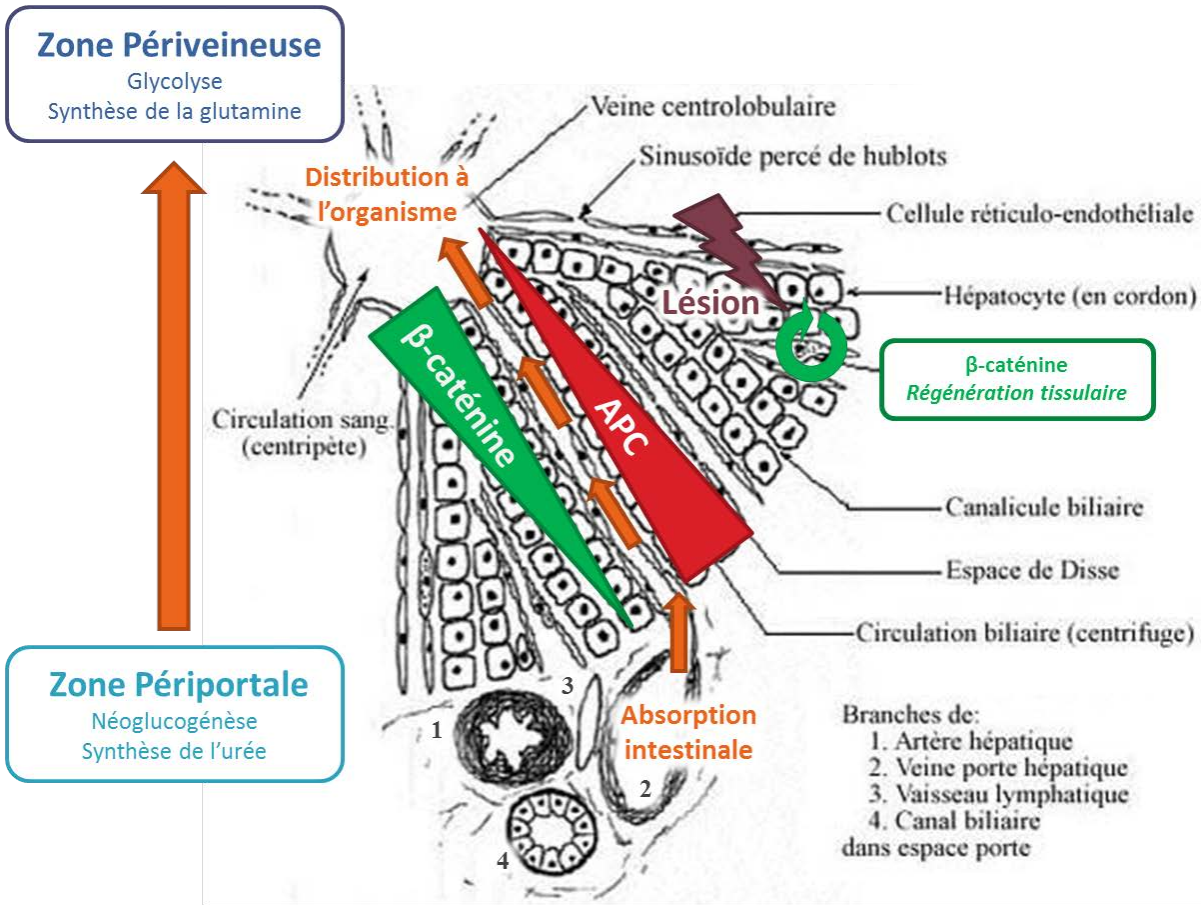


Figure 11 : Rôles physiologique de la voie Wnt/β-caténine dans le foie. La β-caténine participe à la zonation hépatique, phénomène par lequel le foie se retrouve divisé en zones fonctionnelles. Le gradient de β-caténine, inversement proportionnel à celui d'APC permet l'activation et la répression de certaines fonctions associées aux différentes zones. Enfin, l'apparition de lésions entraine une sur-activation de la voie Wnt/β-caténine dans le but de régénérer le tissu lésé.

Néanmoins, la délétion de la β-caténine n'affecte pas le système hématopoïèse/lymphopoïèse chez la souris, suggérant une compensation à ce niveau par l'homologue de la β-caténine, la plakoglobine (Cobas et al., 2004).

Au vu de l'importance de la β-caténine dans ces tissus, certaines dérégulations de ces fonctions ont également été reportées dans certaines leucémies ou certaines tumeurs des follicules pileux (Jamieson et al., 2004 ; Lo Celso et al., 2004).

D'autre part, la β-caténine semble participer à la production de la matrice osseuse. En effet, l'inactivation du co-récepteur LRP5 entraîne des défauts importants de densité osseuse impliquant un défaut de prolifération et de maturation des ostéoblastes (Kato et al., 2002).

C. Physiologie hépatique et carcinome hépatocellulaire

1. Rôles de la β-caténine

La β-caténine participe à la croissance, à la mise en place et au bon fonctionnement du tissu hépatique. Pendant la croissance de l'enfant, la β-caténine participe, comme au cours du développement embryonnaire, à la prolifération des hépatocytes (Apte et al., 2007).

Par la suite, la voie Wnt/β-caténine est un composant essentiel dans la zonation hépatique, évènement permettant notamment le bon fonctionnement métabolique du foie (Benhamouche et al., 2006) (Figure 11). Cette zonation permet l'apparition d'un gradient d'expression génique du centre du lobule hépatique dans les hépatocytes périveineux vers l'extérieur du lobule dans les hépatocytes périportaux. On observe une expression différentielle de la β-caténine et d'APC, permettant l'expression progressive des deux programmes géniques des deux types d'hépatocytes. La β-caténine permet notamment l'expression ou la répression de certains gènes spécifiques aux deux types d'hépatocytes. Par exemple, elle favorise la synthèse de la glutamine tandis qu'elle réprime la synthèse de l'urée observée dans les hépatocytes périportaux (Cadoret et al., 2002).

De même, la β-caténine régule le métabolisme du glucose en augmentant l'expression de certaines enzymes du cycle de Krebs comme l'Isocitrate déshydrogenase (Idh3a) ou la dihydrolipoamide acétyltransférase (Dlat), favorisant ainsi la glycolyse, tandis qu'elle diminue l'expression des enzymes de la néoglucogénèse (Phosphoénolpyruvate carboxykinase (PEPCK) ou fructose-1,6-bisphosphatase (FBPase) (Chafey et al., 2009 ; Benhamouche et al., 2006).

D'autre part, la voie Wnt/β-caténine participe à la régénération des tissus hépatiques (Tan et al., 2006, Sekine et al., 2007). En effet, le foie possède l'aptitude de se renouveler entièrement et rapidement à partir de cellules quiescentes en faisant intervenir l'activité transcriptionnelle de la β-caténine alors que les autres organes nécessitent des cellules souches ou progénitrices pour se régénérer (Fausto et al., 2006).

2. Carcinome hépatocellulaire

Le carcinome hépatocellulaire est le 8ème cancer le plus répandu dans le monde avec 500.000 nouveaux cas rescencés par an (El-Serag, 2011). Il est généralement développé en réponse à une lésion hépatique à partir d'hépatocytes matures ou progéniteurs (Sell et Leffert, 2008) et est influencé par de nombreux facteurs tels que la consommation d'alcool.

Ce cancer est également favorisé par certaines pathologies métaboliques telles que le diabète de type 2 (Davila et al., 2005).

Il existe deux grands types de carcinomes hépatocellulaires : (1) les carcinomes associés à une forte instabilité chromosomique et portant des mutations des suppresseurs de tumeur p53 ou axine ; (2) les carcinomes associés à une faible instabilité chromosomique possédant des mutations de la β-caténine (Laurent-Puig et al., 2001). Les dérégulations de la β-caténine représentent entre 26 et 40 % des carcinomes hépatocellulaires (De la coste et al., 1998). Néanmoins, ces tumeurs présentent un aspect peu prolifératif ainsi qu'un maintien des marqueurs de polarisation (Cavard et al., 2008), il s'agit d'un cancer à bon pronostic.

Observées plus rarement, entre 5 et 10 %, des mutations de l'axine provoquent des cancers hépatiques à mauvais pronostic (Taniguchi et al., 2002) au même titre que les mutations d'APC observées dans de très rares cas de carcinomes héréditaires hépatocellulaires FAP (Familial Adenomatous Polyposis) (Su et al., 2001).

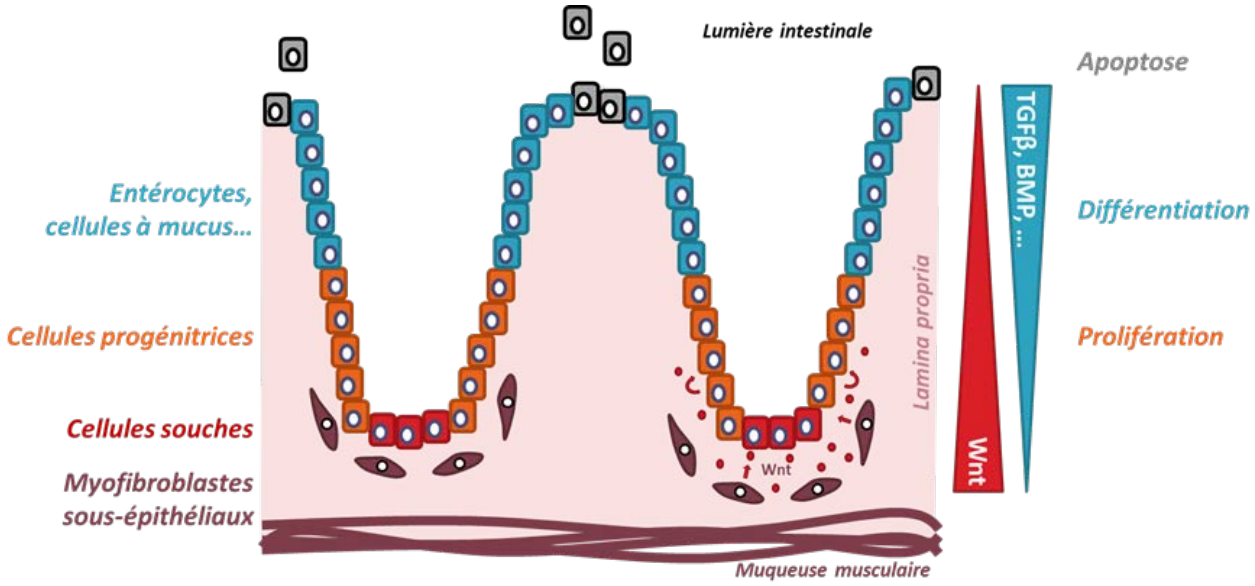


Figure 12 : Renouvellement de l'épithélium intestinal. Les myofibroblastes sous-épithéliaux, mais également les cellules progénitrices elle-mêmes, produisent des facteurs Wnt, entraînant la stimulation de la voie de signalisation Wnt/β-caténine au fond des cryptes. La disparition progressive de cette stimulation entraîne la différentiation progressive des cellules en entérocytes ou en cellules à mucus. Enfin, le flux continu le long des cryptes se termine par l'apoptose des cellules et leur libération dans la lumière intestinale.

BMP: Bone Morphogenetic Protein; TGFβ : Transforming Growth Factor β.

Dans le foie, une des cibles de la β-caténine, la glutamine synthétase, est quasi-systématiquement surexprimée (90%) lorsqu'une mutation de la β-caténine est observée (Audard et al., 2007). Ce marqueur a notamment permis de montrer que les mutations de la β-caténine apparaissent préférentiellement dans les carcinomes hépatiques avancés, l'inscrivant plutôt dans la phase de croissance tumorale (Osada et al., 1999). De plus, il semble que la simple surexpression de la β-caténine ne soit pas suffisante pour induire la cancérisation, confortant son implication dans un stade plus tardif de la progression tumorale (Cadoret et al., 2001 ; Tan et al., 2005).

D. Renouvellement des épithéliums et développement des cancers colorectaux

1. Renouvellement de l'épithélium intestinal

La voie Wnt/β-caténine est particulièrement importante dans le renouvellement des cryptes intestinales. L'épithélium du tractus digestif est composé de cryptes (et de villosités pour l'intestin grêle) renouvelées en moyenne tous les 3 à 5 jours, créant un flux de cellules du bas des cryptes vers le haut. Le fond des cryptes possèdent un bourgeon de cellules souches intestinales au cycle cellulaire très lent, qui échappent à ce flux et fournissent ainsi les cellules progénitrices capables de proliférer très rapidement et de renouveler l'épithélium intestinal (Pinto et Clevers, 2005) (Figure 12). Elles participent également à la réparation de l'épithélium après l'apparition d'une lésion. La voie Wnt/β-caténine est activée physiologiquement dans ces lignées progénitrices par la sécrétion de facteurs Wnt principalement par les myofibroblastes intestinaux subépithéliaux à proximité des cellules progénitrices (Powell et al., 1999 ;Madison et al, 2005). De plus, l'inhibition du complexe β-caténine/TCF4 empêche la prolifération des cellules épithéliales fœtales et adultes, montrant que l'intégrité de ce complexe est indispensable au renouvellement de ces cellules (Korinek et al. , 1998). Les cellules progénitrices ont une prolifération qui diminue au fur et à mesure de leur progression vers le haut des cryptes.

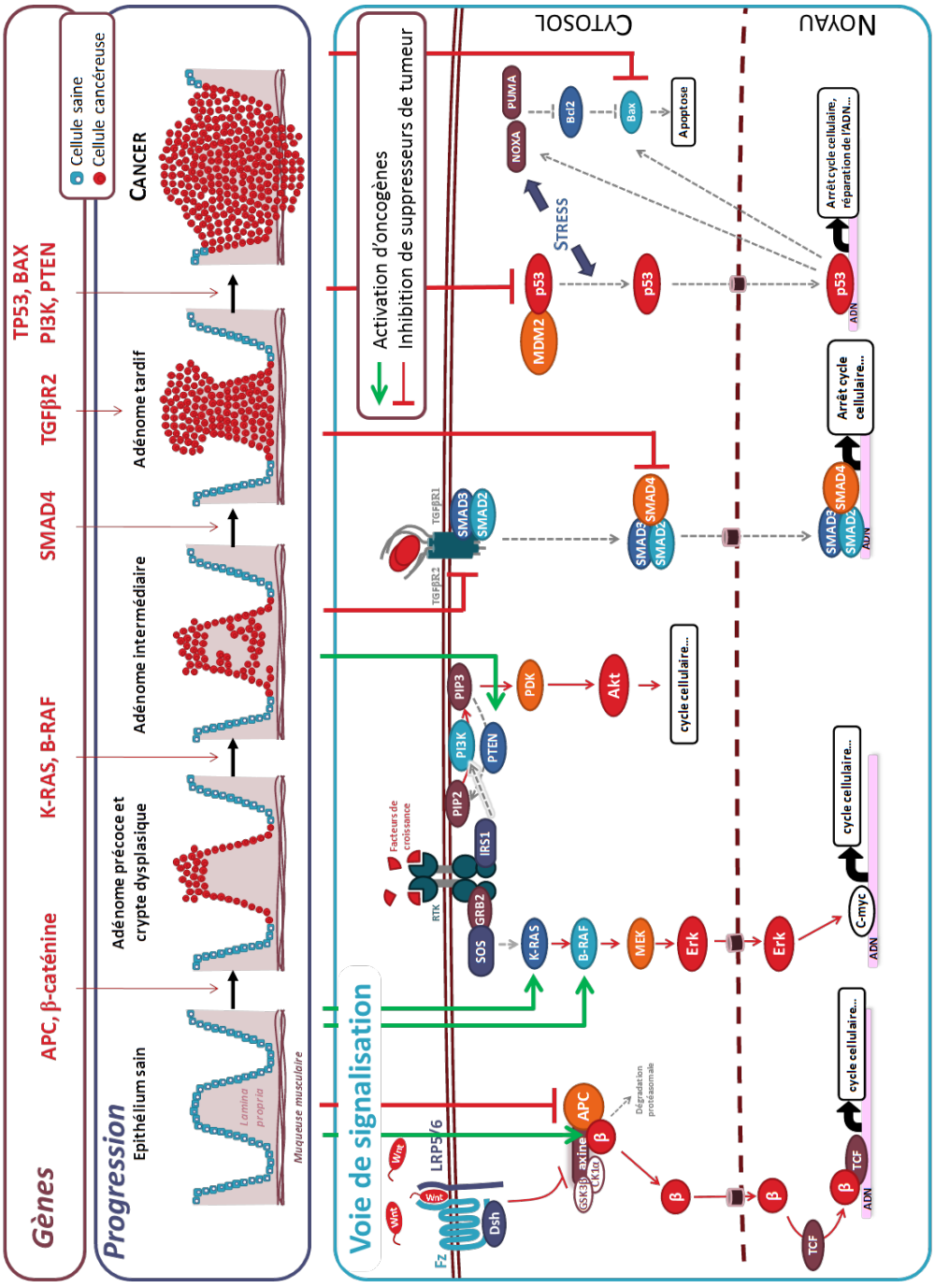


Figure 13 : Carcinogénèse colique. La séquence de Fearon et Vogelstein définie les mutations apparaissant au cours de la cancérogénèse colique et rectale. Les voies de signalisation associées aux différentes protéines mutées sont représentées en dessous.

Akt/PKB: Protein Kinase B ; APC : Adenomatous polyposis Coli; β: β -caténine ; Bcl-2–associated X protein ; CK1α: Caseine kinase 1α ; Dsh : Dishevelled ; GRB2 : Growth factor receptor-bound protein 2 ; GSK3β: Glycogène synthase kinase 3β ; IRS1 : Insulin receptor substrate 1 ; MDM2 : murine double minute 2 ; PDK1: Phosphoinositide-Dependent Kinase 1 ; PI3K : Phoshatidylinositol 3 kinase; PIP2 : PhosphatidylInositol (4,5) bisPhosphate ; PIP3 : PhosphatidylInositol (3, 4, 5) triPhosphate ; PTEN : Phosphatase and TENsin homolog ; SOS : Son of sevenless ; TCF : T-Cell Factor ; TGFβ : Transforming Growth Factor β.

Elles entament alors leur différenciation cellulaire en entérocytes ou cellules sécrétrices de mucus. En haut des cryptes, les différentes cellules entrent en apoptose et sont libérées dans la lumière intestinale. Il reste néanmoins à savoir comment la voie Wnt/β-caténine est inhibée au cours de leur progression le long des cryptes. Pour cela, plusieurs hypothèses ont été émises : l'une concernant la voie TGFβ, l'autre, la voie BMP (Bone Morphogenic Protein)/Smad. La voie du TGFβ possède une activité anti-oncogénique, permettant notamment *via* son effecteur TAK1 (Transforming growth factor β–activated kinase 1), l'inactivation du complexe β-caténine/TCF par sa dissociation de l'ADN (Ishitani et al., 1999). En bref, TAK1 activerait NLK (NEMO-like kinase) qui, en phosphorylant le TCF/LEF, inhiberait son interaction avec la β-caténine.

D'autre part, tout comme la voie du TGFβ, la voie BMP montre également un fort marquage en haut des cryptes (Haramis et al., 2004 ; Hardwick et al., 2004). Cette voie de signalisation permettrait l'inhibition de la voie PI3K, diminuant ainsi le signal β-caténine (He et al., 2004).

2. Carcinogénèse colique

Les cancers colorectaux (CCR) constituent la deuxième cause de décès par cancer chez l'homme après le cancer du poumon et la troisième cause de décès chez la femme après le cancer du sein et du poumon (Source : Dynamique d'évolution des taux de mortalité des principaux cancers en France). Ils sont majoritairement sporadiques (environ 75% des cas) mais il existe également des prédispositions génétiques entraînant des mutations constitutives. A cela, les altérations génétiques acquises au cours de l'existence renforcent l'émergence de ces cancers. Le développement des CCR suit classiquement une séquence de mutations décrit pour la première fois en 1990 par Fearon et Vogelstein (Fearon et Vogelstein, 1990) (Figure 13). Ces mutations interviennent dans différentes voies de signalisation selon l'ordre suivant : voie Wnt/β-caténine, voie associée aux récepteurs des facteurs de croissance, voie du TGFβ et enfin voie de réparation de l'ADN p53.

La voie Wnt/β-caténine constitue la phase d'initiation du cancer colorectal. Lorsque cette voie est mutée, les autres voies ne sont plus capables de freiner le signal β-caténine/TCF dans les cellules progénitrices entraînant leur progression le long des cryptes et leur prolifération incontrôlée. Les mutations des autres voies de signalisation accentuent le phénotype cancéreux, promeuvent l'invasion tumorale des tissus adjacents et enfin entraînent la phase métastatique.

Les mutations d'APC constituent la majeure partie des cancers colorectaux sporadiques (60%) (Powell et al., 1992) et héréditaires (Familial adenomatous polyposis) (Groden et al., 1991). On observe néanmoins des mutations de l'axine (Liu et al., 2000) ainsi que de la β-caténine (Morin et al., 1997). Les cellules cancéreuses possèdent donc le même programme de prolifération que les progénitrices : stimulation du cycle cellulaire (c-myc, cycline D1), migration cellulaire (MMPs, Nr-CAM), survie cellulaire (Survivine), croissance (FGF, Gastrine), angiogénèse (VEGF), etc… (Tableau 2). Ces cellules ne sont pas limitées dans leur croissance, entrainant une prolifération incontrôlées et la perte du gradient de différentiation des cryptes. On observe au cours du processus de cancérisation une dysplasie des cryptes qui progresse et envahit les tissus sous-jacents jusqu'à la dissémination des cellules cancéreuses dans la circulation sanguine. Une auto-stimulation par les facteurs Wnt est également observée à la base des cryptes, accentuant d'autant plus le phénotype.

3. Alimentation, diabètes et cancérisation colorectale

On sait depuis de nombreuses années que l'alimentation est un facteur aggravant de la cancérisation colique et rectale. Freudenheim et collaborateurs en 1990, puis Benito et collaborateurs en 1991, établissent un lien entre la prise alimentaire, et particulièrement le taux de glucides, et le risque de cancers colorectaux (Freudenheim et al., 1990 ; Benito et al., 1991).

De même, Bostick et collaborateurs en 1994, démontrent que la consommation de saccharose ainsi que l'obésité augmentent le risque de développer un cancer colorectal de près de 1,5 fois (Bostik et al., 1994). La prise alimentaire influencerait donc directement le développement du cancer (Gnagnarella et al., 2008 ; Key et Spencer, 2007 ; Michaud et al., 2005).

On suppose également un rôle indirect de l'alimentation sur le développement de certains cancers, ceci par la mise en place préalable de pathologies métaboliques majeures, comme le diabète de type 2 (Dong et al., 2011 ; Hauner et al., 2012). Le diabète, plus particulièrement, est un élément aggravant la cancérisation colique et rectale. En effet, le diabète de type 2 double la probabilité de développer ce type de cancer (Ahmed et al., 2006 ; Khaw et al., 2004 ; Pais et al., 2009). De plus, les patients atteints d'un cancer colorectal montrent une insulinémie de près deux fois supérieure à celle de patients sains (Yam et al., 1996).

Néanmoins, les mécanismes moléculaires sous-jacents reliant alimentation et/ou désordres métaboliques au cancer colorectal restent flous.

Une première explication proviendrait du phénomène de résistance à l'insuline. En effet, l'hyperinsulinémie influencerait le développement du cancer, d'une part, par l'augmentation d'IGF1 et d'autre part, par la stimulation des cellules anormalement exposées à l'insuline (cf publication n°1). Les quelques pistes proposées sont néanmoins insuffisantes pour expliquer l'impact de ces désordres métaboliques sur le cancer. En effet, le seul fait d'une résistance à l'insuline n'explique pas pourquoi le développement du cancer est ainsi influencé par un désordre métabolique tel que le diabète de type 2. La *O*-GlcNAcylation permettrait de relier alimentation et désordres métaboliques à la cancérisation. Les conséquences de désordres métaboliques impliqueraient, par les variations brutales de glycémie, des modifications subséquentes de *O*-GlcNAcylation qui affecteraient l'activité d'oncogènes et/ou de suppresseurs de tumeur.

Récemment, une étude globale de la *O*-GlcNAcylation dans le cancer du côlon a été réalisée (Mi et al., 2011). Les tissus cancéreux coliques montrent un marquage intense des protéines *O*-GlcNAcylées en comparaison aux tissus sains, soutenant l'implication de la modification post-traductionnelle dans les processus de cancérisation. La *O*-GlcNAcylation promeut, *in vitro*, l'agressivité et l'invasion tumorale. Néanmoins, aucune étude n'a permis de cibler la *O*-GlcNAcylation d'une protéine en particulier dans ce type de cancer (Mi et al., 2011).

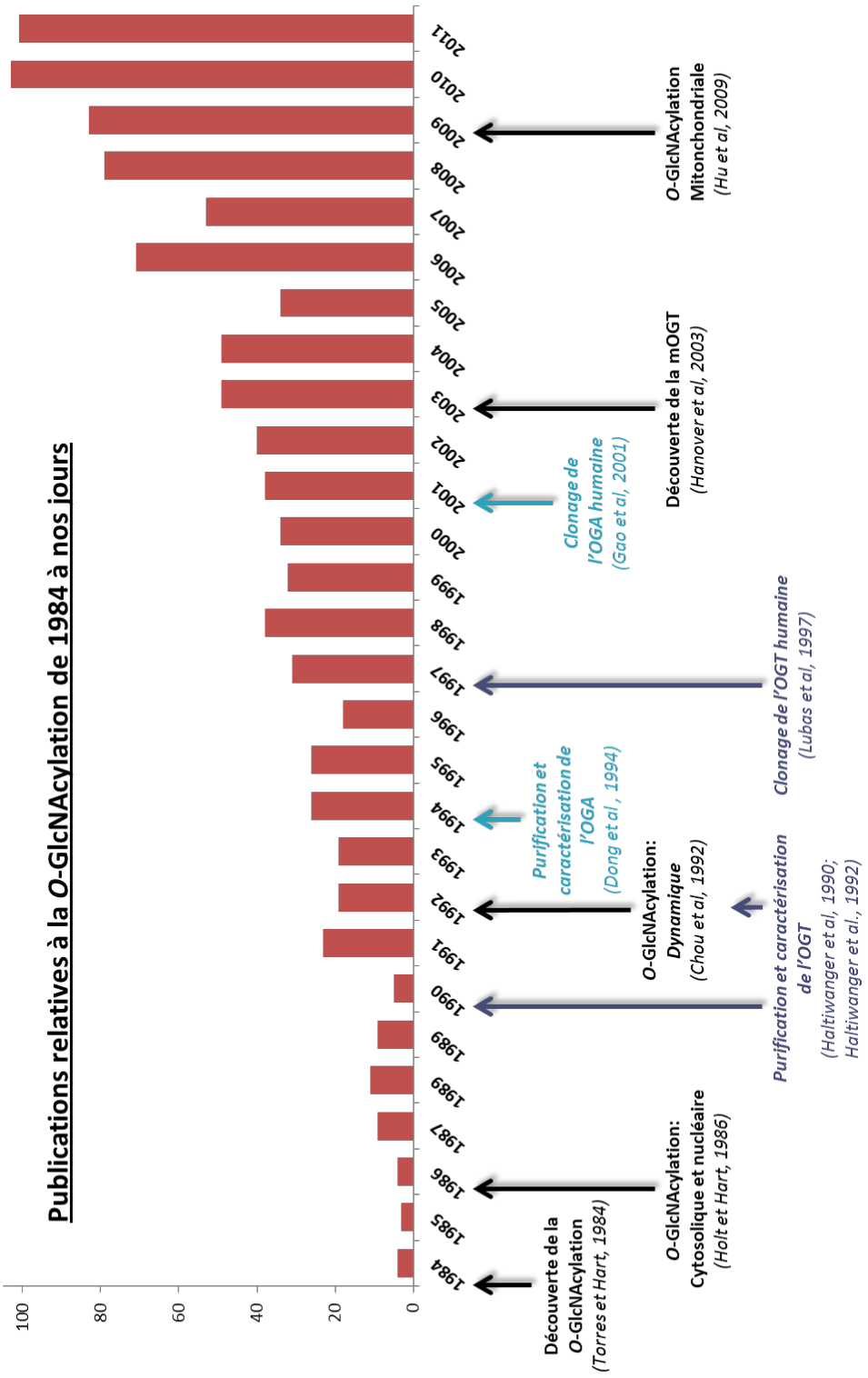


Figure 14 : Historique et publications relatifs à la *O*-GlcNAcylation. OGA : *O*-GlcNAcase ; (m)OGT: *O*-GlcNAc Transférase (mitochondriale). Source Scopus- Mots clés : "O-GlcNAcylation OR O-GlcNAc OR O-linked N-acetylglucosamine".

IV. La *O*-GlcNAcylation

Au cours de ces 30 dernières années, la O-GlcNAcylation a démontré toute son importance dans de nombreux processus cellulaires physiologiques et pathologiques. Depuis sa découverte en 1984, plusieurs équipes de recherche se sont appliquées à décrire et à caractériser l'implication de cette modification post-traductionnelle dans divers mécanismes cellulaires. Cette partie a pour but d'introduire la O-GlcNAcylation.

A. A la découverte de la *O*-GlcNAcylation

1. Petit historique…

Les modifications post-traductionnelles permettent à l'organisme d'affecter à une même protéine plusieurs fonctions biologiques, soit plus de 500 millions d'activités différentes pour seulement 26.500 protéines. Ce processus de modification, réalisé par des enzymes spécifiques, consiste en l'ajout covalent d'une entité peptidique, lipidique, glucidique ou d'une autre nature sur une protéine. Comme son nom l'indique, cet ajout s'effectue en aval de la synthèse protéique. Parmi ces modifications, on peut citer la phosphorylation, la glycosylation, la SUMOylation, l'acétylation, l'ubiquitinylation, la nitrosylation, la palmitoylation, la farnésylation, la méthylation, l'ADP-ribosylation, l'hydroxylation, l'oxydation…etc. On estime à plus de 4000 le nombre de modifications post-traductionnelles différentes dans le monde du vivant.

Parmi celles-ci, la phosphorylation et la glycosylation sont les deux modifications les plus abondantes et sans doute les mieux décrites. Néanmoins, jusqu'au début des années 80, tout semblait les opposer. Il était en effet considéré que la glycosylation était une modification stable, restreinte à certains compartiments cellulaires (*Reticulum* endoplasmique, Golgi et face externe de la membrane plasmique) contrastant avec la phosphorylation, modification dynamique permettant à la cellule de s'adapter rapidement en réponse à des stimuli divers...

On sait aujourd'hui que la glycosylation occupe une place importante parmi les modifications post-traductionnelle puisqu'une part non-négligeable du génome (2-4%) code ces processus de glycosylation, et que près de 50 % des protéines totales sont glycosylées (Davies et al., 2005).

A partir des années 80, la glycosylation a pris une dimension totalement différente avec la découverte de la *O*-GlcNAcylation par Carmen Torres et Gerald Hart (Torres et Hart, 1984) (Figure 14). Cette étude a permit la mise en évidence fortuite d'un nouveau type de glycosylation beaucoup plus simple structuralement que celles connues à l'époque. En effet, le marquage radioactif des extrémités GlcNAc de glycoconjugués portés à la surface des lymphocytes a démontré que certaines protéines portaient un simple résidu de N-acétylglucosamine ancré sur une sérine ou thréonine.

Depuis cette première publication, de nombreux groupes de recherche ont étudié la *O*-GlcNAcylation, augmentant rapidement le nombre d'articles scientifiques en rapport avec ce sujet (Figure 14). Avec la *O*-GlcNAcylation, un nouveau mode de régulation de toute une pléthore de processus physiologiques est apparu.

Comment la *O*-GlcNAcylation est-elle passée inaperçue aux yeux des scientifiques pendant si longtemps, alors que la première liaison covalente entre un sucre et une protéine était connue depuis 1962 (Neuberger, Marshall, Yamashina, Cunningham, 1962) ? Tout d'abord, l'ajout d'un résidu de N-acétylglucosamine de seulement 203 Da sur la protéine n'entraîne pas de changement migratoire en SDS-PAGE. Donc, malgré les toutes les analyses de protéomique, cette modification est passée longtemps sous silence. Par la suite, les analyses des modifications par spectrométrie de masse ont été rendues difficiles de par le caractère labile de cette modification. Enfin, la présence de nombreuses hydrolases libérées dans des conditions de stress ou de lyse cellulaire réduisent le maintien de cette modification sur les protéines.

Heureusement, la production d'anticorps spécifiques des enzymes responsables de la modification, OGT et OGA, et du résidu *O*-GlcNAc, ainsi que l'évolution des techniques de détection de cette modification ont permis une avancée considérable dans la compréhension des mécanismes de *O*-GlcNAcylation des protéines.

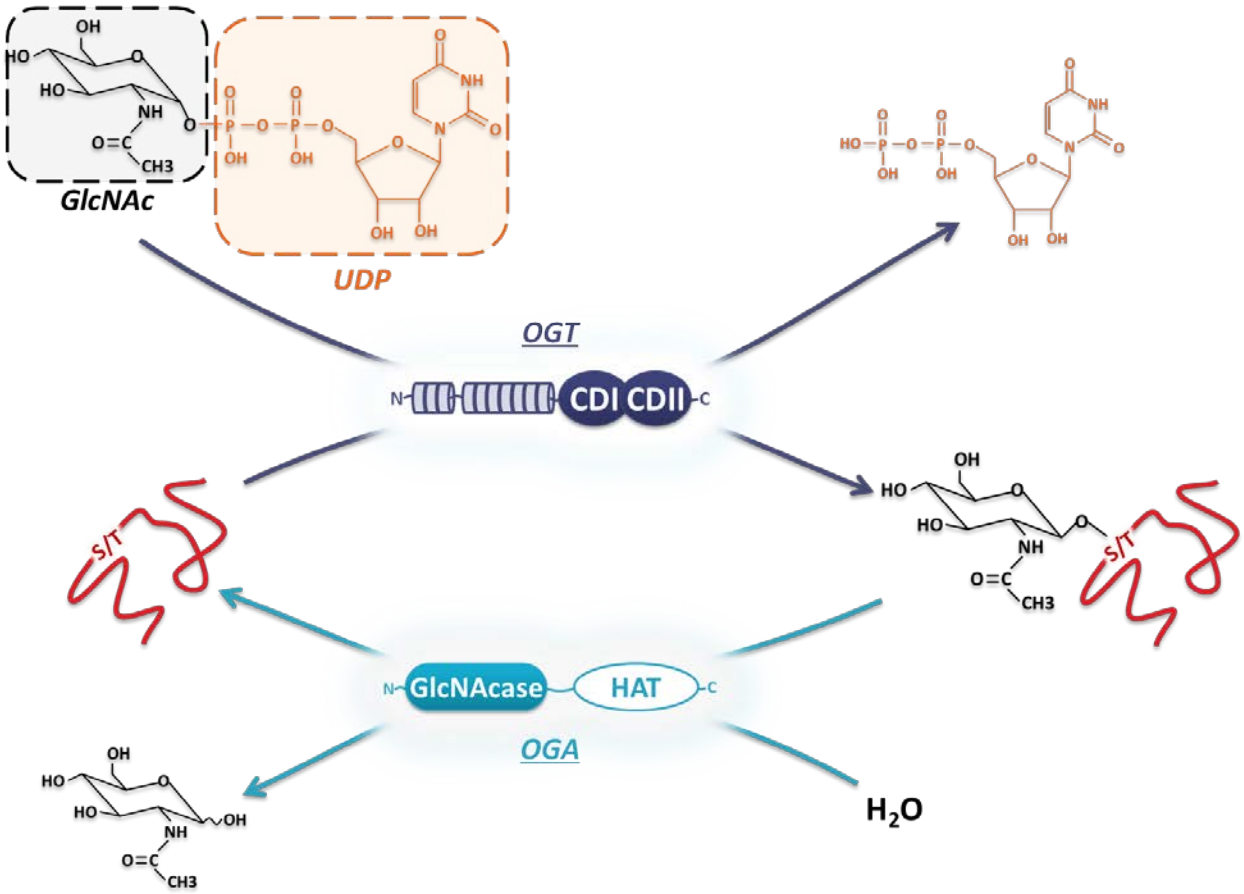


Figure 15 : Le dynamisme de la *O*-GlcNAcylation. La *O*-GlcNAc transférase ajoute un résidu de N-acétylglucosamine sur une sérine ou une thréonine à partir de l'UDP-GlcNAc. La *O*-GlcNAcase hydrolyse ce même résidu.

CD : Catalytic domain ; HAT : Histone Acétyl transférase ; OGA : O-GlcNAcase ; OGT : O-GlcNAc Transférase ; UDP : Uridine Di-Phosphate.

Une partie de ces différentes techniques a fait l'objet d'une publication jointe en annexe :

Lefebvre T, Drougat L, **Olivier-Van Stichelen S**, Michalski JC and Vercoutter-Edouart AS (2012) Antibodies and Activity Measurements for the Detection of *O*-GlcNAc Transferase and Assay of its Substrate, UDP-GlcNAc. *Methods Mol Biol (In press)*

2. Une modification universelle et essentielle

Au fil des découvertes, la *O*-GlcNAcylation a été identifiée dans de nombreux organismes, du plus simple au plus complexe: virus (*Cytomegalovirus humain*) (Greis et al., 1994), bactéries (*Listeria monocytogenes*) (Schirm et al., 2004), parasites (*Toxoplama gondii*) (Perez-Cervera et al., 2011), nématodes (*Caenorabitis elegans*) (Hanover et al., 2005), plantes (*Arabidopsis Thaliana*) (Swain et al., 2001), insectes (*Drosophilia melanogaster*) (Kelly et Hart , 1989) et mammifères (Torres et Hart, 1984). La *O*-GlcNAcylation est par conséquent une modification post-traductionnelle quasi-universelle.

La *O*-GlcNAcylation est essentielle à la vie. En effet, le « Knock-out » du gène de l'OGT entraîne la létalité des embryons et la mort des cellules souches embryonnaires à un stade précoce (Shafi et al., 2000). Le même effet est observé lorsque qu'on inhibe la $GlcNH_2$-6-P-acétyltransférase, une enzyme impliquée dans la synthèse de l'UDP-GlcNAc, substrat de l'OGT, démontrant une fois de plus le caractère essentiel de cette modification (Boehmelt et al., 2000b).

La conservation de la *O*-GlcNAcylation dans l'évolution, ainsi que son caractère ubiquitaire et essentiel, en fait une modification post-traductionnelle d'importance pour le règne du vivant.

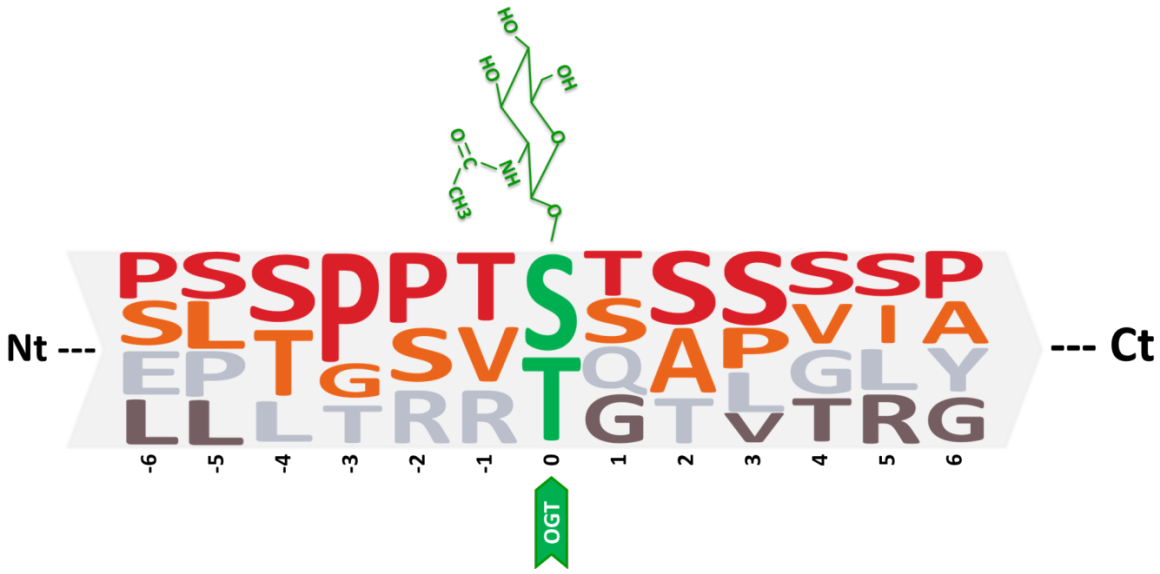


Figure 16 : Séquence préférentielle de *O*-GlcNAcylation.

A : alanine ; E : acide glutamique ; G : Glycine ; I : isoleucine ; L : leucine ; OGT : O-GlcNAc Transférase ; P : proline ; Q : glutamine ; R : arginine ; S : sérine ; T : thréonine ; V : valine ; Y : tyrosine.

A. Les caractéristiques de la *O*-GlcNAcylation

1. Une modification simple…

En comparaison avec les autres types de glycosylation, la *O*-GlcNAcylation est une modification simple. En effet, les glycosylations classiques constituent des modifications complexes, nécessitant de nombreuses étapes catalysées par un nombre important d'enzymes. La *O*-GlcNAcylation, quant à elle, ne consiste qu'en l'addition d'un seul résidu de N-acétylglucosamine en anomérie β sur l'extrémité hydroxylée d'une sérine ou d'une thréonine (Figure 15).

Cette modification très simple concerne à l'heure actuelle près d'un millier de protéines identifiées (Source : dbOGAP: Database of O-GlcNAcylated Proteins and Sites) et pourrait potentiellement affecter toutes les protéines cytosoliques, nucléaires et mitochondriales portant un résidu de sérine ou de thréonine. A ce jour, près de 500 sites de *O*-GlcNAcylation ont été identifiés. Ce nombre est en constante augmentation avec l'évolution et la sophistication des technologies de détection et d'identification des sites de *O*-GlcNAcylation. Néanmoins, la cartographie des sites de *O*-GlcNAcylation est difficile par l'absence de séquence consensus stricte pour cette modification. Il existe tout de même des séquences favorisant la *O*-GlcNAcylation comme par exemple la présence de sérines/thréonines en tandem ou encore la présence d'une séquence proline-valine en amont de l'acide aminé hydroxylé à modifier (Chalkey et al., 2009) (Figure 16). Cette variabilité de séquence est due au fait qu'une seule enzyme, la *O*-GlcNAc transférase, catalyse l'ajout du résidu de GlcNAc sur les protéines, alors qu'il existe plus de 500 kinases responsables des processus de phosphorylation (Manning et al., 2002).

2. …et dynamique…

Phosphorylation et *O*-GlcNAcylation font toutes deux parti de processus dynamiques. En effet, l'étude des cytokératines 8 et 18 (Chou et al., 1992), et de l'α-cristalline (Roquemore et al., 1996) a montré que la demi-vie de la *O*-GlcNAcylation était nettement inférieure à la demi-vie de ces protéines.

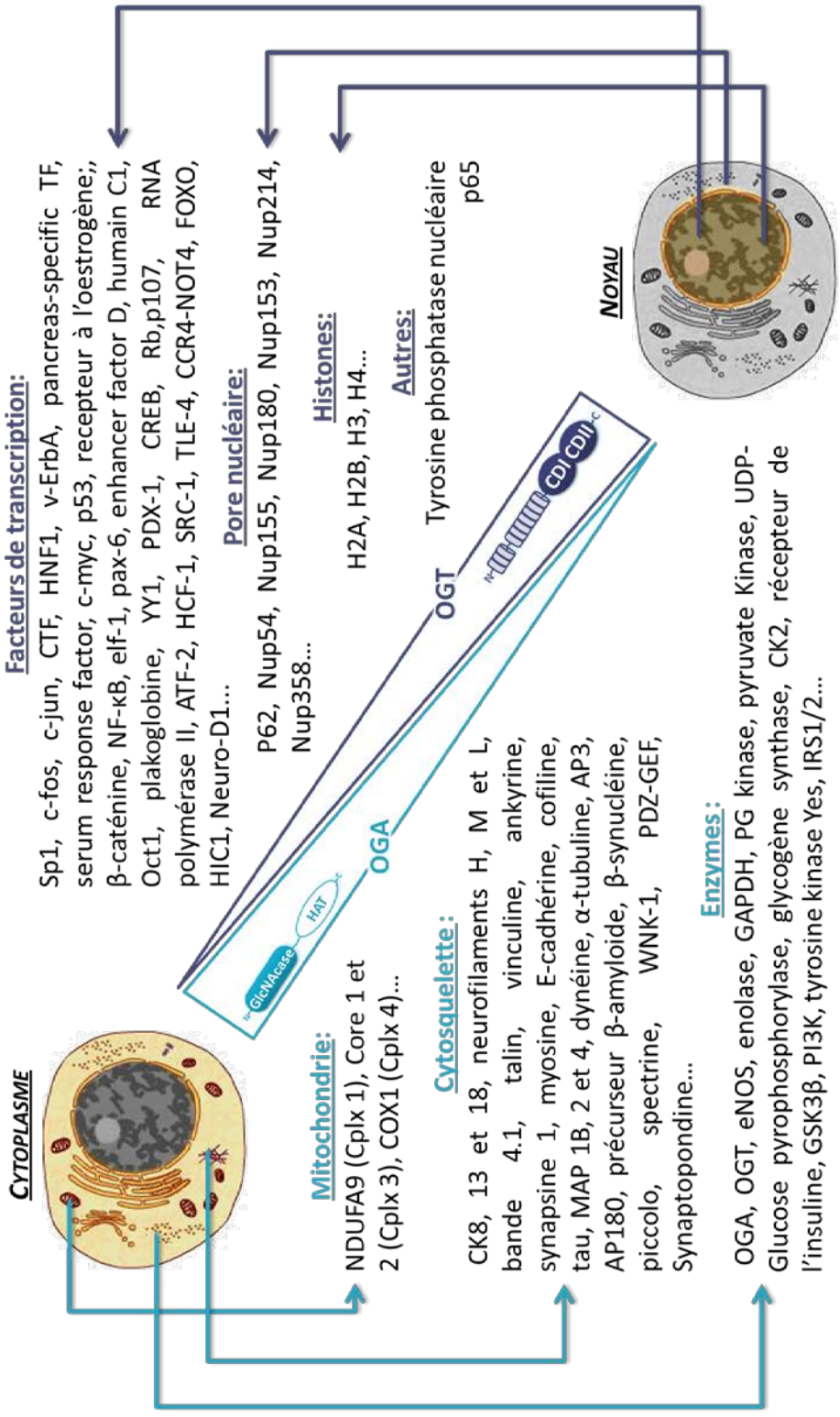


Figure 17 : Répartition des protéines *O*-GlcNAcylées et des enzymes au sein de la cellule. Les protéines *O*-GlcNAcylées se répartissent différemment au sein de la cellule, du cytosol au noyau en passant par la mitochondrie. Les enzymes OGT et OGA montrent également une répartition différente du noyau au cytoplasme.

Les protéines ainsi modifiées subiraient plusieurs cycles de *O*-GlcNAcylation/dé-*O*-GlcNAcylation au cours de leur existence. Cette caractéristique de la *O*-GlcNAcylation permet, entre autres, de réguler de façon transitoire des mécanismes biologiques fondamentaux en réponse à un stimulus extérieur.

3. …des protéines intracellulaires.

Au niveau subcellulaire, la *O*-GlcNAcylation est retrouvée majoritairement dans le noyau et le cytosol (Holt et Hart, 1986 ; Hanover et al., 1987). Dans le noyau, elle modifie un nombre important de facteurs de transcription, d'histones et de protéines du pore nucléaire (Hanover et al., 1987 ; Jackson et al., 1988, Sakabe et al., 2010). Au niveau du cytosol, la *O*-GlcNAcylation est plus diffuse et affecte les protéines du cytosquelette (Chou et al., 1992, Walgren et al., 2003 ; Cieniewski-Bernard et al., 2004 ; Ramirez-Correa et al., 2008), les enzymes du métabolisme (Cieniewski-Bernard et al., 2004), de la phosphorylation (Gandy et al., 2006 ; Robles-Flores et al., 2008 ; Dias et al., 2009), et les enzymes de la *O*-GlcNAcylation en elles-mêmes (Kreppel et al., 1997 ; Lazarus et al., 2006) (Figure 17). Plus récemment, il a été démontré qu'il existait également des protéines *O*-GlcNAcylées au niveau de la mitochondrie (Hu et al., 2009) ainsi qu'une mOGT (mitochondrial OGT) localisée spécifiquement dans cet organite (Hanover et al., 2003).

On peut supposer que la localisation des protéines *O*-GlcNAcylées est due à la répartition des enzymes de la *O*-GlcNAcylation, l'OGT étant retrouvée majoritairement au noyau, et l'OGA au cytosol (Okuyama et Marshall, 2003). Le nucléosome est quant à lui une exception puisqu'il est enrichi en OGA (Zeidan et al., 2010).

A l'échelle de l'organisme, la *O*-GlcNAcylation est retrouvée dans toutes les cellules de tous les tissus mais en proportion inégale néanmoins. Les tissus montrant la plus forte concentration en protéines *O*-GlcNAcylées sont également les plus grands consommateurs de glucose comme le cerveau et le foie (Okuyama et Marshall, 2003 ; Gao et al., 2001).

B. OGT/OGA: les deux font la paire!

L'OGT et l'OGA sont les deux enzymes responsables du cycle *O*-GlcNAcylation/dé-*O*-GlcNAcylation. L'OGT et l'OGA ont été purifiées et caractérisées respectivement en 1990 (Haltiwanger et al., 1990) et 1994 (Dong et al., 1994). Le clonage de l'OGT en 1997 (Lubas et al., 1997) puis de l'OGA en 2001 (Gao et al., 2001) a permis la mise au point d'un large éventail d'outils parmi lesquels des inhibiteurs puissants (Ac-5SGlcNAc, Thiamet-G, NButGt, dérivés de GlcNAcstatine…) et l'application de la technologie de l'ARN interférent .

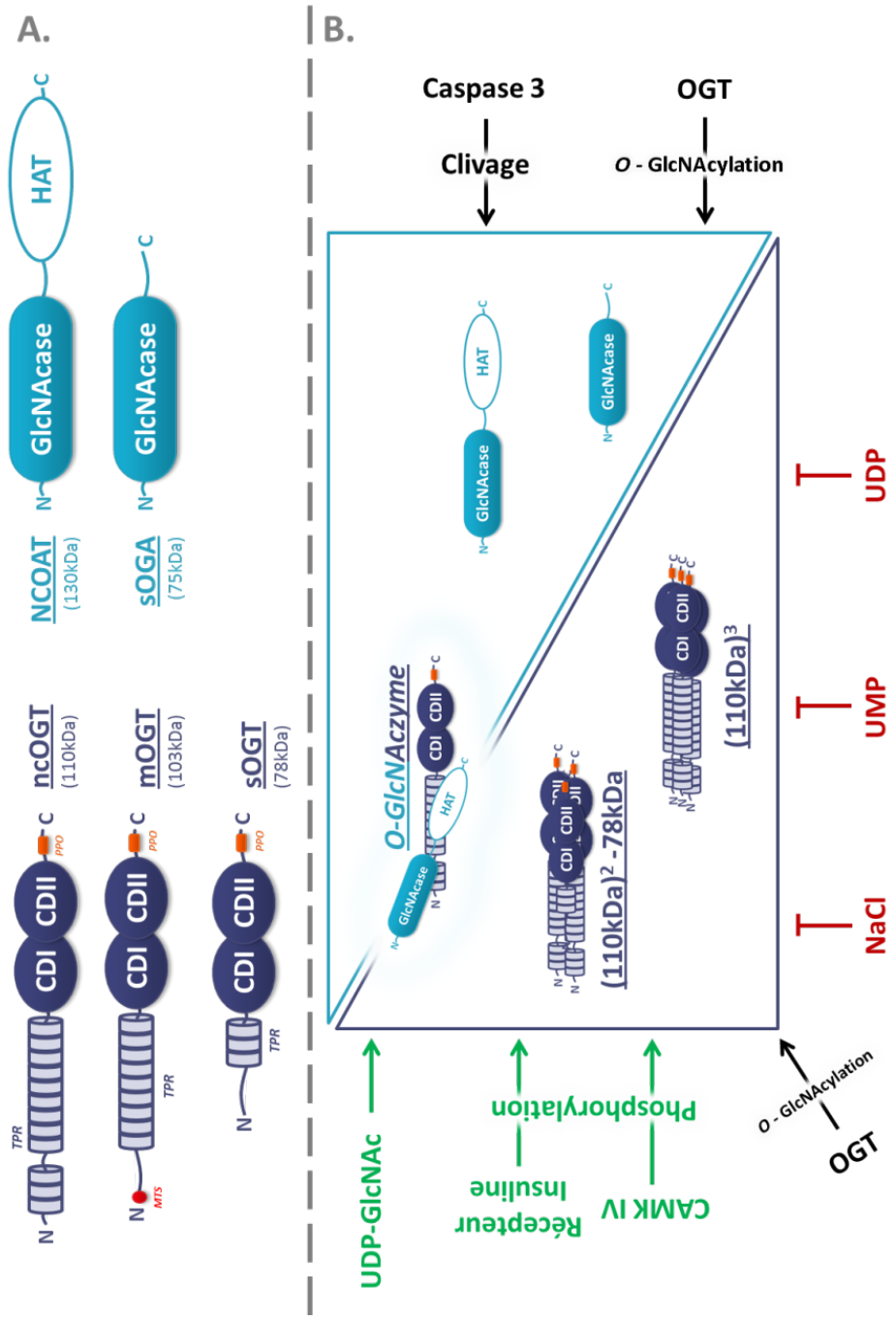
1. L'OGT en quelques mots

a) Structure

L'OGT ou uridine diphospho-N-acétylglucosamine: peptide β-N-acétylglucosamine transférase, a été purifiée en 1990 à partir d'extrait cytosolique de réticulocytes de lapin (Haltiwanger et al., 1990), bien qu'elle fut qualifiée beaucoup plus tard de majoritairement nucléaire (Okuyama et Marshall, 2003). Elle fut également isolée du foie de rat, ce qui permit de la caractériser (Haltiwanger et al., 1992).

Cette enzyme catalyse l'addition du résidu de N-acétylglucosamine en β sur les groupements hydroxyles des sérines et thréonines à partir d'UDP-GlcNAc, pour lequel elle présente une très haute affinité (Km=545nM) (Haltiwanger et al., 1992). Cette caractéristique permet à l'UDP-GlcNAc néosynthétisé d'être utilisable par l'OGT car les concentrations en nucléotide-sucre sont beaucoup plus faibles dans le cytosol que dans les compartiments réticulaire et golgien. Pour mémoire, les Km des GlcNAc transférases du *reticulum* endoplasmique et du Golgi sont plus élevés que celui de l'OGT.

Le clonage de l'OGT a mis en évidence la position du gène codant l'enzyme sur le chromosome X à proximité du centromère (position Xq13.1), région considérée comme fragile et associée à la maladie de Parkinson (Shafi et al., 2000 ; Nolte et al., 2003). Le gène de l'OGT est également situé à proximité du « X-inactivation center» (XIC), et particulièrement du gène Xist, qui code un ARN non traduit induisant l'inactivation d'un des deux chromosome X chez la femelle (Lin et al., 2007).



<u>Figure 18 :</u> Les enzymes de la *O*-GlcNAcylation : structure et régulation. A) Les différentes isoformes de l'OGT et de l'OGA. B) les différents complexes enzymatiques et leur régulation. On distingue en vert : les facteurs influençant positivement l'activité des deux enzymes, en rouge : les facteurs les influençant négativement. En noir sont notés les facteurs dont l'impact sur l'activité des enzymes n'est pas encore défini.

CAMKIV : Calcium/calmodulin-dependent protein Kinase IV ; CD : catalytic domain ; HAT : Histone Acétyl Transférase ; mOGT : mitochondrial O-GlcNAc Transferase ; MTS : mitochondrial target sequence ; ncOGT : nuclear and cytoplasmic O-GlcNAc Transferase ; sOGA : Short O-GlcNAcase ; sOGT : Short O-GlcNAc Transferase ; PPO : PIP-binding activity of OGT ; TPR : tetratricopeptide repeat ; UDP/UMP : uridine Di/mono-phosphate.

Par cette proximité, l'OGT, et la *O*-GlcNAcylation semblent jouer également un rôle de premier plan dans l'embryogénèse (cf partie IV. F.1.d).

Différents variants d'épissage permettent à l'enzyme de s'exprimer sous trois formes : la ncOGT (110 kDa), la sOGT (78 kDa) et la mOGT (103 kDa) (respectivement « nucleocytoplasmic », « short » et « mitochondrial ») (Figure 18A). Ainsi, on observe des multimères de l'OGT comme par exemple un complexe hétérotrimérique formé de deux sous-unités de ncOGT et d'une sous-unité de sOGT dans le foie de rat (Haltiwanger et al., 1992). Néanmoins, l'expression des isoenzymes et la composition en sous-unités des multimères varient en fonction des organes. Par exemple, on observe une forte proportion de sOGT dans le rein, le muscle et le foie, tandis que d'autres tissus comme le cerveau et le pancréas ne contiennent que des homotrimères de sous-unités de 110 kDa (Kreppel et al., 1997). La mOGT, quant à elle, est retrouvée uniquement au niveau de la mitochondrie et permet la modification des protéines de la chaîne respiratoire (Hanover et al., 2003 ; Hu et al., 2009).

Chacune des isoformes de l'OGT contient deux domaines fonctionnels séparés par une région flexible. On note également la présence de régions flexibles au niveau des extrémités N- et C- terminales pouvant porter respectivement, une séquence d'adressage à la mitochondrie pour la mOGT (MTS : Mitonchondrial Target Sequence, acides aminés de 1-20) (Love et al., 2003) ou un domaine PPO d'interaction avec les phosphoinositides (PIP-Binding activity of OGT) (Yang et al., 2008). Par homologie de séquence, ce dernier domaine (PPO : 958-1001 pour la ncOGT) est présent sur toutes les isoformes de l'OGT, sans que l'on comprenne l'intérêt de cette séquence, particulièrement pour la mOGT, même si les PIPs y sont également présents (Pasupathy et al., 1999).

La partie C-terminale porte l'activité enzymatique de l'OGT (Lubas et Hanover, 2000). Ce domaine catalytique montre de nombreuses similarités avec d'autres glycosyltransférases (GalNAc transférase) et, par homologie de séquence, est subdivisé en deux sous-domaines : le domaine catalytique I (CDI) porte le site de liaison à l'UDP et l'activité catalytique tandis que le domaine CDII est une région "lectin-like" (Roos et Hanover, 2000).

La région N-terminale porte des répétitions de 34 acides aminés appelées répétitions tétratricopeptides (TPRs), caractérisées par la séquence W-L-G-Y-A-F-A-P (Lamb et al., 1995). Le nombre de TPRs caractérise les différentes isoformes de l'OGT : la ncOGT en contient 12, la mOGT, 9 et la sOGT, seulement 2 (Hanover et al., 2003).

Ces répétitions serviraient, d'une part à la multimérisation de l'OGT et, d'autre part, à l'interaction avec ses nombreux partenaires en vue de leur O-GlcNAcylation (Iyer et Hart, 2003). La perte de certains TPRs entraîne une diminution de l'activité glycosyltransférase (Lubas et Hanover, 2000). Par ailleurs, par similarité de structure avec l'importine α, ces domaines TPRs permettraient de transloquer l'OGT au noyau (Jínek et al., 2004). Elle pourrait interagir directement avec les protéines du pore nucléaire, justifiant ainsi son abondance nucléaire (Okuyama et Marshall, 2003).

b) Régulation et modulation

(1) Régulation de son activité : interactions et modifications post-traductionnelles

L'OGT possède elle-même des modifications post-traductionnelles comme la phosphorylation, la *O*-GlcNAcylation ou encore la S-nitrosylation, permettant de réguler son activité très finement.

En effet, l'OGT est tyrosine-phosphorylée par le récepteur de l'insuline après fixation de l'hormone (Whelan et al., 2008). Cette stimulation corrèle avec une relocalisation de l'enzyme au niveau de la membrane plasmique, son interaction avec le phosphatidylinositol-3, 4, 5-triphosphate (PIP_3) et la *O*-GlcNAcylation de certains substrats (Yang et al., 2008). Une autre interaction de l'OGT avec le PIP_3 a également été démontrée au niveau nucléaire, activant ainsi l'activité transcriptionnelle de PDX1 (Pancreatic and duodenal homeobox 1) par *O*-GlcNAcylation (Kebede et al., 2012).

L'OGT est également phosphorylée par la CAMK IV (calcium/calmodulin-dependent protein kinase IV) (Song et al., 2008b). Dans le cadre de sa régulation, les phosphorylations augmentent considérablement l'activité de l'OGT en réponse à des stimuli variés.

L'OGT est elle-même *O*-GlcNAcylée suggérant une boucle de rétro-contrôle de cette enzyme (Kreppel et al., 1997).

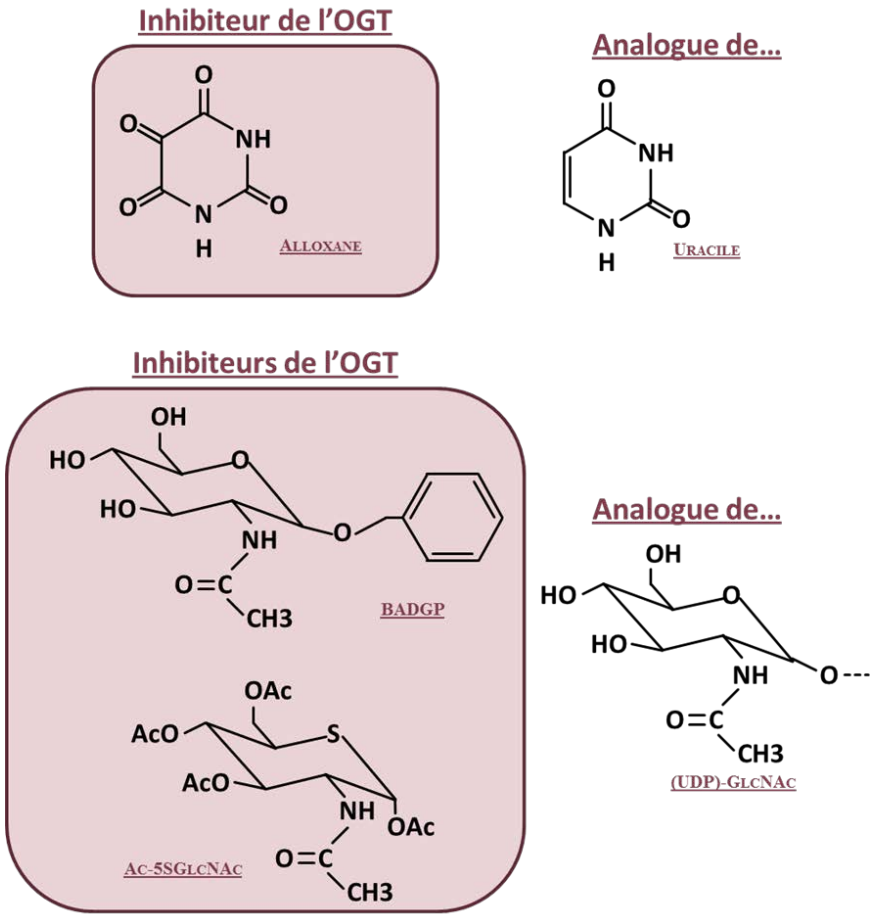


Figure 19 : Les inhibiteurs de la *O*-GlcNAc transférase. Ac-5SGlcNAc : 2-acétamido- 1,3,4,6-tétra-O-acétyl-2-désoxy-5-thio-α-D-glucopyrannose ; BADGP : Benzyl 2-acétamido-2-désoxy-α-D-galactopyranoside ;

OGT : O-GlcNAc transférase ; UDP-GlcNAc : Uridine Di-Phospho-N-acétylglucosamine.

Une modification atypique peut également affectée l'OGT : la S-nitrosylation. Cette modification disparaît après stimulation par les LPS (lipopolysaccharides) et permet l'activation de la réponse innée par les macrophages (Ruy et al., 2011).

D'autres modes de régulation dépendants des conditions physiologiques de la cellule permettent également de réguler l'activité de l'OGT.

Lisa Kreppel et Gerald Hart ont montré que l'OGT module son activité en fonction de la concentration en UDP-GlcNAc, en adaptant son Km de 6 à 217 µM (Kreppel et Hart, 1999). Par contre, une forte concentration en UDP inhibe l'OGT, tout comme l'UMP et le NaCl (Haltiwanger et al., 1992 ; Okuyama et Marshall, 2003) (Figure 18B).

Un point, non des moindres, reste à éclaircir : comment l'OGT acquiert sa spécificité de substrat ? Quelques pistes sont actuellement proposées. L'interaction de l'OGT avec certains partenaires pourrait l'orienter vers la modification d'autres protéines. Par exemple, l'interaction de l'OGT avec GRIF1 ($GABA_A$ Receptor-Interacting Factor 1), Trak1 (ou OIP106-OGT interacting Protein 106) (Iyer et al., 2003), MYPT1 (Myosin phosphatase targeting subunit 1) (Cheung et al., 2008), mSin3A (Yang et al., 2002), p38MAPK (Cheung et Hart, 2008) ou encore PGC1α (PPAR gamma coactivator 1α) (Housley et al., 2009) n'a pas pour (seul) but leur *O*-GlcNAcylation, ce qui suggère un rôle d'accompagnement de l'OGT vers d'autres cibles comme la nucléoporine p62, l'ARN polymérase II, le neurofilament H ou encore FOXO.

(2) Modulation artificielle de l'activité de l'OGT

La cristallisation de l'OGT a permis la conception de nouveaux inhibiteurs (Clarke et al., 2008 ; Martinez-Fleites et al., 2008). Ainsi l'alloxane, un analogue structural de l'uracile, fut le premier inhibiteur de l'OGT (Konrad et al., 2002). Par la suite, le BADGP (benzyl-2-acétamido-2-désoxy-alpha-d-galactopyranoside) fut utilisé afin d'inhiber l'OGT même s'il s'est avéré qu'il n'était pas spécifique de cette glycosyltransférase (D'Alessandris et al., 2004). Enfin plus récemment, l'acétyl-5SGlcNAc s'est révélé être un excellent inhibiteur de l'OGT (Gloster et al., 2011). Il s'incorpore à la voie de sauvetage de l'UDP-GlcNAc pour former de l'UDP-5SGlcNAc qui, de par sa haute affinité pour l'OGT, inhibe préférentiellement l'OGT plutôt que les autres glycosyltransférases (Figure 19).

2. La *O*-GlcNAcase en quelques mots

a) Structure

La β-N-acétylglucosaminidase (OGA ou *O*-GlcNAcase) a été purifiée en 1994 à partir de rate de rat (Dong et al., 1994). En fait, elle fut découverte en 1974 en tant qu'hexosaminidase C qui, contrairement aux autres hexosaminidases lysosomales A et B agissant à pH acide, est cytosolique et active à pH neutre (Braidman et al., 1974). Elle a ensuite été clonée et séquencée en 2001 (Gao et al., 2001) à partir d'extraits de cerveau humain, ce qui a permis de rapprocher sa séquence de celle du gène *MGEA5* (Meningioma-Expressed Antigen 5) codant une hyaluronidase et induisant une réponse immunitaire chez les patients atteints de méningiome (Heckel et al., 1998).

Le gène de l'OGA est situé sur le chromosome 10 en position 10q24 (Gao et al., 2001), locus associé à la maladie d'Alzheimer (Myers et al., 2000) et à la prédisposition au diabète de type 2 chez certaines populations (Farook et al., 2002 ; Lehman et al., 2005).

Même si les publications décrivant la *O*-GlcNAcase sont nettement moins nombreuses que celles portant sur l'OGT, elles nous permettent néanmoins d'avoir des informations sur la structure et la fonction de cette enzyme.

L'OGA est subdivisée en deux domaines : un domaine N-terminal catalytique similaire aux hyaluronidases et un domaine C-terminal HAT (Histone AcétylTransférase), homologue à ceux de type GCN5 (Schultz et Pils, 2002). Ce domaine possède une activité *in vitro* qui n'a pas encore été validée *in vivo* (Toleman et al., 2004). Ce domaine serait capable de lier les histones par une structure en doigts de zinc (Toleman et al., 2004). Ces deux domaines sont séparés par une région de liaison qui possède un site de clivage par la caspase 3 (Asp413) (Butkinaree et al., 2008). Néanmoins, malgré ce clivage apoptotique, les deux domaines resteraient associés et l'enzyme conserverait son activité.

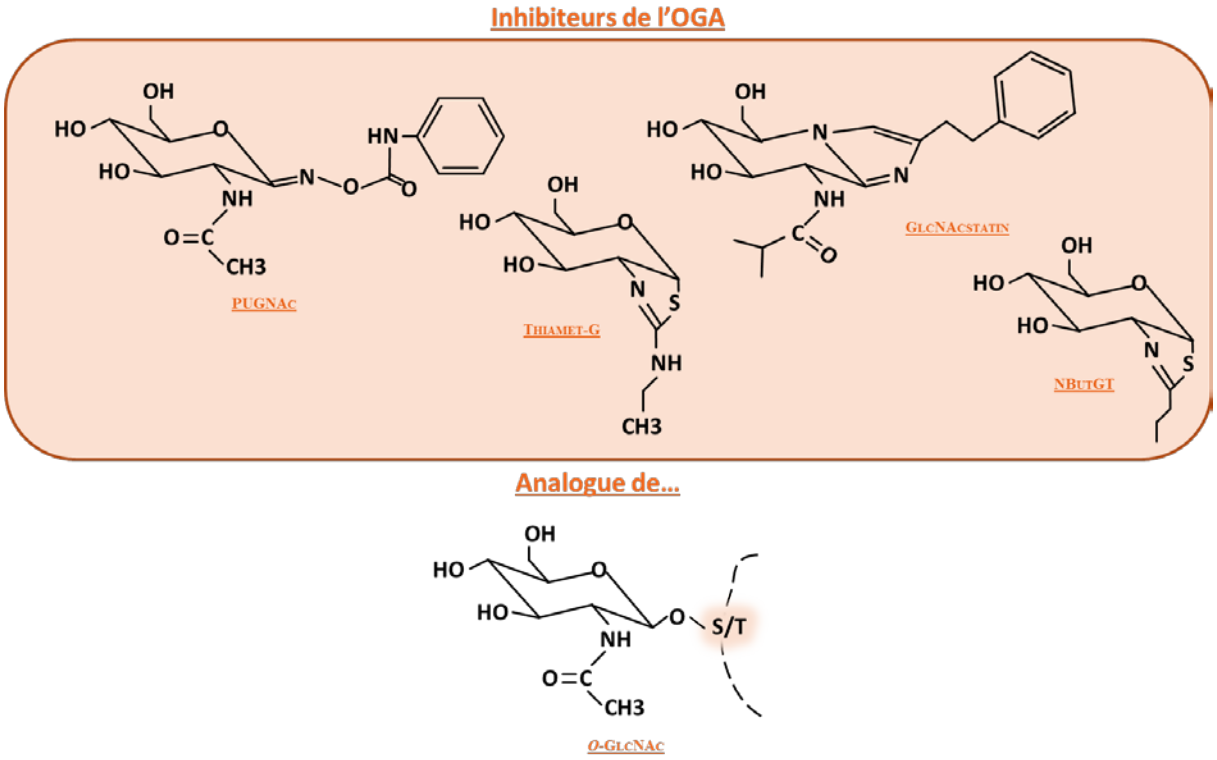


Figure 20 : Les inhibiteurs de la *O*-GlcNAcase. GlcNAcstatine : N-((5R,6R,7R,8S)-6,7-Dihydroxy-5-(hydroxymethyl)-2-phénéthyl-5,6,7,8- tétrahydroimidazo [1,2-a]pyridin-8-yl) acétamide; NButGT : 1,2-didésoxy-2'-propyl-alpha-D-glucopyranoso-[2,1-D]-Delta 2'-thiazoline ; PUGNAc : O-(2-acétamido-2-désoxy-D-glucopyranosylidène)amido-N-phénylcarbamate ; Thiamet G : 1,2-Dideoxy-2'-éthylamino-alpha-D-glucopyranoso-[2,1-d]-DELTA2'-thiazoline.

OGA : O-GlcNAcase.

L'OGA est retrouvée sous deux isoformes de 130 kDa (NCOAT, Nuclear and Cytoplasmic *O*-GlcNAc transferase and AcetylTransferase) et de 75 kDa (sOGA, short OGA), la seconde isoforme résultant d'un variant d'épissage codant une protéine amputée de son domaine HAT (Comtesse et al., 2001) (Figure 18A). Contrairement à l'OGT, la *O*-GlcNAcase ne semble pas se multimériser mais ses différentes isoformes montrent des localisations différentes au sein de la cellule : nucléaire pour la forme de 75 kDa et majoritairement cytosolique pour la forme de 130 kDa (Comtesse et al., 2001).

b) Régulation et modulation

Comme l'OGT, l'OGA est également modifiée par *O*-GlcNAcylation (Lazarus et al., 2006), mais le rôle de cette modification sur l'enzyme n'est pas encore connu.

Certains partenaires de l'enzyme ont également été décrits comme Hsc110, Hsc70, la culine ou la calcineurine, mais sans pour autant savoir si ces interactions jouent un rôle dans la régulation de son activité (Gao et al., 2001) (Figure 18B). Il a été montré récemment que la forme courte de l'OGA jouait un rôle plus particulier dans la formation et la mobilisation des gouttelettes lipidiques. Elle participerait notamment, de par cette localisation particulière, à la dégradation protéasomale des protéines de surface de ces gouttelettes (Keembiyehetty et al., 2011). Des données cristallographiques sur l'OGA ont permis la mise au point d'inhibiteurs très spécifiques et puissants (Rao et al., 2006). Le PUGNAc a été l'inhibiteur le plus utilisé pendant de nombreuses années (Haltiwanger et al., 1998) même s'il s'est avéré qu'il inhibait l'OGA au même titre que les hexosaminidases A et B (Kim et al., 2006a).

D'autres inhibiteurs de plus en plus spécifiques et efficaces ont fait leur apparition par la suite : la GlcNAcstatine (Dorfmueller et al., 2006), le Thiamet G (Yuzwa et al., 2008) et le NButGT (Macauley et al., 2005 ; Macauley et al., 2008) étant les plus connus (Figure 19). De plus, certains de ces inhibiteurs ont prouvé leur capacité à traverser la barrière hémato-encéphalique et à cibler le cerveau. Ces inhibiteurs sont par conséquent d'un intérêt tout particulier pour l'étude de la *O*-GlcNAcylation dans les processus de neurodégénération (Yuzwa et al., 2008).

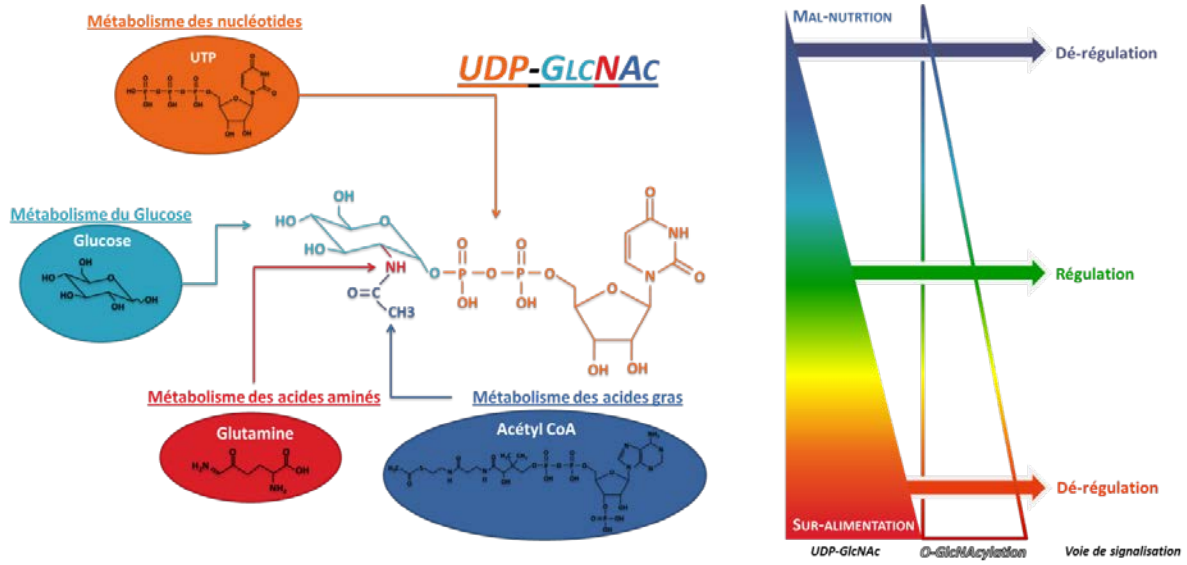


Figure 21 : L'UDP-GlcNAc : un carrefour métabolique. Pour former l'UDP-GlcNAc, la voie de biosynthèse des hexosamines incorpore au fur et à mesure des éléments provenant de divers métabolismes, lui conférant le statut de senseur nutritionnel. Ainsi, en fonction de l'apport alimentaire, du contexte nutritionnel, l'UDP-GlcNAc et par conséquent la *O*-GlcNAcylation modulent l'activation des voies de signalisation.

3. En complexe pour plus d'efficacité

La NCOAT et la ncOGT sont capables d'interagir entre elles par le biais de leur région respective 336-548 et 1-248, formant un complexe appelé *O*-GlcNAczyme (Wisenhunt et al., 2006). Le *O*-GlcNAczyme s'associe entre autres à des histones désacétylases au sein de complexes de répression transcriptionnelle (Wisenhunt et al., 2006) . On retrouve également un complexe OGT/OGA avec la kinase Aurora B et la protéine phosphatase 1B; cette interaction permet de modifier certaines protéines comme la vimentine (Slawson et al., 2008). L'association de l'OGT et de l'OGA avec des enzymes régulant d'autres modifications post-traductionnelles (phosphorylation, acétylation, ubiquitinylation, méthylation….) augmente les combinaisons possibles de modifications des protéines cibles et permet à la cellule de s'adapter plus efficacement en réponse aux stimuli extérieurs.

C. La voie de biosynthèse des hexosamines

1. Du Glucose à l'UDP-GlcNAc

Pour son activité catalytique, l'OGT nécessite la fixation de deux substrats, la protéine à *O*-GlcNAcyler et le donneur de N-acétylglucosamine, l'UDP-GlcNAc. Ce nucléotide-sucre représente un carrefour métabolique, au croisement du métabolisme du glucose (Glc), des acides aminés (Glutamine, acides aminés glucogéniques et cétogéniques), des acides gras (acétyl-CoA) et des nucléotides (UTP) (Wang et al., 1998) (Figure 21). Cette particularité confère à l'UDP-GlcNAc une fonction de senseur métabolique permettant de refléter l'état nutritionnel de la cellule. *Via* l'utilisation de l'UDP-GlcNAc, la *O*-GlcNAcylation se comporte, également comme un senseur nutritionnel, permettant de retranscrire l'état général d'un tissu sur la signalisation et le métabolisme cellulaire.

La synthèse de l'UDP-GlcNAc à partir du glucose s'effectue par une succession de réactions enzymatiques regroupée sous l'appelation de voie de biosynthèse des hexosamines (Figure 22).

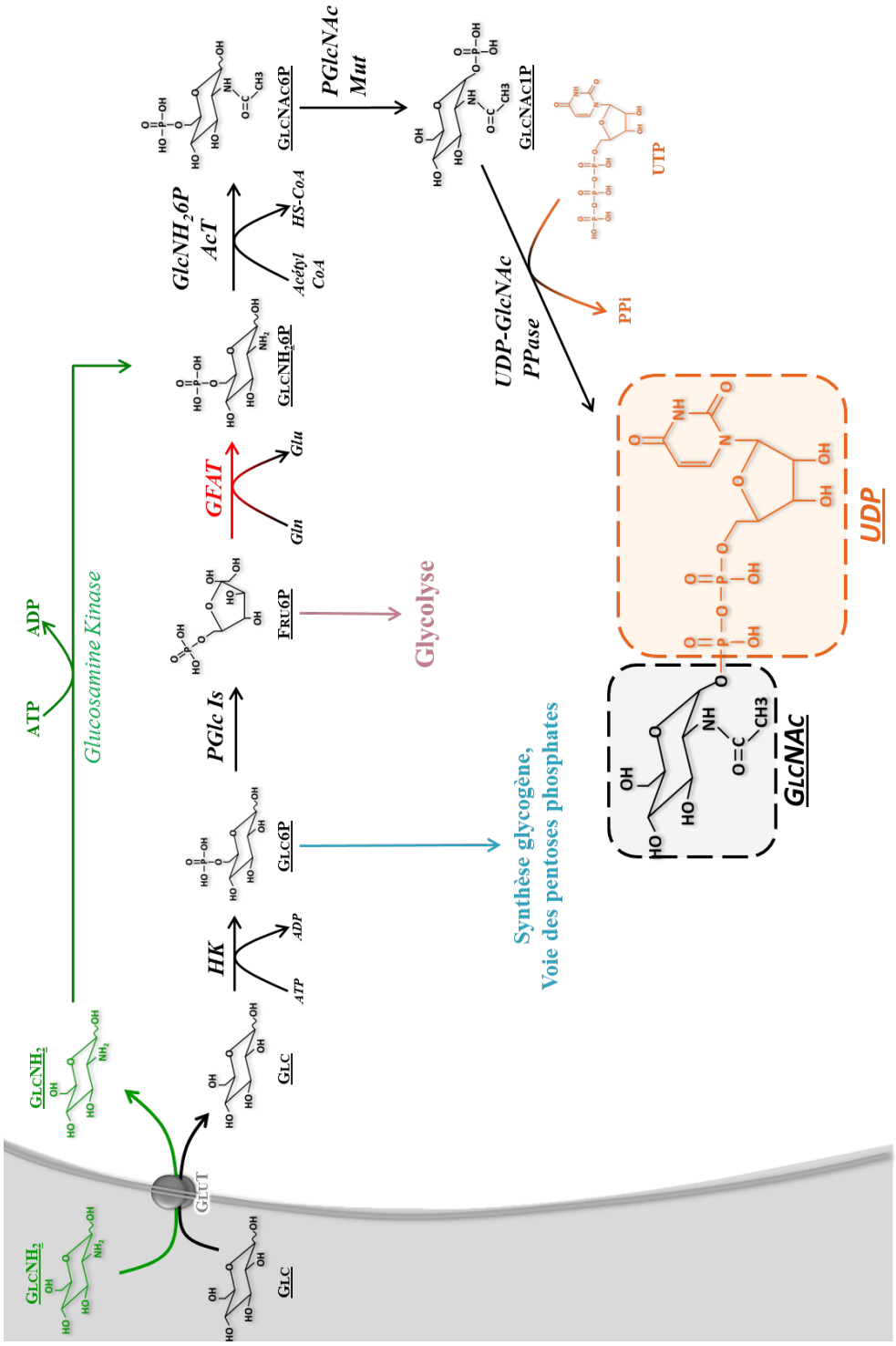


Figure 22 : La voie de biosynthèse des hexosamines.

Fru : Fructose ; PGlc Is: glucose-6-phosphate isomérase ; GFAT: glutamine: fructose-6-phosphate amido transférase ; Glc : Glucose ; $GlcNH_2$: Glucosamine; $GlcNH_2$6P AcT: glucosamine-6-phosphate acétyltransférase ; PGlcNAc Mut: N-acétylglucosamine phosphate mutase ; Gln : Glutamine; Glu : glutamate; GluT: Glucose transporter ; HK: hexokinase ; UDP : Uridine Di-Phosphate UDP-GlcNAc PPase: UDP-N-acétylglucosamine pyrophosphorylase ; UTP : Uridine Tri-Phosphate.

En premier lieu, le glucose entre dans la cellule par des transporteurs GLUT (Glucose Transporter), transporteurs prenant également en charge le transport d'autres monosaccharides : par exemple la glucosamine emprunte les transporteurs GLUT1, 2 ou 4 (Uldry et al., 2002), le fructose est quant à lui internalisé par GLUT5, GLUT2 et GLUT11 (Wood et Trayhurn, 2003). Ces transporteurs sont tissus-spécifiques. Par exemple, les adipocytes utilisent le transporteur GLUT4, qui, nous le verrons plus tard, joue un rôle essentiel dans l'apparition de la résistance à l'insuline. A l'inverse, GLUT2 est plutôt exprimé dans le foie et les cellules β du pancréas, et GLUT3 dans le cerveau (Bell et al., 1990).

Dès son entrée dans la cellule, le glucose est converti en glucose-6-phosphate. Il emprunte ensuite différentes voies métaboliques pour, par exemple, fournir du glycogène au niveau hépatique ou musculaire ou entrer dans la voie des pentoses phosphate. Celui-ci peut être également converti en fructose-6-phosphate pour emprunter, soit la voie de la glycolyse, soit, pour 2 à 3 % de celui-ci, la voie de biosynthèse des hexosamines (Bouché et al., 2004).

L'importance de cette voie métabolique a été mise en évidence dans plusieurs études qui ont démontré son implication dans la mise en place de la résistance à l'insuline associée au diabète de type 2 (Marshall et al., 1991b). En effet, le phénomène de résistance à l'insuline nécessite, en plus de l'insuline, du glucose et de la glutamine, tous deux membres essentiels de cette voie de biosynthèse (Traxinger et Marshall, 1989). Des études suggèrent que l'UDP-GlcNAc serait responsable en partie de la résistance à l'insuline, soit par modification des protéines intracellulaires par *O*-GlcNAcylation (Vosseller et al., 2002), soit par modification des protéines extracellulaires par *N*-glycosylation (Ohtsubo et al., 2005 ; Mendelsohn et al., 2007). Dans tous les cas, l'importance de cette voie métabolique a été révélée dans de nombreux organismes et modèles cellulaires. Par exemple, il a été démontré que la GFAT (glutamine: fructose-6-phosphate amido transférase) murine était l'enzyme limitante de cette voie métabolique (Marshall et al., 1991a), permettant de réguler le flux de glucose entrant dans la voie en fonction des concentrations en UDP-GlcNAc de la cellule (Kornfeld et al., 1964). De même, cette enzyme permet de retranscrire l'état nutritionnel de la cellule par incorporation de la glutamine, essentielle à la synthèse d'UDP-GlcNAc (Marshall et al., 1991a).

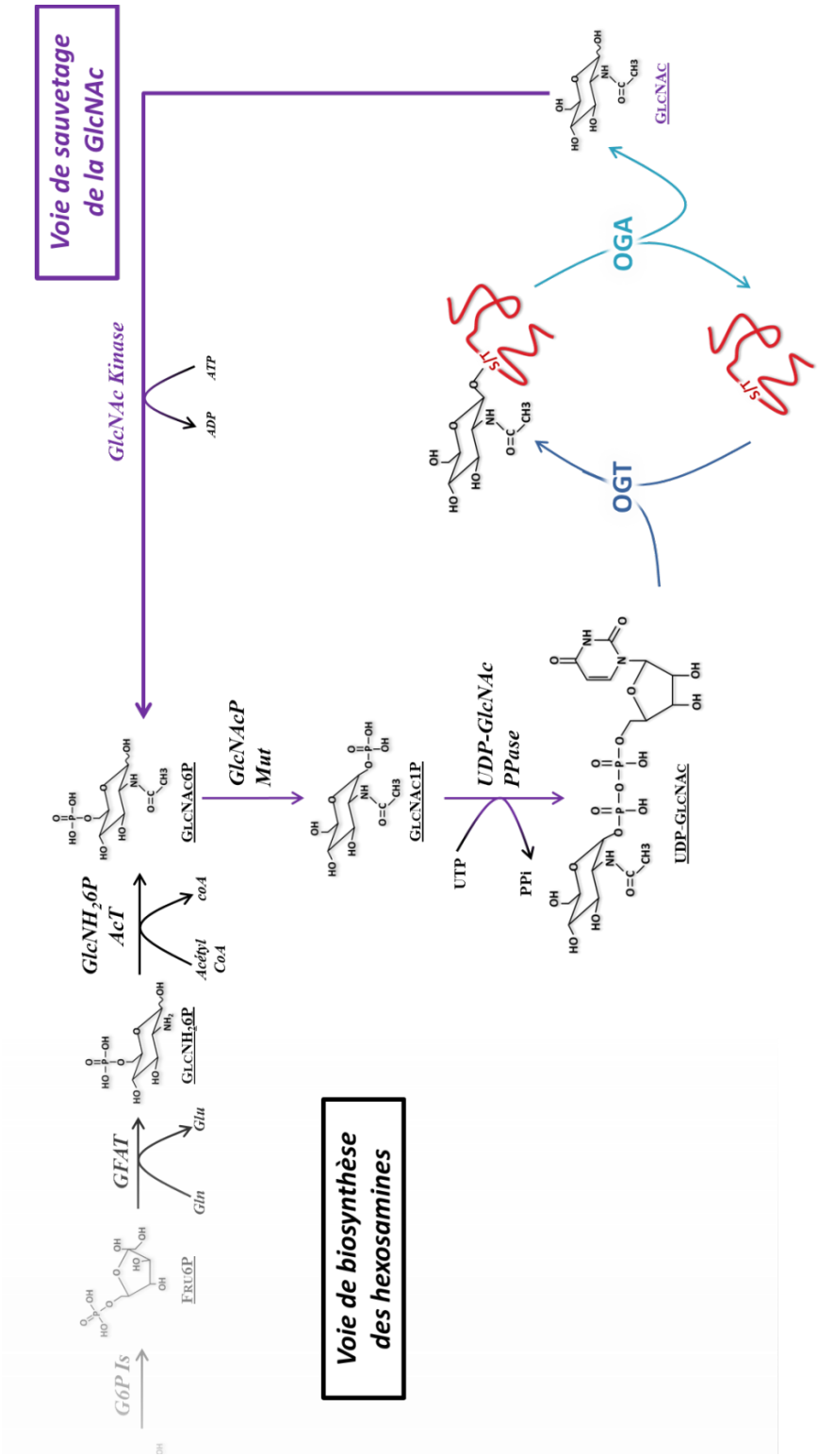


Figure 23 : la voie de sauvetage de la GlcNAc.

GlcNAcP Mut: N-acétylglucosamine phosphate mutase ; UDP-GlcNAc PPase: UDP-N-acétylglucosamine pyrophosphorylase ; UTP : Uridine Tri-Phosphate

Il existe deux isoformes de la GFAT réparties différemment au niveau tissulaire : la GFAT1 est retrouvée majoritairement dans le cœur, le placenta, les ovaires et les testicules tandis que la GFAT2 est présente en abondance dans le système nerveux central (Oki et al., 1999).

L'utilisation de la glucosamine a permis également d'approfondir les connaissances sur cette voie métabolique. En effet, la glucosamine, après phosphorylation, rejoint la voie des hexosamines sous la forme de glucosamine-6-phosphate et entraîne une dérégulation totale de la production d'UDP-GlcNAc, normalement finement régulée par la GFAT. Son rôle dans l'apparition de la résistance à l'insuline, en outrepassant le contrôle effectué par la GFAT, a d'ailleurs été clairement démontré (Marshall et al., 1991b).

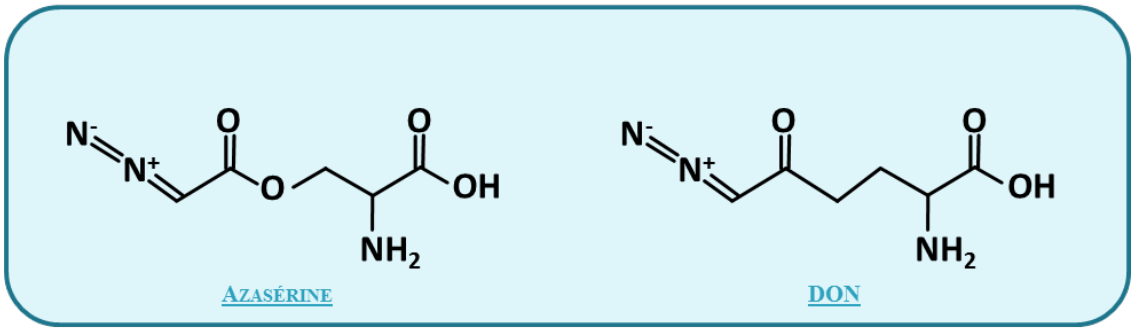
Une des enzymes de cette voie métabolique, la Glucosamine-6-phosphate acétyltransférase, est essentielle puisque son inactivation par recombinaison homologue entraîne une mort embryonnaire précoce (7.5 jours) (Boehmelt et al., 2000b). Elle permet notamment d'établir le lien entre la voie des hexosamines, le métabolisme des acides gras et la voie de sauvetage de l'UDP-GlcNAc (Boehmelt et al., 2000a).

2. La voie de sauvetage de l'UDP-GlcNAc

Il existe également une voie connexe à la voie des hexosamines permettant de synthétiser l'UDP-GlcNAc en circuit fermé : il s'agit de la voie de sauvetage de l'UDP-GlcNAc. Cette voie permet la récupération de la N-acétylglucosamine hydrolysée par l'OGA et sa réincorporation dans la voie de biosynthèse des hexosamines. La GlcNAc hydrolysée est phosphorylée par la GlcNAc kinase et réintégrée sous la forme de GlcNAc-6-Phosphate dans la voie des hexosamines (Figure 23).

Néanmoins, cette voie de sauvetage ne supporte pas une carence trop importante en glucose comme le montre l'étude de Boehmelt. En effet, la voie de biosynthèse des hexosamines reste indispensable à la synthèse d'UDP-GlcNAc et au bon fonctionnement cellulaire malgré l'existence de cette voie de sauvetage fournissant le même produit (Boehmelt et al., 2000b).

Inhibiteurs de GFAT



Analogue de…

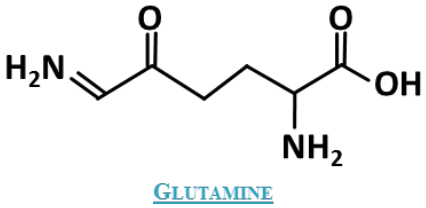


Figure 24 : Les inhibiteurs de la GFAT. DON : 6-Diazo-5-Oxo Norleucine.

GFAT : Glutamine : Fructose-6-phosphate amidotransférase.

Par contre, cette voie de secours permettrait de maintenir un niveau suffisant d'UDP-GlcNAc sur une courte période après dérégulation de la GFAT ou en cas de défaillance dans le métabolisme de la glutamine.

De plus, des analyses *in silico* ont montré qu'il existerait une interaction entre la GlcNAc kinase et la glucosamine-6-phosphate acétyltransférase, dans le but de maintenir une quantité suffisante d'UDP-GlcNAc dans la cellule quel que soit l'état nutritionnel (NAGK protein-STRING interaction network).

3. Régulation et modulation de la voie de biosynthèse des hexosamines

La régulation de la GFAT, enzyme limitante de la voie de biosynthèse des hexosamines, a été très étudiée au cours de ces dernières années.

On sait que le produit final de cette voie, l'UDP-GlcNAc (comme c'est le cas pour la majorité des voies métaboliques), en est un inhibiteur suggérant une boucle de rétro-contrôle, (Kornfeld et al., 1964 ; Kornfeld,1967). D'autre part, le produit de l'enzyme, la glucosamine-6-phosphate serait également capable de diminuer son activité (Broschat et al., 2002).

La phosphorylation de la sérine 243 par AMPK et CAMKII augmente l'activité de la GFAT en réponse aux variations physiologiques (AMP, Ca^{2+}) (Li et al., 2007). La phosphorylation de l'enzyme par PKA sur la sérine 205 régulerait également son activité mais avec un effet différent sur l'une ou l'autre des isoformes de l'enzyme : effet activateur pour GFAT2 et inhibiteur pour GFAT1 (Chang et al., 2000 ; Hu et al., 2004).

La difficulté première d'obtenir des inhibiteurs efficaces d'OGT a été palliée pendant plusieurs années par l'existence d'inhibiteurs de la synthèse de l'UDP-GlcNAc. Deux inhibiteurs sont tout particulièrement utilisés : le DON (6-Diazo-5-Oxo-L-Norleucine) et l'Azasérine (*O*-Diazoacétyl-L-serine) (Marshall et al., 1991b) (Figure 24). Ce sont deux analogues structuraux de la glutamine non métabolisables qui bloquent la GFAT, induisant ainsi une chute des niveaux d'UDP-GlcNAc et, par voie de conséquence, de *O*-GlcNAcylation. D'autre part, et comme l'ont démontré Stephen Marshall et collaborateurs, la glucosamine permet d'outre passer la régulation imposée par la GFAT.

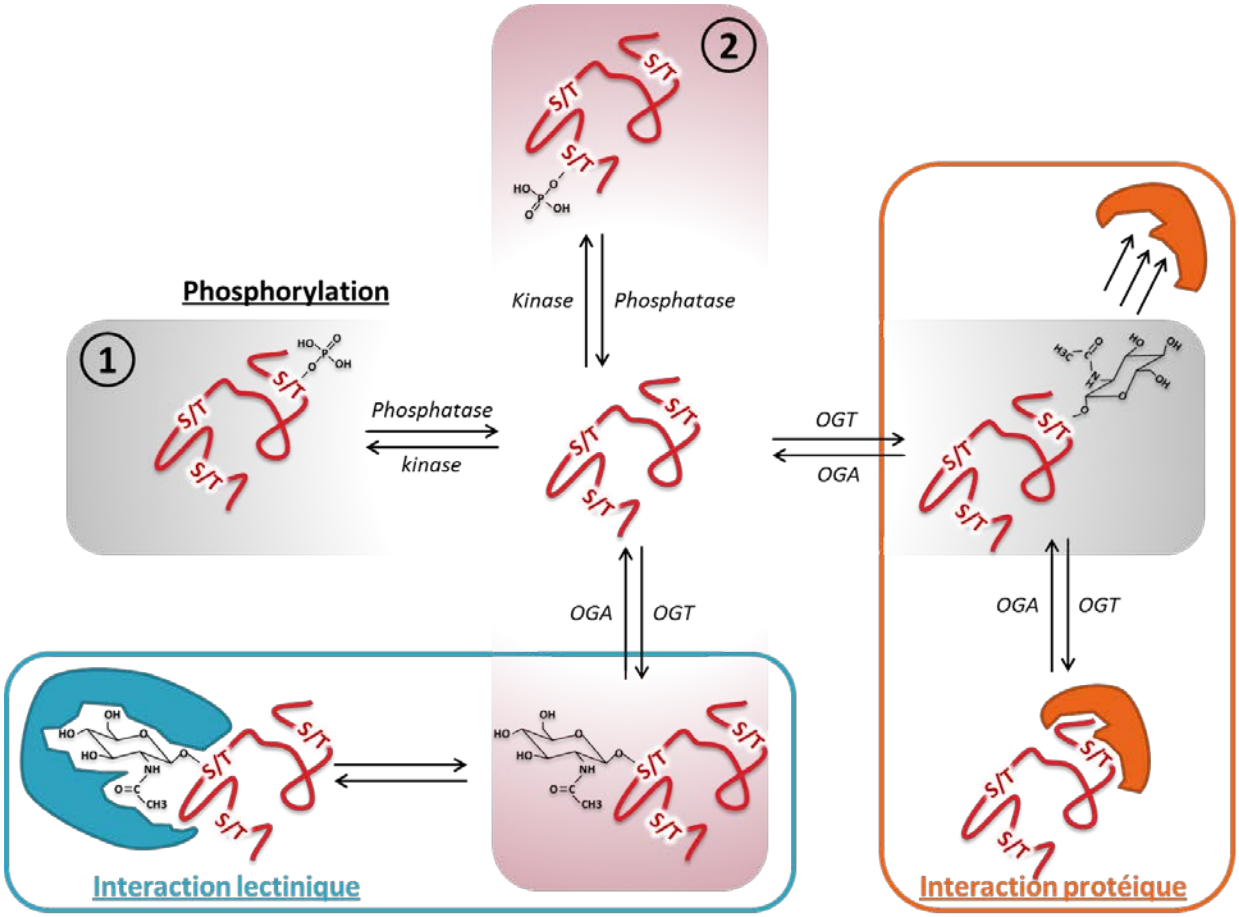


Figure 25 : La *O*-GlcNAcylation : quels changements pour la protéine ? La *O*-GlcNAcylation peut entrer en compétition avec la phosphorylation sur un même résidu d'acide aminé (1) ou sur des résidus adjacents (2). L'ajout d'un résidu de GlcNAc peut également moduler les interactions protéiques ou encore entrainer une activité de type lectinique.

La glucosamine est donc un composé particulièrement efficace utilisé pour augmenter "artificiellement" les niveaux d'UDP-GlcNAc même si sa conversion en glucosamine-6-phosphate entraîne des déplétions rapides en ATP critiques voire létaux pour la cellule (Marshall et al., 1991a).

Enfin plus récemment, l'étude de la voie de sauvetage a permis l'élaboration d'un inhibiteur efficace d'OGT, l'acétyl-5SGlcNAc (Figure 24). La péracétylation de ce composé lui permet de pénétrer efficacement dans la cellule, les groupements acétyles sont alors rapidement hydrolysés par des estérases. L'inhibiteur est ensuite phosphorylé par la GlcNAc kinase puis intégré à la voie de biosynthèse des hexosamines (Figure 23). Sa transformation en UDP-5SGlcNAc bloque le site catalytique de l'OGT (Gloster et al., 2011).

D. *O*-GlcNAcylation : quels changements pour la protéine ?

La O-GlcNAcylation entraîne des changements conformationnels locaux offrant des surfaces d'interaction différentes pour une mutitude de partenaires. La demi-vie de la protéine mais également sa localisation subcellulaire peuvent alors subir des modifications profondes : par conséquent, l'activité de la protéine O-GlcNAcylée est modifiée (Figure 25).

1. Phosphorylation et *O*-GlcNAcylation

La *O*-GlcNAcylation et la phosphorylation possèdent de nombreux points communs. En effet, ces deux modifications peuvent occuper les mêmes groupements hydroxyles des résidus de sérine ou de thréonine, les deux modifications s'excluant mutuellement l'une-l'autre (compétition de sites). En effet, au cours d'un traitement par l'acide okadaïque, un inhibiteur de phosphatases, on observe une diminution des protéines sous leur forme *O*-GlcNAcylée (Lefebvre et al., 1999), alors que l'inhibition des kinases PKA ou PKC (« Protein Kinase » A ou C) augmente de manière conséquente la *O*-GlcNAcylation de ces mêmes protéines (Griffith et al., 1999).

Néanmoins, Wang et al. ont démontré que l'inhibition de GSK3β, une kinase modifiant un très large éventail de protéines, se traduit par une augmentation de la *O*-GlcNAcylation de certaines protéines (majoritairement des protéines de choc thermique et cytosquelettiques) et par une diminution de la *O*-GlcNAcylation d'autres protéines (principalement des facteurs de transcription et des protéines liant l'ARN) (Wang et al., 2007). Il existe donc une relation complexe entre phosphorylation et *O*-GlcNAcylation, soutenue par l'existence de complexes OGT/OGA/kinases/phosphatases (Slawson et al., 2008 ; Cheung et al., 2008).

On note l'existence de nombreux exemples de compétitions site-spécifique entre ces deux modifications (Figure 25 -1) comme c'est le cas par exemple pour c-myc (Chou et al., 1995a), le récepteur aux œstrogènes β (Cheng et al., 2001), eNOS (endothelial Nitric Oxid Synthase) (Du et al., 2001) ou encore l'ARN polymérase II (Comer et al., 2001). Cette compétiton peut également apparaître sur des résidus adjacents (Figure 25-2). C'est le cas pour la vimentine (Slawson et al., 2008), p53 (Yang et al., 2006), CAMKIV (Dias et al., 2009), FOXO1 (Housley et al., 2008) ou encore Snail1 (Park et al., 2010). Il peut exister également des régions distinctes pour ces deux modifications au niveau de la protéine comme c'est le cas pour les kératines 8 et 18 (Chou et al., 1992 ; Omary et al., 1998).

Enfin, les deux modifications peuvent également cohabiter sur la même molécule comme pour IRS1 (Ball et al., 2006) ou MCC (Myosin Cardiac Chain) (Ramirez-Correa et al., 2008) par exemple.

2. Interactions protéiques

De par sa taille, la *O*-GlcNAcylation peut entraîner un encombrement stérique modifiant les interactions entre protéines (Figure 25). C'est le cas pour le facteur de transcription Sp1 sous sa forme *O*-GlcNAcylée qui diminue fortement ses interactions avec HoloSp1, TAF110 (TATA-binding-protein Associated Factor 110) et SREBP2 (Sterol Regulatory Element Binding Protein 2) (Roos et al., 1997 ; Lim et Chang 2010). Les protéines CREB et YY1 perdent également leurs interactions respectives avec TAFII130 et pRb (protéine du Rétinoblastome) suite à leur *O*-GlcNAcylation (Lamarre-Vincent et al., 2003 ; Hiromura et al., 2003). Inversement, certaines interactions protéiques se font préférentiellement sous la forme *O*-GlcNAcylée, c'est le cas pour l'interaction STAT5-CBP (C-terminal Binding Protein) (Gewinner et al.,2004).

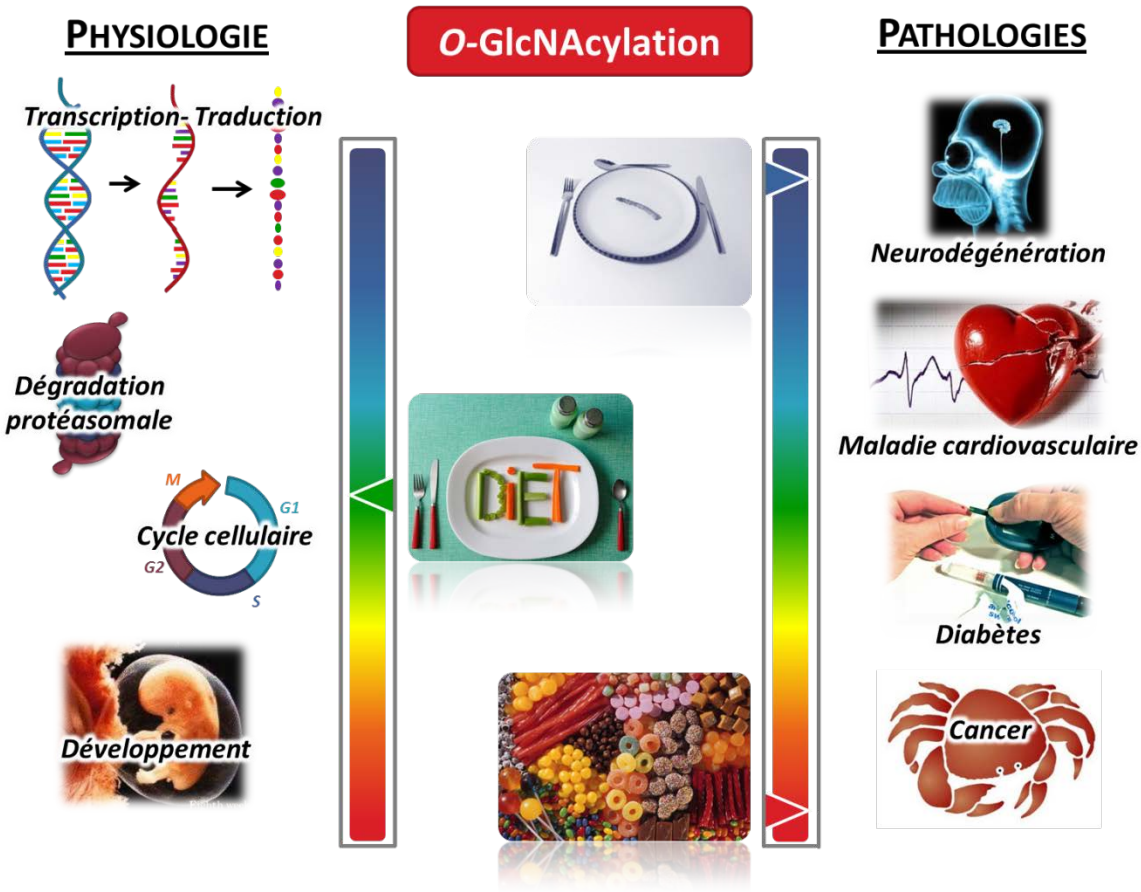


Figure 26 : Les fonctions physiologiques et dérégulations pathologiques associées à la *O*-GlcNAcylation. L'apport alimentaire entraine des changements dans les niveaux de *O*-GlcNAcylation. Des variations raisonnables de ses niveaux permettraient de réguler les processus physiologiques majeurs tandis que des changements trop extrêmes engendreraient l'émergence de pathologies.

On observe également une interaction de type lectinique reconnaissant spécifiquement le résidu sucré de GlcNAc (Figure 25). La capacité de certaines protéines à développer des propriétés lectiniques, comme les Hsc70 et Hsp70, engendrerait une fonction de protection des protéines *O*-GlcNAcylées, en réponse notamment à un stress cellulaire (Lefebvre et al., 2001 ; Guinez et al., 2004; Guinez et al., 2007).

E. *O*-GlcNAcylation : entre physiologie et pathologie

La *O*-GlcNAcylation permet de retranscrire l'état nutritionnel de la cellule sur ses activités intracellulaires essentielles. Elle intervient donc dans toute une pléthore de processus physiologiques par la modification de milliers de protéines impliquées dans la transcription, la dégradation protéique ou encore le cycle cellulaire et le développement embryonnaire. Néanmoins, dans des conditions de dérèglement d'ordre nutritionnel, cela pourrait se répercuter sur une modification anormale des protéines et une possible dérégulation des voies de signalisation entrainant sur le long cours la pathologie (Figure 26).

1. *O*-GlcNAcylation dans un contexte physiologique

Transcription, traduction et dégradation protéique sont des processus fondamentaux régulant des processus plus complexes comme le cycle cellulaire ou encore le développement embryonnaire. La O-GlcNAcylation intervient à différents niveaux de ces processus vitaux afin d'en assurer le bon fonctionnement.

a) Transcription et traduction

Du fait d'une expression particulièrement élevée au niveau nucléaire, des travaux pionniers sur la *O*-GlcNAcylation ont suggéré son implication très forte dans les phénomènes de transcription ou de remodelage de la chromatine (Jackson and Tjian, 1988 ; Kelly and Hart, 1989). On sait aujourd'hui que la *O*-GlcNAcylation modifie entre autres les histones (Sakabe et al., 2010 ; Zhang et al., 2011b).

Au niveau transcriptionnel, la *O*-GlcNAcylation joue un rôle essentiel en modifiant le domaine C-terminal de l'ARN polymérase II (Kelly et al., 1993). Le complexe de pré-initiation serait composé d'une forme *O*-GlcNAcylée de l'ARN polymérase II qui basculerait vers la forme phosphorylée au cours de la phase d'élongation (Comer et Hart, 1999 ; Comer et Hart, 2001).

Récemment il a été démontré que l'OGT était codé par le gène *Super sex combs* (*Sxc*) à proximité du locus Xist (Lin et al., 2007). Ce gène fait partie du groupe Polycomb (PcG) codant des répresseurs transcriptionnels intervenant au cours du développement (Sinclair et al., 2009 ; Gambetta et al., 2009). Il est intéressant de noter que les éléments de réponse Polycomb, site de fixation sur l'ADN des protéines du même nom, sont eux-mêmes particulièrement enrichis en protéines *O*-GlcNAcylées, appuyant un peu plus le caractère fondamental de l'OGT et soulignant le rôle de la *O*-GlcNAcylation dans la régulation de la transcription au cours du développement (Love et al., 2010).

Quelques équipes se sont également penchées sur l'effet de la *O*-GlcNAcylation sur la traduction et ont montré que les activités des facteurs d'initiation de la traduction eIF2 (eucaryotic chain Initiation Factor 2) et d'élongation EF1 (Elongation Factor 1) sont modifiés par *O*-GlcNAcylation (Datta et al., 1989, Dehennaut et al., 2008b). De nombreuses protéines du complexe ribosomal sont également *O*-GlcNAcylées (sous-unités 40S, 60S et 80S) (Dehennaut et al., 2008b). Bien qu'on ignore le rôle de cette modification sur l'activité ribosomale, elle permettrait la stabilisation de ses sous-unités (60S et la 80S) (Zeidan et al., 2010).

b) La dégradation des protéines

Le système Ubiquitine-Protéasome régule la dégradation des protéines et libère la cellule de protéines mal conformées et potentiellement toxiques, ou de protéines en fin de vie. A ce niveau, la *O*-GlcNAcylation intervient à différents niveaux. En premier lieu, de nombreuses études ont montré un lien entre *O*-GlcNAcylation et demi-vie protéique, reliant l'augmentation de la demi-vie de la protéine à sa *O*-GlcNAcylation. Ceci a été démontré sur des protéines telles que Sp1, le récepteur aux l'œstrogènes β, la plakoglobine et p53 (Han et Kudlow, 1997 ; Cheng et al., 2000 ; Hatsell et al., 2003 ; Yang et al., 2006).

D'autre part, la *O*-GlcNAcylation modifie la sous-unité catalytique 20S du protéasome 26S (Sumegi et al., 2003) et inhibe le complexe macromoléculaire *in vitro* par diminution de l'activité de sa sous-unité régulatrice 19S (Zhang et al., 2003).

Enfin, E1 (ubiquitin-activating enzyme), une des enzymes du système d'ubiquitinylation, est elle-même *O*-GlcNAcylée, et une augmentation de son *O*-GlcNAcylation corrèle avec un niveau élevé d'ubiquitinylation des protéines suggérant un lien étroit entre les deux modifications post-traductionnelles (Guinez et al., 2008).

c) Le cycle cellulaire

L'implication de la *O*-GlcNAcylation dans la progression du cycle cellulaire a été proposée très tôt du fait que de nombreuses protéines sont *O*-GlcNAcylées en réponse aux facteurs mitogènes dans les lymphocytes T (Kearse et Hart, 1991). Cette stimulation entraîne une augmentation rapide et transitoire de la *O*-GlcNAcylation des protéines nucléaires, concomitante à une diminution pour les protéines cytosoliques. Le rôle essentiel de la *O*-GlcNAcylation dans le contrôle du cycle cellulaire a également été démontré sur l'ovocyte de Xénope où l'inhibition de l'OGT entraîne le blocage de la reprise méiotique en réponse à la progestérone (Dehennaut et al., 2007, 2008a).

De plus, l'inhibition de l'OGA et l'augmentation subséquente de la *O*-GlcNAcylation ralentie la progression en phase S ou G2/M tandis que l'inhibition de la GFAT, et donc la diminution de l'UDP-GlcNAc, accélère ces mêmes phases et ralentit la progression en phase G1 (Slawson et al., 2005). Récemment, il a été démontré que l'OGA est nécessaire dans le maintien de l'intégrité du génome au cours du cycle cellulaire, limitant le vieillissement dû notamment aux nombreuses duplications de l'ADN (Yang et al., 2012).

Enfin, Slawson et collaborateurs ont observé une forte concentration d'OGT au niveau du midbody, fragment résiduel de la cytokinèse. A ce niveau, l'OGT est en complexe avec l'OGA, la kinase Aurora B et PP1 afin de modifier des protéines de structure comme la vimentine, et participe de ce fait à la séparation des cellules filles (Slawson et al., 2008).

Toutes ces observations tendent à prouver que le dynamisme de la *O*-GlcNAcylation impose une régulation complexe au cycle cellulaire en modifiant toute une panoplie de régulateurs parmi lesquels Erk2 et Sp1 (Dehennaut et al., 2008b ; Vicart et al., 2006).

Sur ce point, l'étude de la *O*-GlcNAcylation au cours de la transition G1/S a fait l'objet d'une publication jointe en annexe :

Drougat L, **Olivier-Van Stichelen S**, Mortuaire M, Foulquier F, Lacoste AS, Michalski JC, Lefebvre T, Vercoutter-Edouart AS (2012) Characterization of *O*-GlcNAc Cycling and Proteomic Identification of Differentially *O*-GlcNAcylated Proteins during G1/S transition. *BBA General subject 1820 (12):1839-1848*

Dans cette étude, notre équipe a montré une diminution générale de la *O*-GlcNAcylation au cours de la transition G1/S, due probablement à une augmentation de l'activité de la *O*-GlcNAcase. Nous avons également identifié 61 protéines différentiellement *O*-GlcNAcylées au cours du cycle parmi lesquelles les protéines régulatrices de la réplication de l'ADN, MCM (MiniChromosome Maintenance protein) 3, 6 et 7, renforçant plus encore l'implication de la *O*-GlcNAcylation dans le cycle cellulaire.

d) Le développement embryonnaire

L'implication de la *O*-GlcNAcylation dans l'embryogenèse a été mise en évidence par le Knock-out létal de l'OGT chez des souris au stade embryonnaire (Shafi et al., 2000). De même, le Knock-out inductible de l'OGT entraîne la mort des tissus cibles et de l'embryon quelques jours après son implantation (O'Donnell et al., 2004).

L'étude des niveaux de *O*-GlcNAcylation au cours de l'embryogénèse de Xénope (*Xenopus* laevis) a montré qu'il existait, tout au moins jusqu'à l'organogenèse, des variations de cette modification post-traductionnelle (Dehennaut et al., 2009).

Chez le poisson zèbre, des anomalies sévères de développement ont été observées en réponse à la modulation de l'expression des deux enzymes du cycle de la *O*-GlcNAcylation, OGA et OGT (Webster et al., 2009).

On observe de plus, chez cet organisme une variation d'expression des six différents transcrits (var1/6) de l'OGT (zOGT) au cours de l'embryogénèse. Ces trois études, réalisées sur deux modèles distincts, soulignent le caractère crucial de l'OGT au cours du développement (Sohn et Do, 2005).

2. *O*-GlcNAcylation dans un contexte pathologique

Cette partie a fait l'objet d'une publication, jointe en annexe :

Lefebvre T, Dehennaut V, Guinez C**, Olivier S**, Drougat L, Mir AM, Mortuaire M, Vercoutter-Edouart AS, Michalski JC. (2010) Dysregulation of the nutrient/stress sensor *O*-GlcNAcylation is involved in the etiology of cardiovascular disorders, type-2 diabetes and Alzheimer's disease. *Biochim Biophys Acta 2010 Feb; 1800(2):67-79*

a) La neurodégénération

La position de l'OGA sur un locus lié à la maladie d'Alzheimer, 10q24.1-10q24.3, et de l'OGT sur un locus lié à la maladie de Parkinson, Xq13.1, laisse entrevoir une implication majeure de la *O*-GlcNAcylation dans les processus de neurodégénération (Shafi et al., 2000 ; Gao et al., 2001 ; Nolte et al., 2003).

Dans les neurones de patients Alzheimer, on observe un défaut de régulation du métabolisme du glucose se répercutant probablement sur des défauts de *O*-GlcNAcylation de la cellule (Alexander et al., 2002 ; Liu et al., 2009). Ce phénomène est d'ailleurs assimilé à un diabète de type 3, car il regroupe des caractéristiques à la fois du diabète de type 1 et du diabète de type 2 (Kröner, 2009). Comme attendu, ce défaut métabolique entraîne une diminution conséquente des niveaux de *O*-GlcNAcylation dans ces mêmes neurones (Liu et al., 2009). Parmi les protéines impliquées dans cette pathologie, la *O*-GlcNAcylation de Tau a été la plus étudiée. La dégénérescence neurofibrillaire est un évènement important dans le développement de la maladie et est due à l'agrégation de la protéine Tau hyperphosphorylée.

Le traitement de neurones en culture par des agents favorisant ou diminuant la phosphorylation se répercute sur la *O*-GlcNAcylation de Tau (Griffith et Schmitz, 1999 ; Lefebvre et al., 2003 ; Liu et al., 2004), ce qui laisse supposer, par le biais d'une compétition directe ou indirecte entre *O*-GlcNAcylation et phosphorylation, une régulation très complexe de l'activité de Tau (Liu et al., 2004). Plusieurs sites de *O*-GlcNAcylation de Tau ont par ailleurs été identifiés (S400, S409, S412, S413) (Yuzwa et al., 2011, Smet-Nocca et al., 2011). Une diminution de la *O*-GlcNAcylation de Tau entraînerait donc son hyper-phosphorylation et l'initiation de la neurodégénérescence (Liu et al., 2009).

D'autres protéines, importantes dans cette maladie, sont également modifiées par *O*-GlcNAcylation comme le précurseur β-amyloïde mais sans que l'on comprenne pour l'instant le rôle de la modification (Griffith et al., 1995).

Enfin, quelques équipes ont essayé d'enrailler le processus de neurodégénération par l'augmentation artificielle de la *O*-GlcNAcylation chez la souris, induisant ainsi une diminution de l'agrégation et de la progression de la maladie (Yuzwa et al., 2008). Pour cela, des souris ont subi un traitement à base de Thiamet-G, induisant une augmentation des niveaux de *O*-GlcNAcylation et la diminution subséquente de la phosphorylation de Tau, particulièrement dans les régions corticales affectées par l'agrégation de tau dans la maladie d'Alzheimer. Ce dernier point permet notamment d'entrevoir des possibilités de traitement de la maladie d'Alzheimer.

En ce qui concerne les dystonies, quelques explications ont été proposées quant au lien entre *O*-GlcNAcylation et parkinsonisme via l'association de l'OGT avec THAP1 (THanatos-Associated Protein 1), dont la mutation favorise l'émergence de ce groupe de pathologies (Mazars et al., 2010).

b) Les maladies cardiovasculaires

Il a été montré que la *O*-GlcNAcylation possède des propriétés cardioprotectrices, prévenant notamment les dommages tissulaires engendrés suite à un infarctus, par exemple (Chatham et Marchase, 2010 ; Fülop et al., 2007 ; Jones, 2005). Néanmoins, l'augmentation prolongée des niveaux de *O*-GlcNAcylation de cardiomyocytes entraîne l'apparition de défauts liés à la diminution de SERCA2a (SarcoEndoplasmic Reticulum Ca^{2+}-ATPase 2a), protéine qui contribue au bon fonctionnement du muscle cardiaque (Clark et al., 2003).

Inversement, la surexpression de l'OGT dans des cardiomyocytes isolés de souris diabétiques, entraîne l'amélioration de la fonction contractile (Hu et al., 2005).

Il existe donc un effet protecteur à court terme de la *O*-GlcNAcylation sur les dommages cardiaques, mais également un effet pervers de cette modification en réponse à une exposition prolongée à de fortes concentrations en glucose similaires à celles retrouvées chez les patients diabétiques.

c) La cancérisation

La *O*-GlcNAcylation modifie l'activité ou la demi-vie de nombreux oncogènes ou suppresseurs de tumeur, suggérant son implication dans les processus de cancérisation, notamment dans des conditions de déséquilibre nutritionnel. Par exemple, l'oncogène c-myc, surexprimé dans la majorité des lymphomes (lymphomes de Burkitt en particulier) et dans un tiers des cancers du sein et du colon, est *O*-GlcNAcylé sur la thréonine 58, un site de phosphorylation par ailleurs fréquemment muté (Chou et al., 1995a ; Chou et al., 1995b). Le suppresseur de tumeur p53, muté dans 50 % des cancers, est lui aussi régulé par *O*-GlcNAcylation (Yang et al., 2006), de même que le récepteur aux œstrogènes β impliqué dans le développement des tumeurs mammaires (Cheng et Hart, 2001). Enfin, d'autres oncogènes ou suppresseurs de tumeurs sont modifiés, et par conséquent potentiellement régulés, par *O*-GlcNAcylation, c'est le cas de HIC1 (Lefebvre et al., 2004b), pRb (Wells et al., 2011), IKK (IκB kinase) (Kawauchi et al., 2009) ou encore la β-caténine (Lefebvre et al., 2004a).

Par ailleurs, plusieurs études réalisées sur différents types de cancers ont permis de souligner l'importance de la *O*-GlcNAcylation au cours de la progression tumorale et métastatique. Une augmentation quasi-systématique des niveaux de *O*-GlcNAcylation est observée dans les tissus cancéreux en comparaison aux tissus sains: sein (Caldwell et al., 2010 ; Gu et al., 2010), poumons, colon (Mi et al., 2011a) et foie (Zhu et al., 2011). L'inhibition de la *O*-GlcNAcylation permet d'ailleurs de limiter la progression tumorale dans ces différents tissus. De plus, la *O*-GlcNAcylation de la E-cadhérine et des MMPs (Matrix MetalloProtéinases) promeut l'invasion métastatique (Gu et al., 2010 ; Mi et al., 2011a).

On peut également supposer qu'un déséquilibre nutritionnel affectant les niveaux de *O*-GlcNAcylation, altère les caractéristiques des protéines impliquées dans la régulation du cycle cellulaire et participe ainsi à la prolifération incontrôlée caractéristique des cellules tumorales.

V. Alimentation, syndromes métaboliques et *O*-GlcNAcylation

Les désordres alimentaires et/ou l'apparition de désordres métaboliques comme le diabète ou l'obésité engendrent une augmentation chronique de la glycémie. Comme décrit dans la partie précédente, la O-GlcNAcylation est directement influencée par des variations de concentration en glucose. Nous allons ici décrire les différentes altérations qui permettent de moduler durablement la O-GlcNAcylation et les processus dans lesquels elle intervient.

A. *O*-GlcNAcylation et glycémie

Une étude du département Américain d'agriculture a récemment démontré que la consommation de sucre a augmenté de près de 20 % au cours de ces vingt dernières années, favorisant de ce fait l'incidence de pathologies liées à cette consommation en hausse (Haley et al., 2005). Une consommation chronique de sucres en grande quantité modifie durablement la glycémie de l'organisme (Brito et al., 2007). Les niveaux de *O*-GlcNAcylation, dépendant directement de la voie de biosynthèse des hexosamines et donc de la glycémie, sont par conséquent influencés par cette altération. A l'opposé, on sait que l'OGT adapte son activité en fonction des concentrations en UDP-GlcNAc, lui permettant d'assurer une activité de *O*-GlcNAcylation minimale même lorsque les concentrations en nucléotide sucre sont basses (Kreppel et Hart, 1999). Par conséquent, l'augmentation des niveaux d'UDP-GlcNAc entraine légitimement une *O*-GlcNAcylation accrue des protéines.

Figure 27 : Les différentes définitions des syndromes métaboliques. En jaune, critères prenant en compte la glycémie.

Néanmoins, l'exposition d'adipocytes à des concentrations croissantes de glucose montre une augmentation maximale de 30 % des niveaux d'UDP-GlcNAc, pour une concentration en glucose de 365 % supérieure à la normale (Bosch et al., 2004). Ceci suggère que d'autres éléments restant à définir freinent la production d'UDP-GlcNAc.

Malgré tout, on peut citer le rétrocontrôle de la GFAT par le surplus en UDP-GlcNAc. Il faut également souligner la nécessité d'avoir d'autres nutriments, acides aminés, acides gras ou nucléotides en quantité suffisante pour pourvoir aux stocks d'UDP-GlcNAc. Un régime riche a donc pour conséquence l'augmentation globale des niveaux d'UDP-GlcNAc.

Dans ce sens, Medford et collaborateurs ont démontré qu'une consommation à long terme basé sur un régime occidental (« western diet »), saturé en sucres et en acides gras, entraînait une augmentation des protéines *O*-GlcNAcylées (Medford et al., 2012). Cette même augmentation est observée chez des rats soumis à un régime riche en lipides (Li et al., 2005).

Le régime alimentaire impacterait de ce fait sur la *O*-GlcNAcylation de toute une pléthore de protéines : des désordres alimentaires et métaboliques dérégueleraient, par ce biais, des processus biologiques essentiels.

B. *O*-GlcNAcylation et syndromes métaboliques

Lié le plus souvent au mode de vie mais aussi à certains facteurs génétiques, le syndrome métabolique se caractérise, entre autres, par une dérégulation chronique de la glycémie. Le syndrome métabolique est considéré comme un problème de santé publique majeur des sociétés occidentales. Ce type de syndrome a été décrit pour la première fois par Kylin, il y a près de 80 ans (Nilsson, 2005). Ces désordres sont caractérisés par une hypertension, une hyperglycémie et une obésité abdominale, critère rajouté par Vague en 1979 (Vague et al., Edited by Mancini et al., London Academic Press, 1979). A l'heure actuelle, les syndromes métaboliques sont définis par différentes organisations telles que l'organisation mondiale de la santé, la fédération internationale du diabète, le groupe Européen d'étude de la résistance à l'insuline ou encore le programme national Américain d'information sur le cholestérol (Zimmet et al., 2005) (Figure 27). On retrouve une constante dans les différentes définitions des syndromes métaboliques : l'hyperglycémie à jeun (Figure 27- Jaune).

Du fait d'une augmentation quasi-systématique des taux de glucose, les syndromes métaboliques sont potentiellement associés à une augmentation anormale de *O*-GlcNAcylation.

Au-delà des syndromes métaboliques, le diabète de type 2, l'obésité, les troubles et les accidents cardiovasculaires sont également des pathologies associées à des variations de *O*-GlcNAcylation (cf partie IV.F.2).

On observe particulièrement que l'incidence des désordres métaboliques corrèle considérablement avec le risque de développer un cancer ou une pathologie neurodégénérative (Barone et al., 2008 ; Aronson, 2008 ; Brownlee et al., 2001).

C. La *O*-GlcNAcylation et le diabète de type 2 : une relation complexe

Le diabète de type 2 est le sujet de toutes les préoccupations depuis de nombreuses années, notamment dans le monde occidental. L'impact de la *O*-GlcNAcylation sur l'étiologie de cette pathologie a également été particulièrement étudié.

Le diabète de type 2 se caractérise principalement par le phénomène de résistance à l'insuline qui se traduit par l'incapacité pour l'organisme de répondre à l'insuline (Figure 28).

En réponse à une augmentation de la glycémie, l'organisme stimule normalement une sécrétion d'insuline par les cellules β du pancréas. L'insuline ordonne aux organes cibles, insulino-sensibles, comme le foie, les muscles ou le cerveau (cellules gliales), de stocker le glucose sous forme de glycogène et/ou de l'utiliser (Wang et al.,2009). La stimulation de la voie de signalisation de l'insuline permet notamment la relocalisation membranaire des transporteurs de glucose GLUT4 au niveau adipocytaire et musculaire, l'entrée du glucose dans la cellule et l'activation de la glycolyse et de la lipogénèse pour stocker le glucose sous la forme de triglycérides (Figure 28 A).

Chez les patients diabétiques, on observe une désensibilisation de ces cellules vis-à-vis de l'insuline (Ferrannini, 1998). Le transporteur GLUT4 n'étant pas acheminé à la membrane, l'organisme n'internalise plus suffisamment le glucose circulant, une augmentation durable de la glycémie est alors observée.

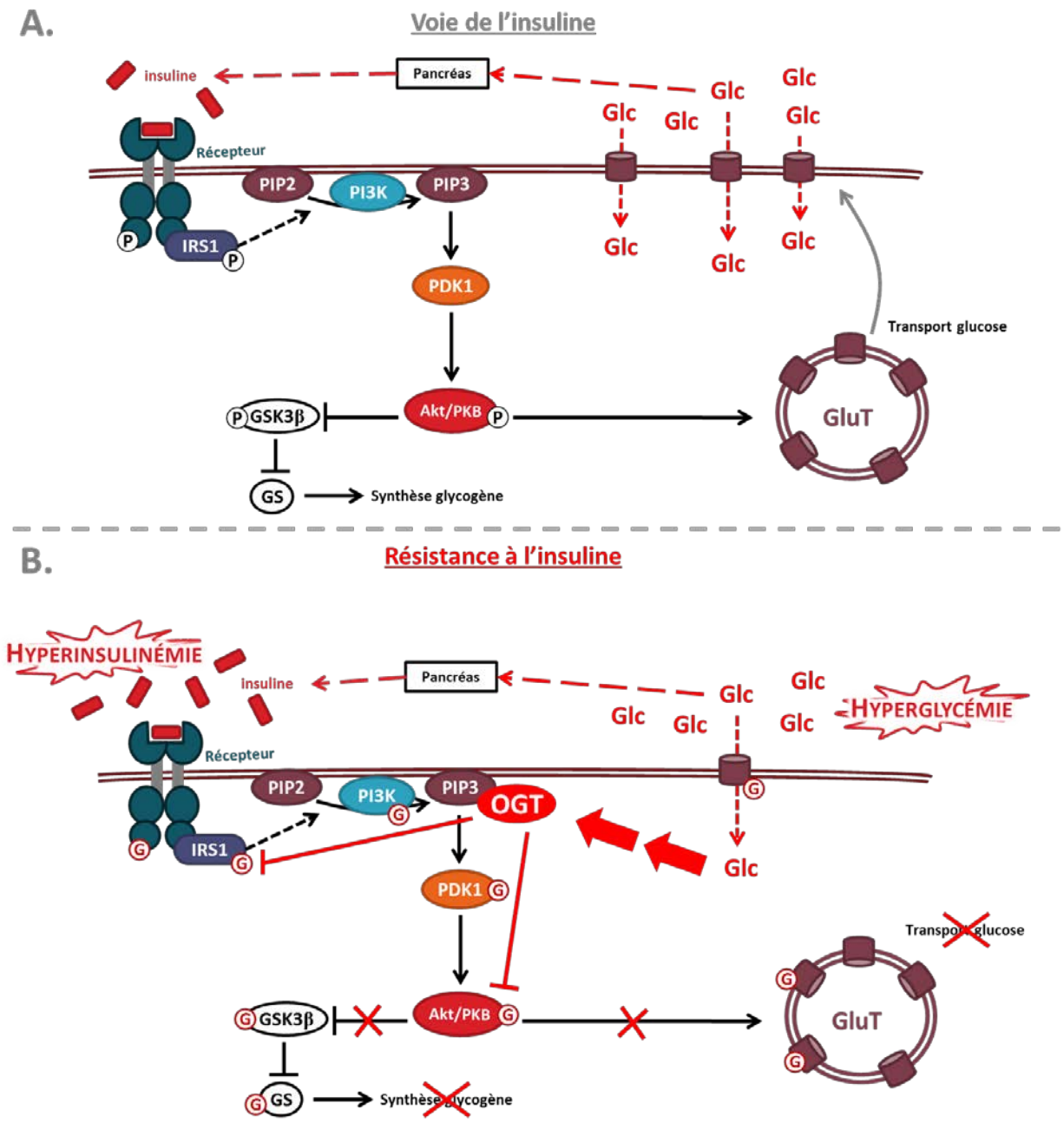


Figure 28 : Voie de signalisation de l'insuline et apparition de la résistance liée à la *O*-GlcNAcylation. A) La voie de signalisation de l'insuline entraîne l'entrée du glucose dans la cellule et son stockage. B) La résistance à l'insuline apparait au cours d'une exposition prolongée des tissus cibles à l'insuline. La *O*-GlcNAcylation de nombreux composants de la voie inhiberait leur activité et entrainerait ainsi une désensibilisation de la cellule en réponse à l'insuline. De plus, la non-internalisation du glucose accentue d'autant plus ce phénomène par le maintien d'une glycémie élevée.

Akt/PKB: Protein Kinase B ; G : GlcNAc ; Glc : glucose ; GS glycogène synthétase ; GSK3β : glycogen synthase kinase 3β ; IRS1 : Insulin Receptor substrate 1 ; OGT : O-GlcNAc transférase ; P : Phosphorylation ; PDK1: Phosphoinositide-Dependent Kinase 1 ; PI3K : Phoshatidylinositol 3 kinase; PIP2 : PhosphatidylInositol (4,5) bisPhosphate ; PIP3 : PhosphatidylInositol (3, 4, 5) triPhosphate .

Les cellules bêta synthétisent et sécrètent plus d'insuline en réponse à l'hyperglycémie, une hyperinsulinémie chronique se met en place avec pour conséquence une auto-amplification de la résistance à l'insuline (Figure 28 B) (Reaven, 1976 ; Yalow et Berson, 1996).

Les mécanismes par lesquels la cellule acquiert cette résistance à l'insuline ne sont pour l'instant pas complétement définis. Néanmoins une partie de la réponse résiderait dans un déséquilibre du dynamisme de la *O*-GlcNAcylation de la voie de l'insuline.

En effet, la glycémie chronique élevée observée chez les patients diabétiques montre une augmentation systématique des niveaux de *O*-GlcNAcylation au sein des cardiomyocytes notamment (Fülöp et al., 2007 ; Marsh et al., 2011). De plus, bon nombre d'effecteurs de la voie de l'insuline sont *O*-GlcNAcylés comme le récepteur de l'insuline (chaîne β), IRS1 (Insulin Receptor substrate 1), PI3K (Phoshatidylinositol 3 kinase), PDK1 (Phosphoinositide-Dependent Kinase 1), Akt/PKB (Figure 28 B) (Ball et al., 2006 ; Klein et al., 2009 ; Whelan et al., 2010 ; Gandy et al., 2006).

L'existence d'une compétition entre la phosphorylation, normalement activatrice de cette voie, et la *O*-GlcNAcylation des protéines citées ci-dessus a permis d'émettre l'hypothèse suivante : au cours d'une stimulation par l'insuline, la phosphorylation d'IRS1, de PI3K et d'Akt/PKB joue un rôle activateur (Figure 28 A) (Holman et Kasuga, 1997). La *O*-GlcNAcylation de ces mêmes protéines bloque la phosphorylation et l'activation de la voie. Ce phénomène est favorisé par un recrutement de l'OGT à la membrane plasmique, *via* son domaine PPO (Yang et al., 2008), et la phosphorylation subséquente d'une de ses tyrosines par le récepteur de l'insuline (Whelan et al., 2008). La *O*-GlcNAcylation, par exemple, du domaine SH2 d'IRS1 diminue fortement son interaction avec PI3K, résultant en une atténuation de la signalisation de l'insuline (Klein et al., 2009). De même, la *O*-GlcNAcylation d'Akt/PKB en réponse à l'insuline diminue, d'une part, sa phosphorylation activatrice sur T308 et, d'autre part, la phosphorylation inhibitrice de GSK3β sur S9, ce qui engendre une forte diminution du signal (Vosseler et al., 2002 ; Gandy et al., 2006). Enfin, la glycogène synthétase et le transporteur GLUT4 sont *O*-GlcNAcylés en réponse à l'insuline, ce qui se traduit par une diminution de l'activité de l'enzyme et de la translocation membranaire du transporteur (Park et al., 2005 ; Parker et al., 2004).

De nombreuses études ont tenté de prouver l'hypothèse selon laquelle une *O*-GlcNAcylation aberrante serait en partie responsable de la résistance à l'insuline.

Par exemple, la surexpression adipocytaire et musculaire de l'OGT induit une hyperinsulinémie chez la souris (Marshall et al., 2004).

Par ailleurs, l'utilisation de PUGNAc ou de glucosamine entraîne un défaut de signalisation de l'insuline (Vosseller et al., 2002) alors qu'un inhibiteur plus spécifique de l'OGA, le NButGT, ne provoque pas cet effet (Macauley et al., 2008).

Une autre étude montre que la diminution de la *O*-GlcNAcylation chez des souris diabétiques les re-sensibilise à l'insuline (Dentin et al., 2008). De même, l'utilisation d'une OGT tronquée et ne possédant plus de domaine PPO, n'entraine pas de résistance à l'insuline (Yang et al., 2008).

Il semble donc bien exister un lien étroit entre diabète, signalisation de l'insuline et *O*-GlcNAcylation. Néanmoins, il semble que la *O*-GlcNAcylation ne soit qu'un phénomène responsable, parmi d'autres, de la résistance à l'insuline.

Afin de mieux comprendre cette relation étroite, notre équipe s'est penchée sur l'étude de la *O*-GlcNAcylation en réponse à l'insuline au niveau des lipides rafts. Cette étude fait l'objet de l'article joint en annexe :

Perez-Cervera Y, Guedri K, Dehennaut V, **Olivier-Van Stichelen S**, Solorzano Mata C, Michalski JC, Foulquier F, Lefebvre T (2012) Insulin signaling controls the expression of *O*-GlcNAc transferase and its interaction with lipid microdomains, *FASEB J* (soumis pour publication)

Dans cette étude, nous avons notamment confirmé que l'OGT était recrutée au niveau de zones particulières de la membrane plasmique appelée radeaux lipidiques ou « rafts », véritables plateformes d'interaction, d'échange et de communication entre le milieu environnant et la cellule. De plus, l'inhibition de la voie de signalisation de l'insuline par la Wortmannine (inhibiteur de la PI3K) empêche le recrutement de l'OGT à la membrane. Ceci appuie une fois de plus la relation complexe entre voie de l'insuline et *O*-GlcNAcylation.

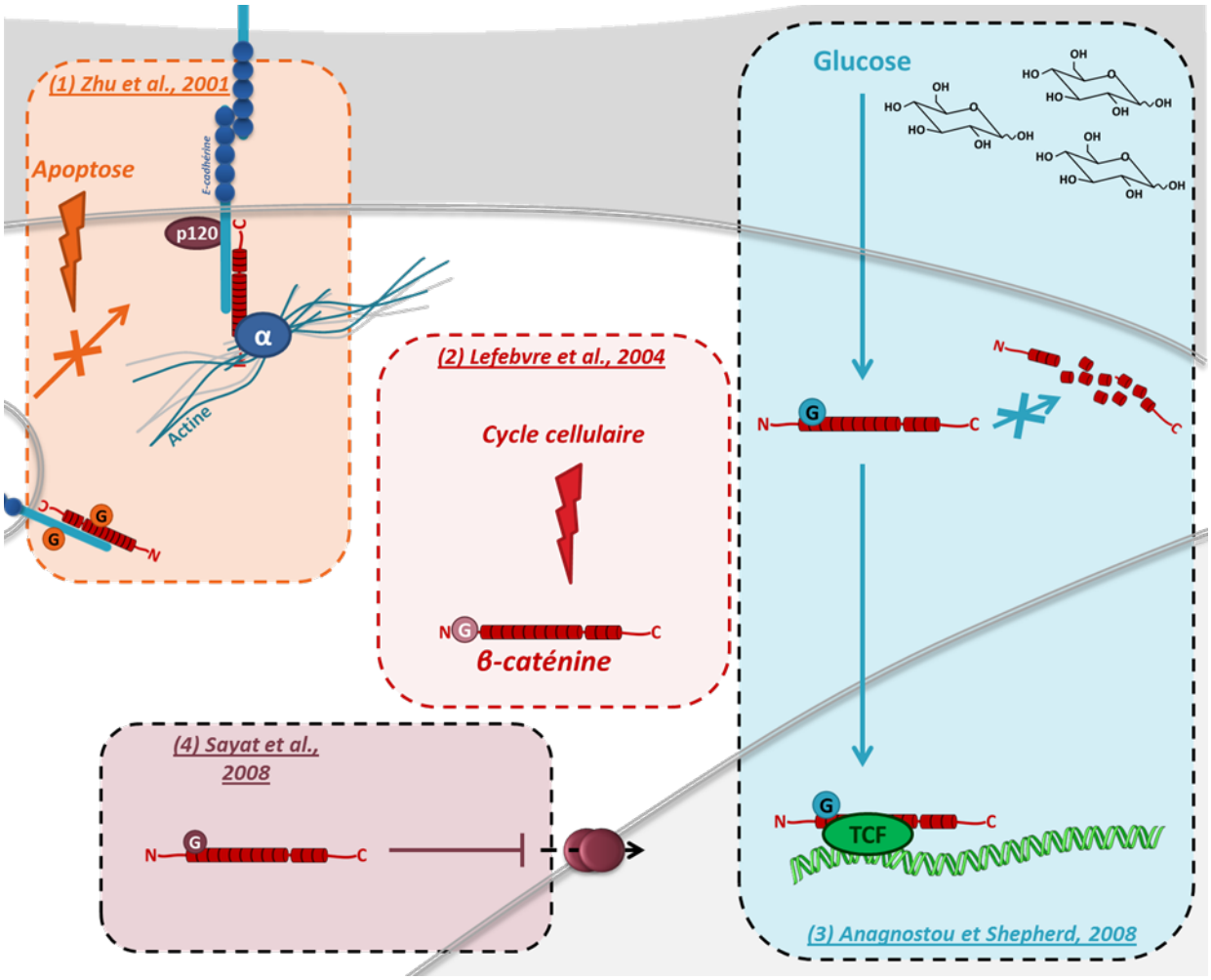


Figure 29 : La *O*-GlcNAcylation de la β-caténine : Etat des lieux. La *O*-GlcNAcylation de la β-caténine a été décrite dans différentes études et dans différents contextes, fournissant ainsi des pistes sur le rôle de la modification.

G : O-GlcNAcylation

VI. Objectif de l'étude : la *O*-GlcNAcylation de la β-caténine

Les éléments reliant désordres métaboliques et cancers colorectaux semblent nombreux et variés mais ne sont pas, pour le moment, parfaitement définis et maîtrisés (voir III.D.3). Dans cette partie, nous proposons de montrer en quoi la O-GlcNAcylation de la β-caténine constituerait un élément supplémentaire permettant d'effectuer un lien entre ces deux groupes de pathologies. Pour cela, nous avons rassemblé les différentes données de la littérature sur la O-GlcNAcylation de la β-caténine et de ses partenaires, ainsi que des pistes sur la fonction de cette modification sur la β-caténine. Enfin, les données statistiques plaident en faveur de l'existence d'une origine moléculaire fondamentale reliant ces problèmes de santé publique majeurs: la β-caténine O-GlcNAcylée semble en rassembler toutes les caractéristiques.

A. La *O*-GlcNAcylation de la β-caténine

La *O*-GlcNAcylation de la β-caténine a été décrite à plusieurs reprises dans des contextes différents (Figure 29):

(1) Dans une étude portant sur l'induction de l'apoptose par la thapsigargine, inhibiteur de SERCA (Sarco/Endoplasmic Reticulum Calcium ATPase), de cellules de carcinome mammaire (MCF7), Zhu et al. ont observé la glycosylation de la β-caténine et d'un de ses partenaires privilégiés, la E-cadhérine (Zhu et al., 2001). La glycosylation de la E-cadhérine limite sa localisation membranaire en bloquant son interaction avec la p120 caténine, stabilisateur des jonctions sans diminuer son interaction avec la β-caténine. Cette étude est la toute première reportant la glycosylation de la β-caténine .

(2) La *O*-GlcNAcylation de la β-caténine a ensuite été observée au cours de la maturation ovocytaire chez le Xénope (*Xenopus laevis*). Il a été démontré que la stimulation des ovocytes par la progestérone était suivie d'une augmentation de *O*-GlcNAcylation des protéines, parmi lesquelles, la β-caténine. L'augmentation de *O*-GlcNAcylation de la β-caténine corrèle avec son accumulation en phase M (Lefebvre et al., 2004a).

(3) Quatre années plus tard, il a été démontré sur des lignées de macrophages, que des concentrations croissantes en glucose modifient l'expression et l'activité transcriptionnelle de la β-caténine (Anagnostou et Shepherd, 2008). La voie de biosynthèse des hexosamines ainsi que les processus de *N*-glycosylation sont en partie responsables de ces modifications. Les auteurs ont souligné un changement de comportement particulièrement intéressant pour des concentrations en glucose situées entre 5 et 20 mM, correspondant respectivement à la concentration physiologique, normoglycémie, et à une condition hyperglycémique.

(4) Enfin, la même année, Sayat et collaborateurs ont montré l'importance de la *O*-GlcNAcylation dans le transport nucléaire de la β-caténine. Contre toute attente, ils ont démontré que la *O*-GlcNAcylation de la β-caténine était inversement proportionnelle à son activité nucléaire dans des lignées prostatiques humaines. Cette étude suggère que la *O*-GlcNAcylation bloque le transport nucléaire de la β-caténine (Sayat et al., 2008).

Ces différentes études démontraient la modification de la β-caténine par un résidu unique de *N*-acétylglucosamine. Le rôle précis de la *O*-GlcNAcylation sur cette protéine restait néanmoins à préciser.

B. Prédiction des sites de *O*-GlcNAcylation de la β-caténine : comparaison avec la plakoglobine

L'étude de protéines de la même famille que la β-caténine, la plakoglobine par exemple, et l'utilisation d'outils de prédiction bioinformatique nous ont apporté certaines informations sur la *O*-GlcNAcylation de la β-caténine (Figure 30).

La plakoglobine (ou γ-caténine) est un homologue de la β-caténine. Elle est capable, via ses domaines armadillo d'interagir avec les cadhérines afin de former des jonctions cellulaires (desmosomes), notamment entre les cellules épithéliales pour assurer la communication cellulaire (Zhurinsky et al., 2000).

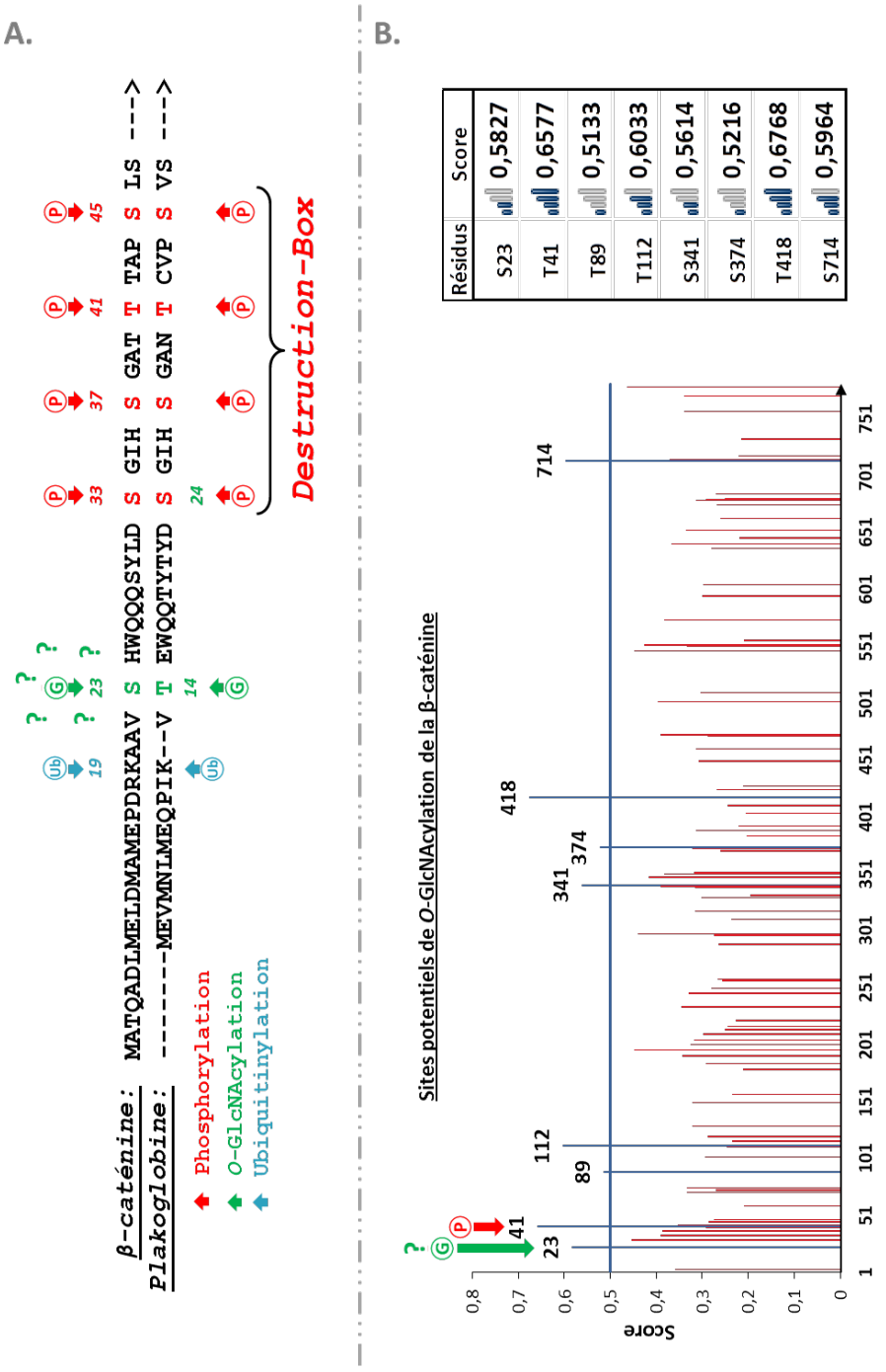


Figure 30 : Les modifications post-traductionnelles N-terminales de la β-caténine et de la plakoglobine (A) et prédiction des sites de *O*-GlcNAcylation de la β-caténine (B). L'étude des modifications post-traductionnelles de la plakoglobine nous offre, par homologie de séquence, des pistes sur la localisation de celles de la β-caténine. En complément, la prédiction *in silico* des sites de *O*-GlcNAcylation par le logiciel YingOYang laisse entrevoir des possibilités quant à la position de la modification sur la β-caténine (Source : http://www.cbs.dtu.dk/services/YinOYang/).

G : O-GlcNAcylation ; P : Phosphorylation ; Ub : Ubiquitinylation

L'extrémité N-terminale de la plakoglobine est très similaire à celle de la β-caténine. On note notamment, la présence d'une "destruction-box" putative mais la séquence de phosphorylation et de prise en charge par le système ubiquitine/protéasome restent à définir (Pasdar et Chlumecky, 1995 ; Sadot et al., 2000). On sait néanmoins que la séquestration au sein du complexe de destruction APC/Axine/GSK3β est nécessaire, suggérant un mécanisme de dégradation identique à celui de la β-caténine (Kodoma et al., 1999 ; Hülsken et al., 1994). L'étude de la *O*-GlcNAcylation de la plakoglobine a permis d'approfondir les connaissances sur la régulation de sa dégradation. En effet, un seul site a été décrit pour la plakoglobine, la thréonine 14 (Figure 29 A) (Hatsell et al., 2003). La modification de ce résidu, situé à proximité de la "destruction-box", affecterait les phosphorylations potentielles de cette dernière et la stabiliserait. Par la suite, Hu et collaborateurs ont démontré que la surexpression de l'OGT stabilise la plakoglobine, renforçant ainsi l'étude de Hatsell (Hu et al., 2006).

Par homologie de séquence avec la β-caténine, la sérine 23 (équivalente de la thréonine 14 de la plakoglobine) semble être un excellent candidat pour la *O*-GlcNAcylation.

De plus, la prédiction des sites de *O*-GlcNAcylation (source: http://www.cbs.dtu.dk/services/YinOYang/) souligne le caractère prioritaire de cette sérine en terme de glycosylation parmi d'autres cibles potentielles localisées au sein de la « destruction–box » (Figure 29 B).

C. Information sur le rôle de la *O*-GlcNAcylation dans les processus de cancérisation : Snail1 et E-cadhérine

La cancérisation est caractérisée par une longue phase d'expansion tumorale, se prolongeant par la dissémination de la tumeur dans l'organisme, appelée phase métastatique. Cette phase nécessite la perte des jonctions d'adhérence, et donc, par conséquent la perte de la E-cadhérine. La voie Wnt/β-caténine participe à la stabilisation de Snail1, un répresseur transcriptionnel de la E-cadhérine (Barralo-Gimeno et Nieto, 2005 ; Zhou et al., 2004).

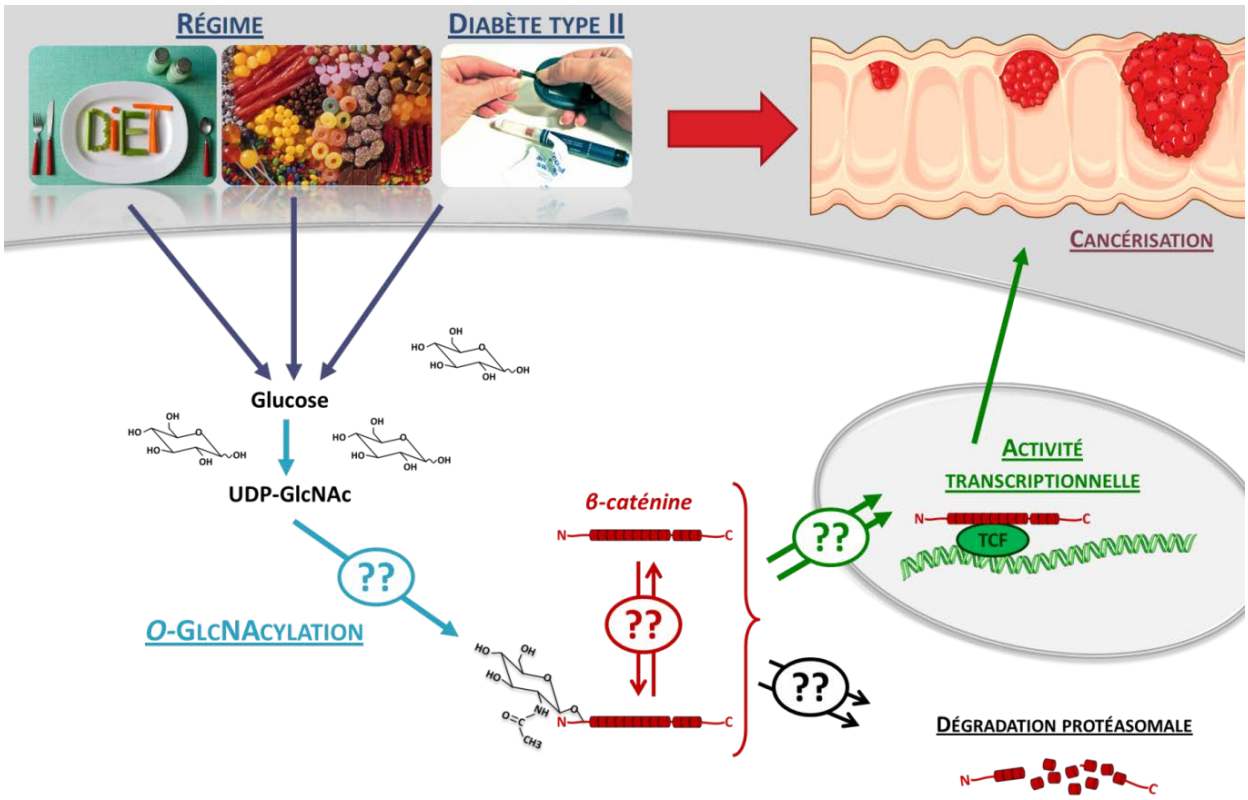


Figure 31 : Influence des désordres métaboliques sur la cancérisation : hypothèses. L'influence de l'alimentation, et des désordres métaboliques, sur la cancérisation pourrait trouver un début d'explication par l'étude de la *O*-GlcNAcylation de la β-caténine, en corrélation directe avec le statut nutritionnel. Néanmoins, il nous faut maintenant connaitre le rôle précis de la *O*-GlcNAcylation de la β-caténine sur sa dégradation et son activité transcriptionnelle afin de comprendre son implication dans les processus de cancérisation.

Dans ce cadre, la *O*-GlcNAcylation de Snail1 sur la sérine 112, en réponse notamment à des variations des concentrations en glucose, permet sa stabilisation par compétition avec la phosphorylation. Cette étude souligne une fois de plus le lien entre désordres métaboliques, *O*-GlcNAcylation et progression tumorale. De plus, la *O*-GlcNAcylation à ce niveau affecte directement la E-cadhérine néosynthétisée, en bloquant sa localisation membranaire, renforçant ainsi un peu plus le phénotype (Zhu et al., 2001). La *O*-GlcNAcylation permettrait donc entre autres d'accentuer la phase métastatique de cancer et serait donc un moyen ici de promouvoir le cancer.

D. Etats des lieux et objectifs

Au commencement de cette étude, nous savions donc que:

- Le développement des cancers colorectaux est fortement influencé par la présence de désordres métaboliques impliquant des variations de glycémie majoritairement.
- La *O*-GlcNAcylation constitue un senseur métabolique permettant de retranscrire l'état glycémique sur les protéines intracellulaires.
- La β-caténine est une protéine intracellulaire impliquée dans la phase d'initiation du cancer colorectal.
- La β-caténine est *O*-GlcNAcylée.

Cette partie introductive du sujet a fait l'objet d'une revue rassemblant les données disponibles et les hypothèses de travail.

Publications n°1:

Olivier S, Mir AM, Michalski JC and Lefebvre T (2011) Signaling and metabolic predispositions linked to the colorectal cancer, *Med Sci (Paris)* May;27(5):514-520

Le but de cette thèse était de (Figure 31) :

- Localiser les sites de *O*-GlcNAcylation de la β-caténine.
- Comprendre la fonction de cette modification sur la β-caténine.
- Etudier l'impact de variations en glucose (temporaire ou lié à un désordre métabolique) sur la β-caténine.
- Corrèler les niveaux de *O*-GlcNAcylation à l'expression de la β-caténine dans des contextes de cancer colique et rectal.

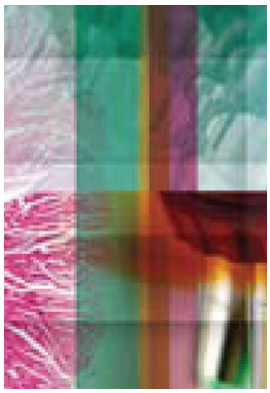
Publication N°1

médecine/sciences 2011 ; 27 : 514-20

Signalisation et prédispositions métaboliques liées au cancer colorectal

Stéphanie Olivier, Anne-Marie Mir, Jean-Claude Michalski, Tony Lefebvre

> La mise en place et la progression du cancer colorectal (CCR) suivent une séquence d'événements rigoureusement orchestrés dans l'espace et dans le temps. Les défaillances ciblant les voies de signalisation responsables de la cancérisation de la muqueuse rectocolique sont bien décrites, et parmi ces voies il apparaît que dans 90 % des cas un dysfonctionnement de la voie Wnt/β-caténine est impliqué dans l'amorce de ce processus. Il a également été décrit que plusieurs facteurs de risque liés à des troubles métaboliques (alimentation, résistance à l'insuline, syndrome métabolique, etc.) prédisposaient certains individus au CCR sans pour autant l'expliquer. Nous proposons que le senseur nutritionnel *O*-GlcNAcylation, entre autres par son rôle dans le contrôle de la voie de l'insuline, puisse être l'élément connectant ces désordres métaboliques et le CCR. <



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Le cancer colorectal (CCR) résulte d'une succession d'altérations génétiques qui affectent certains oncogènes, suppresseurs de tumeur ou gènes de stabilité de l'ADN [32]. Ces altérations sont d'origines diverses. On distingue l'instabilité chromosomique (CIN pour *chromosomal instability*) responsable d'une perte importante du nombre de copies de gènes ou d'un fragment de chromosome, et l'instabilité microsatellitaire (MSI pour *microsatellite instability*), correspondant à une modification de gènes qui contiennent des séquences microsatellites. La séquence de mutations des oncogènes et suppresseurs de tumeur qui conduit à l'altération de voies de signalisation précises définit la progression du CCR : c'est la séquence de Fearon et Vogelstein *(Figure 1)* [1, 2]. Les voies qui activent la prolifération cellulaire sont stimulées (Wnt, RTK [récepteurs à activité tyrosine kinase]) et les voies de réparation de l'ADN, de l'arrêt du cycle cellulaire et de l'apoptose sont inactivées (TGFβ, p53).

Mécanismes moléculaires au service du cancer colorectal

La voie Wnt/β-caténine : l'initiation de la cancérisation

L'altération de la voie Wnt/β-caténine est dans 90 % des cas l'élément initiateur du processus de cancérisation des muqueuses colique et rectale, et correspond à l'apparition d'un adénome précoce (cryptes dysplasiques). Cette voie de signalisation est fondamentale pour le contrôle de l'embryogenèse et le renouvellement de l'épithélium des cryptes intestinales [3]. Au cours de ces processus, les cellules sont stimulées par des facteurs Wnt, ce qui provoque la formation d'un complexe membranaire constitué du récepteur *Frizzled* (Fz) couplé au corécepteur LRP5/6 (*low-density lipoprotein-related receptor protein*) et de *Dishevelled* (Dsh). Après phosphorylation de Dsh et de LRP5/6, l'axine est recrutée à la membrane. Cette protéine d'échafaudage est le facteur-clé du complexe de destruction de la β-caténine : son recrutement à la membrane empêche donc la dégradation de la β-caténine, élément central de la voie Wnt. En absence de stimulation, la β-caténine est séquestrée dans un complexe formé de l'axine, d'APC (*adenomatous polyposis coli*) et des kinases GSK3β (glycogène synthase kinase 3 β) et CK1α (caséine kinase 1α). La β-caténine subit tout d'abord une série de phosphorylations dans une région appelée boîte de destruction (D-*box*), puis est la cible de l'ubiquitine ligase β-TrCp (β-*transducin repeat containing protein*). L'ubiquitinylation de la β-caténine provoque sa dégradation protéasomale. Le recrutement de l'axine à la membrane en réponse aux facteurs Wnt bloque ce processus de dégradation. La β-caténine migre alors dans le noyau où elle s'associe au *T-cell factor* (TCF) pour former un facteur

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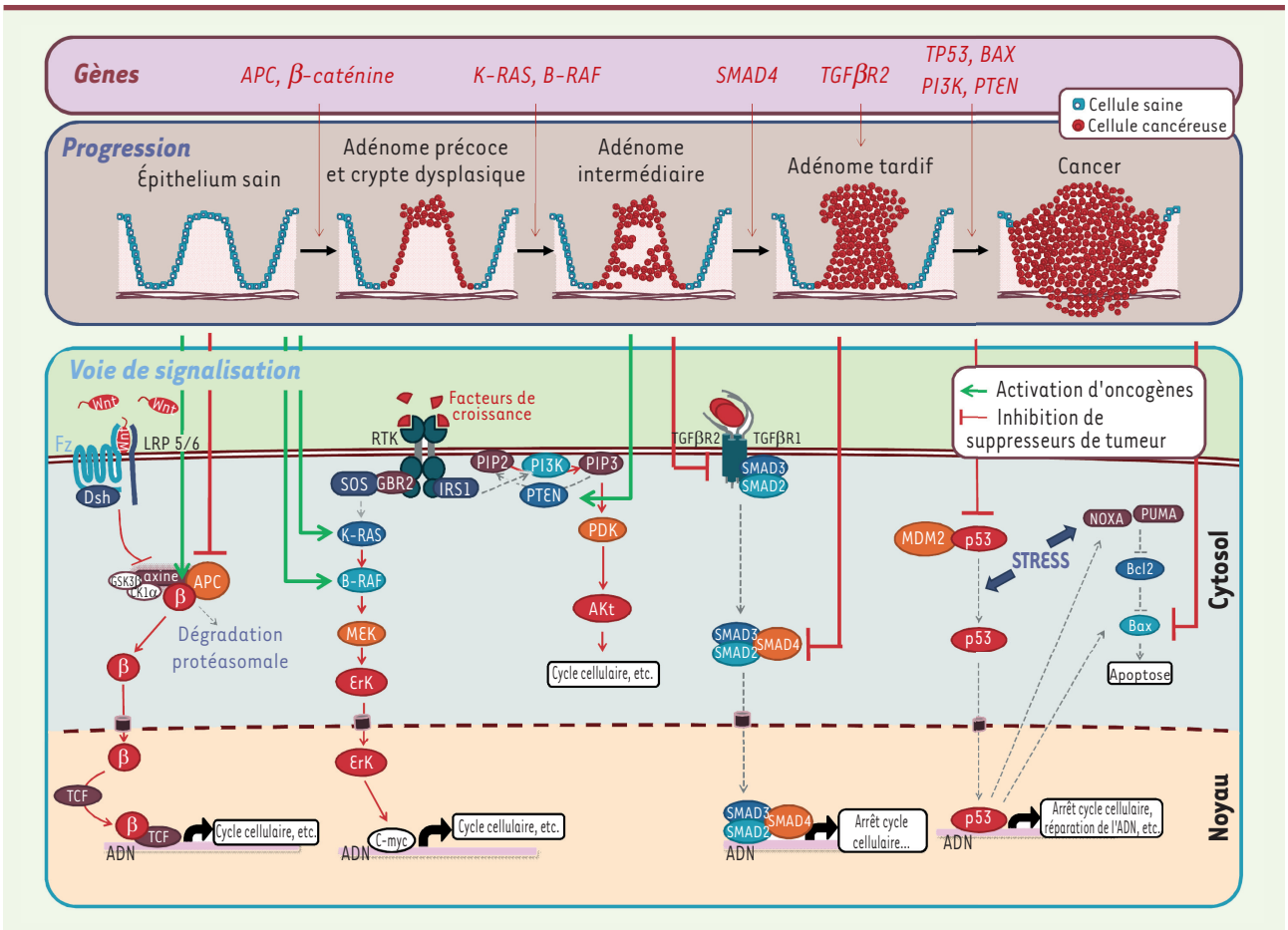

Figure 1. ***Mécanisme moléculaire du CCR.*** La mise en place et la progression du cancer colorectal (CCR) (progression de l'épithélium sain vers l'adénome puis au cancer) sont des processus multi-étapes. Progressivement les cellules des muqueuses colique et rectale sont le siège de mutations ou de défaillances qui permettent la transition d'une étape à l'autre. Dans bon nombre de CCR, c'est la voie Wnt/β-caténine (étape initiatrice) qui est à l'origine de cette longue marche vers le cancer (l'ensemble de ces événements peut mettre entre dix et vingt ans avant d'aboutir à un cancer véritable). Pour les abréviations voir le texte (partie supérieure adaptée de [31]).

de transcription ciblant entre autres les gènes du cycle cellulaire (cycline D1, c-myc, etc.). Les premiers dérèglements observés dans la cancérogenèse colique sont dus pour 85 % des cas à des altérations génétiques d'APC qui conduisent à des formes tronquées de la protéine [4] incapables de s'associer avec la β-caténine ; celle-ci n'étant plus dégradée, la prolifération cellulaire est incontrôlée. On observe également en moindre proportion des mutations au niveau de la D-*box* de la β-caténine empêchant sa phosphorylation et sa dégradation.

La compréhension de ces premiers dérèglements est essentielle pour la détection précoce des CCR et leur traitement.

Les voies de signalisation associées aux récepteurs à activité tyrosine kinase : la progression du CCR

Les récepteurs à activité tyrosine kinase sont des éléments essentiels de la prolifération cellulaire ; ils permettent la transmission au sein de la cellule de l'information portée par des facteurs de croissance tels que l'EGF (*epidermal growth factor*). Deux voies de signalisation majeures sont activées par ces récepteurs : la voie MAPK (*mitogen-activated protein kinase*) et la voie PI3K (phosphatidylinositol 3 kinase)/Akt, stimulant de concert la prolifération cellulaire et inhibant l'apoptose. Ces deux voies sont la cible d'altérations génétiques au cours de la cancérogenèse colique, ce qui entraîne le passage vers un adénome tardif (cryptes dysplasiques). Dans une cellule saine, la phosphorylation des récepteurs à activité tyrosine kinase qui intervient en réponse aux facteurs de croissance permet le recrutement de protéines relais qui possèdent un domaine SH2 (*Src homology 2*), comme SHC (*Src homology 2 domain-containing*), Grb2 (*growth factor receptor-bound protein 2*) ou encore PI3K.

SHC et Grb2, *via* l'oncogène K-Ras, stimulent la voie MAPK constituée de la cascade de kinases suivante : Raf (MAPKKK), MEK (MAPKK) et Erk (MAPK), cette dernière étant la protéine centrale de cette voie [5]. Erk active par phosphorylation différents facteurs de transcription parmi lesquels c-myc et c-fos, impliqués dans la prolifération cellulaire. De plus, Erk entraîne la séquestration de Bad et inhibe la caspase-9, bloquant ainsi les processus apoptotiques. Il est intéressant de noter que ces deux protéines sont également inactivées par la voie PI3K/Akt, ce qui indique une action concertée de ces deux voies de signalisation. Lors de la cancérogenèse colique, des mutations activatrices de K-Ras ou de B-Raf (37 % et 13 % des CCR respectivement) permettent l'activation constitutive de la voie MAPK et la prolifération anarchique des cellules des cryptes intestinales [6, 7]. Celles-ci sont également capables d'échapper à l'apoptose, une caractéristique majeure des cellules cancéreuses. Une autre caractéristique est l'autosuffisance en facteurs de croissance initiée par la stimulation de cette voie (capacité d'activer des gènes codant des facteurs de croissance comme le facteur angiogénique VEGF) [5].

Comme pour les protéines relais, la sous-unité régulatrice p85 de la PI3K est elle aussi capable de se fixer aux résidus phosphorylés de ces récepteurs à activité tyrosine kinase. Après recrutement de la sous-unité catalytique p110, PI3K catalyse la transformation du PIP2 (phosphatidylinositol-4,5-bisphosphate) en PIP3 (phosphatidylinositol-3,4,5-triphosphate). La réaction inverse est catalysée par le suppresseur de tumeur PTEN (*phosphatase and tensin homolog*). L'accumulation de PIP3 recrute des protéines à domaine PH (*pleckstrin homology*) comme PDK1 (*phosphatidylinositol-dependent protein kinase-1*) puis Akt/PKB (protéine kinase B). Après activation par PDK1, PI3K module l'activité de nombreuses cibles impliquées dans l'apoptose et le cycle cellulaire (Bad, procaspase-9, p21, etc.) [8]. De plus, la voie PI3K/Akt interagit avec la voie Wnt/β-caténine en inhibant GSK3β, ce qui souligne les relations entre les différentes voies de signalisation. Parmi les CCR, 30 % ont une mutation activatrice de la sous-unité catalytique de PI3K et 13 % des altérations qui inactivent le suppresseur de tumeur PTEN [9, 10]. Ces deux modifications permettent l'accumulation de PIP3 à la membrane et la stimulation incontrôlée de la voie PI3K/Akt. Les altérations génétiques de ces deux voies sont problématiques, notamment pour le traitement des cancers par des anticorps dirigés contre certains récepteurs de facteurs de croissance (cétuximab : anti-EGFR) [33]. Une mutation de ces voies (Ras, Raf, PI3K) rend le traitement inefficace chez ces patients.

Les mutations du TGFβR2 et de p53 : progression vers le cancer invasif

Ces deux voies de signalisation contrôlent la croissance cellulaire en déclenchant l'apoptose et l'arrêt du cycle cellulaire. Ce sont donc des voies anticancéreuses qui doivent être inactivées pour que la tumeur progresse vers un stade invasif.

Afin de stopper la prolifération cellulaire, la cellule stimule la voie du TGFβ (*transforming growth factor β*) par action autocrine. La fixation du TGFβ à la sous-unité II du récepteur permet la formation d'un complexe hétérodimérique avec la sous-unité I et sa phosphorylation. Une fois activée, cette dernière phosphoryle les facteurs de transcription Smad2 et Smad3, leur permettant de se complexer à Smad4, qui est nécessaire à la translocation nucléaire du complexe. Le complexe Smad2/3/4 s'associe avec d'autres protéines telles que p21 ou la survivine pour les réguler [11]. Cette voie de signalisation doit être désactivée pour la progression tumorale des cellules coliques. Près d'un tiers des CCR possède des mutations de la sous-unité II du récepteur du TGFβ inactivant la transduction du signal [12]. On peut également trouver des mutations inactivatrices des composants en aval de cette voie comme Smad4 (20 % des CCR) [13].

Une des conséquences de la réplication anarchique des cellules cancéreuses est l'apparition de dommages à l'ADN ou de défauts de la division cellulaire. Si elles ne sont pas réparées correctement, ces erreurs déclenchent l'apoptose par le biais de p53, peu exprimée dans la cellule saine puisque dégradée sous l'impulsion de l'ubiquitine ligase MDM2 (*murine double minute 2*). Lors d'un stress (lésions de l'ADN), MDM2 est inhibé, conduisant à l'accumulation de p53. Après avoir été la cible de nombreuses modifications post-traductionnelles, p53 active la transcription de gènes cibles qui, comme pour la voie du TGFβ, ont pour but dans un premier temps l'arrêt du cycle cellulaire puis la mort cellulaire programmée (p21, Puma, Noxa, Bax, etc.) [14]. La mutation de p53 (45 % des CCR) est donc une des dernières étapes avant la métastase des cellules cancéreuses coliques et elle provoque la non-réparation des erreurs de réplication et l'échappement définitif à l'apoptose [15]. Enfin, plus directement et en parallèle, on observe des mutations de protéines-clés de l'apoptose comme Bax [16].

Bien que les acteurs et les mécanismes de la cancérogenèse colique soient bien décrits, les causes des premiers dérèglements menant aux CCR restent incertaines. Néanmoins, plusieurs facteurs de risque sont connus, parmi lesquels le syndrome métabolique ou la suralimentation.

L'émergence du cancer colorectal : importance des facteurs de risque

Alimentation, glucides et CCR

L'alimentation a depuis longtemps été décrite comme un facteur-clé dans l'émergence du CCR. Certains nutriments, comme les glucides, jouent un rôle particulier dans ce processus. En effet, une forte consommation de sucres augmente considérablement la probabilité de déclencher un cancer colorectal [17, 18]. De plus, l'hyperglycémie est une des caractéristiques du syndrome

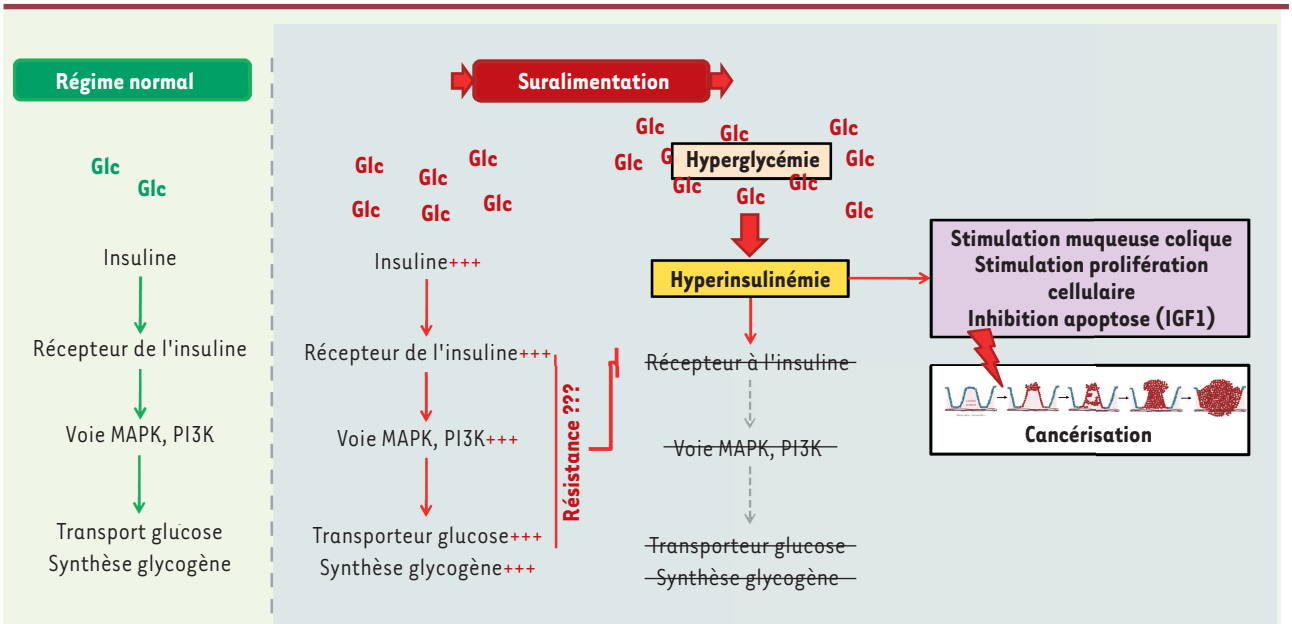

Figure 2. ***La résistance à l'insuline : un facteur favorisant la cancérisation des muqueuses coliques ?*** Un déséquilibre alimentaire serait responsable d'une plus forte propension à développer un cancer colorectal. Dans certaines pathologies telles que le diabète de type 2 mais également chez les patients souffrant de syndrome métabolique, une suralimentation et une exposition prolongée au glucose aboutissent progressivement au phénomène de résistance à l'insuline caractérisée par une défaillance de régulation de certaines voies de signalisation (PI3K et MAPK en particulier). Ces problèmes d'ordre moléculaire se manifestent pour certains d'entre eux par l'émergence de cancers, c'est le cas des cancers colorectaux.

métabolique, pathologie associée aux CCR et favorisant la mise en place de la résistance à l'insuline. Cette résistance, observée notamment chez les diabétiques de type 2, permettrait de faire le lien entre l'hyperglycémie et l'initiation des CCR.

La résistance à l'insuline, un facteur favorisant la tumorigenèse ?

Dans un organisme sain, les cellules β pancréatiques synthétisent l'insuline nécessaire au transport et au stockage du glucose. L'ingestion prolongée ou trop importante de nutriments stimule de manière excessive la voie de l'insuline, provoquant peu à peu une désensibilisation et une diminution de la réponse attendue. Pour compenser cette perte d'efficacité qui mène à l'hyperglycémie, l'organisme synthétise de plus en plus d'insuline : c'est l'hyperinsulinémie. On sait depuis le milieu des années 1990 que des patients atteints de CCR ont une intolérance au glucose et une résistance à l'insuline (taux d'insuline sérique triple de la normale) [19, 20]. De plus, les diabétiques de type 2 ont un risque de déclencher un CCR très supérieur à celui d'individus sains [21]. La question posée est celle du mécanisme moléculaire qui fait le lien entre la résistance à l'insuline et le CCR. L'hyperinsulinémie exerce de nombreux effets néfastes sur l'organisme *(Figure 2)*. Parmi ceux-ci, on note que la surexposition de tissus normalement peu ou pas exposés à l'insuline mais possédant toutefois le récepteur hormonal, c'est le cas du côlon, déclenche l'activation des voies de signalisation impliquées dans la progression tumorale. À cela il faut ajouter que l'insuline favorise la stimulation cellulaire par d'autres facteurs de croissance comme l'IGF-1 (*insulin growth factor-1*) accentuant par conséquent la prolifération cellulaire et l'apparition d'erreurs aberrantes de l'ADN, ce qui se traduit par la cancérisation du tissu [22].

Bien que les relations entre les désordres métaboliques et les CCR soient bien connues aujourd'hui, on peine encore à expliquer le processus intime liant ces deux groupes de pathologies. Pour tenter de comprendre les mécanismes sous-jacents, il semble crucial de mieux maîtriser l'impact de l'alimentation et de la résistance à l'insuline sur la cancérisation. La régulation des voies de signalisation impliquées dans l'étiologie des CCR par la *O*-N-acétylglucosaminylation, une modification post-traductionnelle qui dépend directement du statut nutritionnel de l'organisme, pourrait apporter quelques réponses.

La *O*-GlcNAcylation, senseur nutritionnel de l'organisme

La *O*-GlcNAcylation

La *O*-N-acétylglucosaminylation (*O*-GlcNAcylation) est une glycosylation un peu originale [23] *(Figure 3)*. En effet, contrairement aux glycosylations dites « classiques » (*N*- et *O*-glycosylations complexes) des protéines sécrétées, membranaires ou résidentes du réticulum

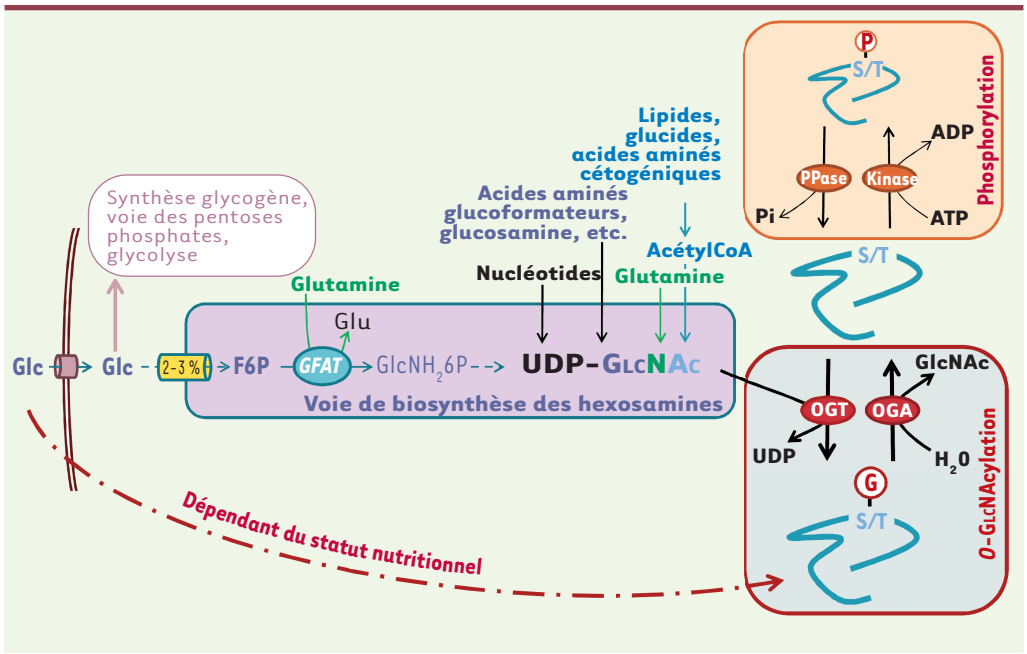

Figure 3. ***La O-GlcNAcylation : un senseur nutritionnel.*** Un niveau très bas de glucose circulant emprunte, après son transport dans la cellule, la voie de biosynthèse des hexosamines. Cette voie aboutit à la formation d'UDP-GlcNAc, un nucléotide-sucre majeur utilisé dans les processus de glycosylations simples et complexes. Parmi ces glycosylations on retrouve la *O*-GlcNAcylation, modification post-traductionnelle réversible et capable de bloquer certains sites de phosphorylation : c'est l'antagonisme phosphorylation/*O*-GlcNAcylation. D'autres sources nutritionnelles ou métaboliques sont également capables de pourvoir aux besoins cellulaires en UDP-GlcNAc (acides aminés, acides gras, nucléotides, etc.). C'est pourquoi ce nucléotide-sucre ainsi que la *O*-GlcNAcylation sont considérés comme des senseurs nutritionnels puisque leur concentration reflète l'état nutritionnel de l'organisme dans son ensemble. Puisque la *O*-GlcNAcylation entre en compétition avec certains sites de phosphorylation, les conditions nutritionnelles d'un organisme ont également un impact sur les processus de phosphorylation-déphosphorylation. Glc, glucose ; F6P, fructose-6-phosphate ; $GlcNH_2 6P$, glucosamine-6-phosphate ; UDP, uridine diphosphate ; UDP-GlcNAc, uridine diphospho-N-acétylglucosamine ; OGT, *O*-GlcNAc transférase ; OGA, *O*-GlcNAcase ; G, *O*-GlcNAc ; P, phosphate ; GlcNAc, N-acétylglucosamine ; Glu, glutamate ; GFAT, glutamine : fructose-6-phosphate amido-transférase ; ADP, adénosine diphosphate ; ATP, adénosine triphosphate ; PPase, protéine phosphatase.

endoplasmique ou du Golgi, la *O*-GlcNAcylation affecte les protéines cytosoliques, nucléaires et mitochondriales. De plus, c'est une modification dynamique qui consiste en l'ajout d'un résidu unique de N-acétylglucosamine (GlcNAc) sur un résidu sérine ou thréonine. Cette versatilité fait de la *O*-GlcNAcylation un compétiteur de la phosphorylation, modification post-traductionnelle avec laquelle elle est souvent comparée. Contrairement aux phénomènes de phosphorylation qui sont contrôlés par plus de 1 000 kinases et plus de 150 phosphatases, le dynamisme de la *O*-GlcNAcylation est régulé par un couple unique d'enzymes. La *O*-GlcNAc transférase (OGT) catalyse l'ajout d'un résidu de GlcNAc sur la protéine à partir d'UDP-GlcNAc, produit final de la voie de biosynthèse des hexosamines. La *O*-GlcNAcase (OGA) hydrolyse quant à elle ce même résidu. Le caractère dynamique de la *O*-GlcNAcylation et l'utilisation de l'UDP-GlcNAc comme substrat en font un senseur nutritionnel pour la cellule. En effet, les niveaux de *O*-GlcNAcylation sont intimement corrélés avec le niveau d'UDP-GlcNAc disponible, qui dépend lui-même du glucose qui entre dans la cellule. Par cet aspect, elle fait le lien entre l'état nutritionnel et la régulation des processus biologiques fondamentaux comme la transcription, la traduction, la signalisation cellulaire, le trafic intracellulaire, le cycle cellulaire ou le développement [23].

La *O*-GlcNAcylation : entre alimentation et résistance à l'insuline

De nombreux éléments de la voie de signalisation de l'insuline sont *O*-GlcNAcylés : chaîne β du récepteur de l'insuline, IRS1/2 (*insulin responsive substrate*), PDK1, p110PI3K, Akt1/2, GSK3, GLUT4. L'hyperglycémie, par le biais de la voie de biosynthèse des hexosamines, augmenterait la *O*-GlcNAcylation des protéines cibles de l'OGT, et plus particulièrement IRS1 et Akt/PKB. En réponse à l'insuline, l'OGT est rapidement recrutée à la membrane par son interaction avec le PIP3 (lui-même s'accumule en cas de suractivation de PI3K ou de défaillance de PTEN, *voir plus haut*). La *O*-GlcNAcylation de IRS1 et Akt, en restreignant leur phosphorylation, bloque leur activité, induisant la résistance à l'insuline [24] (*Figure 4*). Par cette séquence d'événements, la *O*-GlcNAcylation pourrait être un élément faisant le lien entre l'hyperglycémie, la résistance à l'insuline et la cancérisation des cellules coliques.

La *O*-GlcNAcylation affecte de nombreux oncogènes et suppresseurs de tumeur

Il a été décrit que de nombreux facteurs impliqués dans les processus de cancérisation ou dans la régulation du cycle cellulaire étaient *O*-GlcNAcylés. Parmi ceux-ci on retrouve c-myc, oncogène fréquemment surexprimé dans les cancers et muté dans les lymphomes de Burkitt ; il est *O*-GlcNAcylé sur un site de phosphorylation (T58) responsable de sa dégradation [25]. P53 est également

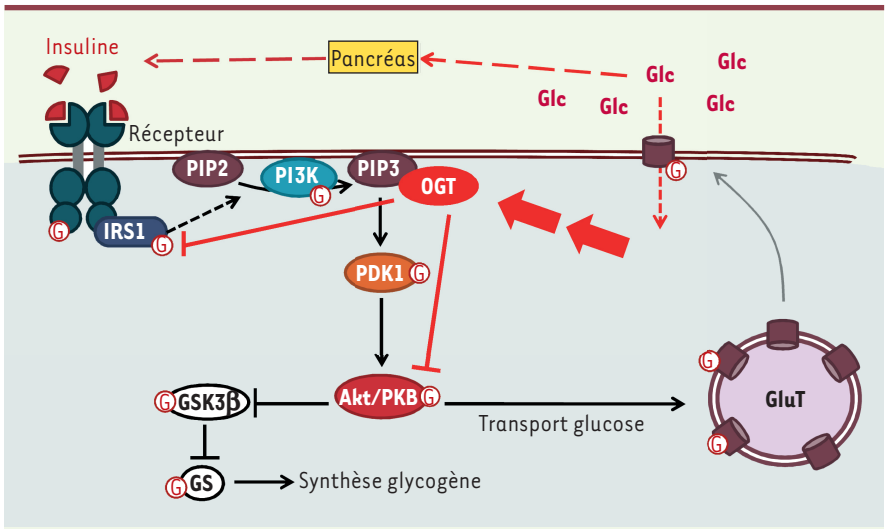

Figure 4. ***La O-GlcNAcylation favorise la désensibilisation de la voie de l'insuline observée dans de nombreux syndromes métaboliques.*** L'insuline, en se fixant sur son récepteur, active la voie PI3kinase. Cette activation se traduit par une série de phosphorylations qui aboutit à une réponse cellulaire, notamment un transport et une utilisation accrus du glucose (mis en réserve sous forme de glycogène). Il a été démontré que l'OGT (*O*-GlcNAc transférase) inactive cette signalisation par *O*-GlcNAcylation de certains composants de cette voie dont Akt/PKB : cette inactivation serait en partie à l'origine du phénomène de résistance à l'insuline. G : *O*-GlcNAc.

O-GlcNAcylé sur un résidu (T149) proche d'un site de phosphorylation (T155) responsable de la dégradation et du changement d'activité de la protéine [26]. Certains composants de la voie MAPK sont *O*-GlcNAcylés comme Erk2 [27]. Une corrélation entre l'augmentation des niveaux de *O*-GlcNAcylation et l'activation de la voie MAPK dans l'ovocyte de xénope a été démontrée [28].

L'oncogène β-caténine est également *O*-GlcNAcylé [29] mais le rôle de sa *O*-GlcNAcylation reste flou. Néanmoins, l'étude d'une protéine similaire, la plakoglobine, qui possède le même système de dégradation (phosphorylation d'une D-*box* conduisant à la dégradation) nous permet d'émettre quelques hypothèses. La plakoglobine est *O*-GlcNAcylée sur un résidu adjacent à la D-*box*, ce qui entraîne une compétition avec la phosphorylation et la stabilisation de celle-ci [30]. Un mécanisme similaire pourrait expliquer l'initiation de la cancérisation de la muqueuse colorectale et permettre d'établir un lien étroit entre la suralimentation, la production excessive d'UDP-GlcNAc (donneur du motif *O*-GlcNAc) par la voie des hexosamines, l'élévation des niveaux de *O*-GlcNAcylation et une suractivation de la voie Wnt/β-caténine par stabilisation aberrante de son élément central : la β-caténine. ◊

SUMMARY

Signaling and metabolic predispositions linked to the colorectal cancer

The setting up and the progression of the colorectal cancer (CCR) follow a sequence of events that are spatio-temporally rigorously orchestrated. The failures that specifically target the signaling pathways responsible for the cancerization of the colorectal mucosa have been well described and among these it seems that a dysregulation of the Wnt/β-catenin pathway is involved in the triggering of near 90 % of the cases. It has been also described that several risk factors linked to metabolic disorders (feeding, insulin resistance, metabolic syndrome, etc.) predispose individuals to CCR but no rational explanations were given. We propose that, since it is implicated in the control of the insulin pathway among other actions, the nutritional sensor *O*-GlcNAcylation may be the element linking these metabolic disorders to CCR. ◊

CONFLIT D'INTÉRÊTS

Les auteurs déclarent n'avoir aucun conflit d'intérêts concernant les données publiées dans cet article.

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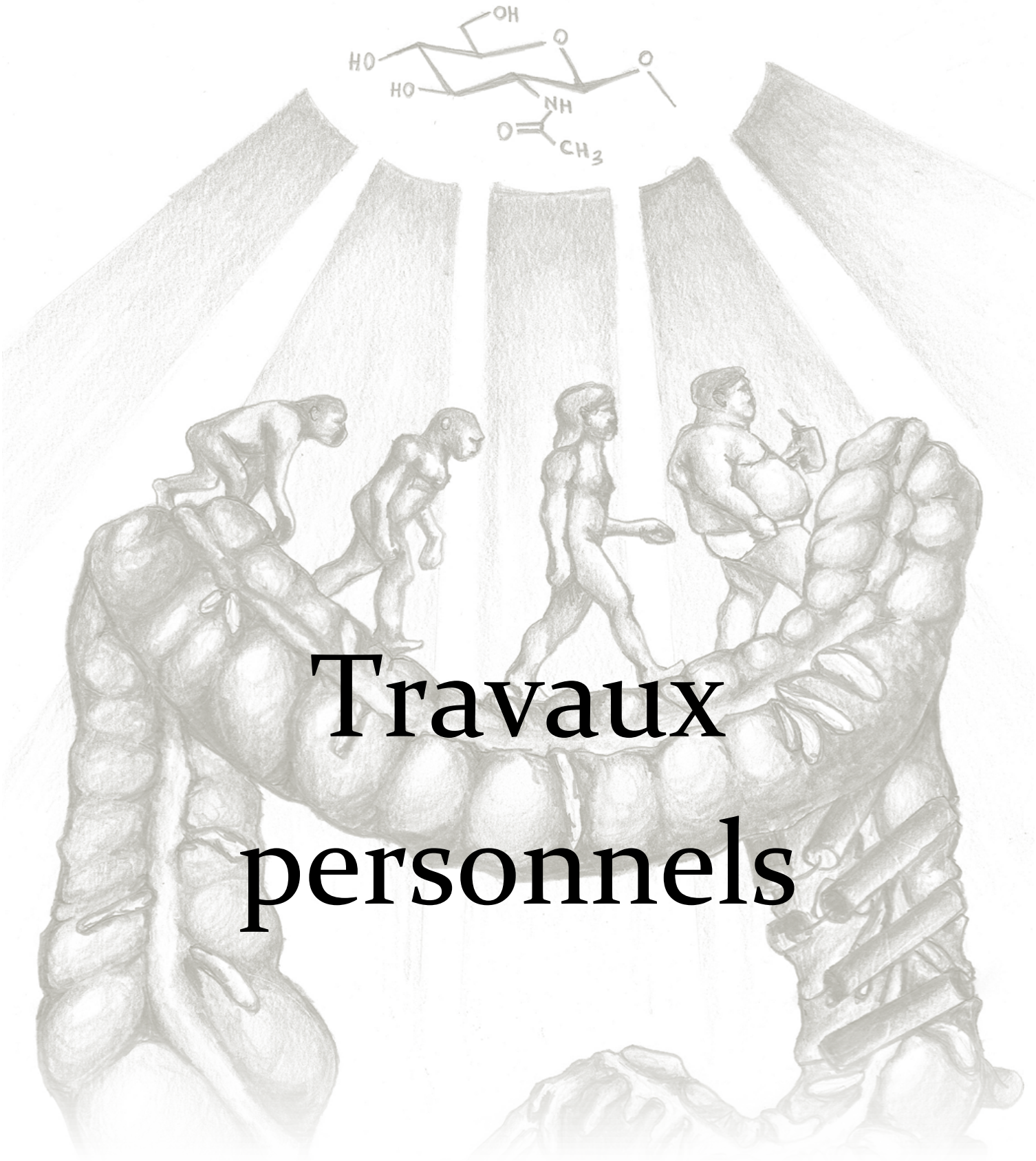
TIRÉS À PART

T. Lefebvre



Travaux personnels

L'ensemble des travaux de recherche ont permis la production de 3 publications scientifiques:

Article publié dans

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First published November 22, 2011; doi:10.1152/ajpendo.00390.2011.

The hexosamine biosynthetic pathway and *O*-GlcNAcylation drive the expression of β-catenin and cell proliferation

Stéphanie Olivier-Van Stichelen,[1] Céline Guinez,[1] Anne-Marie Mir,[1] Yobana Perez-Cervera,[1] Chunming Liu,[2] Jean-Claude Michalski,[1] and Tony Lefebvre[1]

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SHORT COMMUNICATION

Serum-stimulated cell cycle entry promotes ncOGT synthesis required for cyclin D expression

S Olivier-Van Stichelen, L Drougat, V Dehennaut, I El Yazidi-Belkoura, C Guinez, A-M Mir, J-C Michalski, A-S Vercoutter-Edouart and T Lefebvre

Article publié dans FASEB J (2014):

The FASEB Journal • Research Communication

O-GlcNAcylation stabilizes β-catenin through direct competition with phosphorylation at threonine 41

Stéphanie Olivier-Van Stichelen,*,[1] Vanessa Dehennaut,*,[1] Armelle Buzy,† Jean-Luc Zachayus,† Céline Guinez,* Anne-Marie Mir,* Ikram El Yazidi-Belkoura,* Marie-Christine Copin,‡ Didier Boureme,† Denis Loyaux,† Pascual Ferrara,† and Tony Lefebvre*,[2]

Publication N°2

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The hexosamine biosynthetic pathway and *O*-GlcNAcylation drive the expression of β-catenin and cell proliferation

Stéphanie Olivier-Van Stichelen,[1] Céline Guinez,[1] Anne-Marie Mir,[1] Yobana Perez-Cervera,[1] Chunming Liu,[2] Jean-Claude Michalski,[1] and Tony Lefebvre[1]

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Olivier-Van Stichelen S, Guinez C, Mir AM, Perez-Cervera Y, Liu C, Michalski JC, Lefebvre T. The hexosamine biosynthetic pathway and *O*-GlcNAcylation drive the expression of β-catenin and cell proliferation. *Am J Physiol Endocrinol Metab* 302: E417–E424, 2012. First published November 22, 2011; doi:10.1152/ajpendo.00390.2011.— The short half-life protooncogene β-catenin acquires a remarkable stability in a large subset of cancers, mainly from mutations affecting its proteasomal degradation. In this sense, colorectal cancers (CRC) form a group of pathologies in which early steps of development are characterized by an aberrant expression of β-catenin and an uncontrolled proliferation of epithelial cells. Diet has long been described as an influence in the emergence of CRC, but the molecular events that link metabolic disorders and CRC remain elusive. Part of the explanation may reside in hexosamine biosynthetic pathway (HBP) flux. We found that fasted mice being force-fed with glucose or glucosamine leads to an increase of β-catenin and *O*-GlcNAcylation levels in the colon. MCF7 cells possessing intact Wnt/β-catenin signaling heavily expressed β-catenin when cultured in high glucose; this was reversed by the HBP inhibitor azaserine. HBP inhibition also decreased the expression of β-catenin in HT29 and, to a lesser extent, HCT116 cells. The same observation was made with regard to the transcriptional activity of β-catenin in HEK293 cells. Inhibition of HBP also blocked the glucose-mediated proliferation capacity of MCF7 cells, demonstrating that glucose affects both β-catenin expression and cell proliferation through the HBP. The ultimate element conducting these events is the dynamic posttranslational modification *O*-GlcNAcylation, which is intimately linked to HBP; the modulation of its level affected the expression of β-catenin and cell proliferation. In accordance with our findings, we propose that metabolic disorders correlate to CRC via an upregulation of HBP that reverberates on high *O*-GlcNAcylation levels including modification of β-catenin.

glucose; hexosamine biosynthetic pathway; glutamine:fructose-6-phosphate amidotransferase; protooncogene; diet; colorectal cancers

THE WNT/β-CATENIN PATHWAY is fundamental during embryogenesis and for the renewal of the intestinal crypt epithelium (25). During these processes, the main component of this pathway, the protooncogene β-catenin controls cell proliferation by initiating the transcription of its target genes, including cyclin D1 and c-Myc (15, 29). This process is shut down by targeting β-catenin to the 26S proteasome. Wnt/β-catenin signaling is also crucial for cardiac and neuronal development and plays an important part in many disorders affecting these tissues such as cardiac hypertrophy and neurodegenerative diseases. Misregulations in this system are also often observed in hepatoblastoma, hepatocellular carcinoma, and ovarian or pancreatic cancers, but these dysfunctions were more accurately described in colorectal cancers (CRC). Eighty percent of CRC result from genetic alterations of the crucial member of the β-catenin destruction complex adenomatous polyposis coli (APC), preventing the proteasomal degradation of β-catenin (33) that acquires an aberrant stability. This leads to uncontrolled cell proliferation (27). Remarkably, over time, diet appears to be a key factor involved in CRC emergence. Some nutrients like carbohydrates are known to play a critical role in this process, since high-carbohydrate consumption greatly increases the probability of setting up CRC (10, 16). Moreover, some observations have drawn a correlation between CRC and diet; thus, CRC are also considered obesity-associated diseases. Since the 1990s, it has been clearly demonstrated that patients suffering from CRC show intolerance to glucose and resistance to insulin, reflecting a metabolic disorder (24, 31). Type 2 diabetes individuals also have a higher risk of developing CRC than normal individuals (17).

Despite these observations, the molecular mechanism linking a metabolic disorder or overfeeding to CRC remains unknown. To tentatively answer this question, we started with the observation that metabolic syndrome and diet lead to a common event, a punctual or permanent hyperglycemia. On the basis of their dependence on nutritional status, we questioned whether the hexosamine biosynthetic pathway (HBP) and the posttranslational modification (PTM) *O*-GlcNAcylation could contribute to the emergence of CRC (13). Two decades ago, it was determined that the development of insulin resistance requires three key components: glucose, insulin, and glutamine, and that a small percentage (2–3%) of glucose is used to provide UDP-GlcNAc (*N*-acetylglucosamine), the final product of the HBP (21). Other nutrients are also implicated in the makeup of this nucleotide sugar: ketogenic amino acids, glutamine, fatty acids, and sugars (hexoses). UDP-GlcNAc is the donor of the GlcNAc group for glycosylation processes, including *O*-GlcNAcylation. Accordingly, both UDP-GlcNAc and *O*-GlcNAcylation are considered nutritional sensors. Thanks to its privileged position among thousands of PTMs, *O*-GlcNAcylation reflects the cell's nutritional status to the regulation of fundamental biological processes: transcription, translation, cell signaling, intracellular trafficking, cell cycle, and development (35). Thereby, an imbalance in the use of nutrients will have a repercussion in the homeostasis of the tissues, and consequently, pathologies such as cancers may arise. Numerous factors involved in the cancerization processes or cell cycle regulation are *O*-GlcNAcylated: the protooncogenes c-Myc, (7) and β-catenin (19, 26, 37), the tumor suppressor p53 (32), and components of the MAPK or PI3K pathways (8, 34). Last, the modification of *O*-GlcNAcylation

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levels in malignancy and tumoral progression has been identified as crucial to the development of breast, lung, liver, and colon cancers (11, 22, 36). In this study, using refed or glucose/glucosamine force-fed mice, we made the correlation between an increase in β-catenin content and *O*-GlcNAcylation in the colon. We also report a similar observation in vitro by culturing MCF7 cells in a high-glucose medium.

In contrast to glucosamine, azaserine, the inhibitor of the rate-limiting enzyme of the HBP, affects glucose-induced β-catenin expression, its transcriptional activity, and cell proliferation in a dose-dependent manner. Elevation of the *O*-GlcNAcylation content, using the OGA inhibitor NButGT, increases the level of β-catenin and slows down the cell proliferation. The expression of β-catenin was diminished in the colorectal cancer cell line HT29 and slightly in HCT116 by azaserine and another HBP inhibitor, DON (5-oxo-6-diazonorleucine). Moreover, we observed higher levels of β-catenin, OGT, OGA, and *O*-GlcNAcylation in these cells compared with a fetal colon line. Taken together, our observations and results led us to propose that diet and metabolic disorders are predisposition factors for cancers, particularly CRC, by promoting an elevation of β-catenin level and a stimulation of cell proliferation through the HBP.

MATERIALS AND METHODS

Cell culture and transfection. MCF7, HEK293, HT29, and HCT116 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing various concentrations of glucose (0, 1, or 4.5 g/l) for MCF7 and HEK293. CCD841CoN cells were maintained in Eagle's minimum essential medium (EMEM). All cell lines were maintained in a medium supplemented with 10% (vol/vol) fetal calf serum (heat inactivated for the HT29 and the HCT116 cells), 2 mM L-glutamine, 5 IU/ml penicillin, and 50 μg/l streptomycin at 37°C in a 5% (vol/vol) CO_2-enriched humidified atmosphere.

For a TOP/FOP Flash reporter assay, HEK293 cells were transfected with β-galactosidase, TOP-Flash, FOP Flash, and β-catenin-2× Flag vector by the Lipofectamine 2000 (Invitrogen) reagent (2 μl) in six-well plates with 0.2 μg of DNA for 24 h.

shRNA plasmids (29-mer) were purchased from CliniSciences and were used according to the manufacturer's indications.

Drugs. Glucosamine ($GlcNH_2$) was used at a final concentration of 20 mM; NButGT (1,2-dideoxy-2′-propyl-α-D-glucopyranoso-[2,1-D]-Δ-2′-thiazoline) at 100 μM; azaserine [*O*-(2-diazoacetyl)-L-serine] at 50 μM or at the indicated concentration (see text for details); and DON at 10 or 50 μM. Cells were treated for 16 h with the different drugs except as specified in the text.

Lysis and immunoprecipitation. Cells were first washed with 10 ml of cold phosphate-buffered saline (PBS) and lysed on ice with lysis buffer [10 mM Tris·HCl, 150 mM NaCl, 1% Triton X-100 (vol/vol), 0.5% sodium deoxycholate (wt/vol), 0.1% sodium dodecyl sulfate (SDS; wt/vol), and proteases inhibitors, pH 7.4]. Cell extracts were then centrifuged at 20,000 *g* for 10 min at 4°C. Supernatants were first precleared with Sepharose-labeled protein A for 1 h. After the beads were discarded, the supernatants were incubated together with the rabbit polyclonal anti-β-catenin (H102, Santa Cruz Biotechnology) at a final dilution of 1:500 and placed overnight at 4°C. Antibody-bound proteins were recovered after adding 30 μl of Sepharose-labeled protein A for 1 h at 4°C. Beads were gently centrifuged for 1 min and subsequently washed with the following buffers: lysis buffer, lysis buffer supplemented with 500 mM NaCl, lysis buffer-TNE [10 mM Tris·HCl, 150 mM NaCl, and 1 mM EDTA, pH 7.4 (vol/vol)] and finally with TNE alone.

SDS-PAGE, western blotting, and antibody staining. Equal amounts of extracted protein were subjected to Western blotting. Samples were analyzed by 8, 10, or 15% reticulated SDS-PAGE under reducing conditions, and proteins were electroblotted on a nitrocellulose sheet (GE Healthcare). Efficiency of the transfer and equal loading were verified using Ponceau red staining. Membranes were first saturated for 45 min with 5% (*m/v*) nonfatty acid milk in Tris-buffered saline (TBS)-Tween buffer [15 mM Tris·HCl, 140 mM NaCl, and 0.05% Tween 20 (vol/vol), pH 8.0]. Mouse monoclonal anti-*O*-GlcNAc (RL2, Ozyme) was used at a dilution of 1:1,000. Rabbit polyclonal anti-β-catenin H102 (Santa Cruz Biotechnology), and chicken anti-OGA (345, generously provided by Prof. G. W. Hart) were used at a dilution of 1:2,000. Mouse monoclonal anti-tubulin (Santa Cruz Biotechnology), rabbit polyclonal anti-histone 2B (Millipore), and rabbit polyclonal anti-GAPDH (Abcam) were used at a dilution of 1:5,000.

Membranes were incubated with the different antibodies overnight at 4°C and then washed three times with TBS-Tween for 10 min and incubated with either an anti-rabbit or an anti-mouse horseradish peroxidase-labeled secondary antibody at a dilution of 1:10,000 for 1 h. Finally, three 10-min washes were performed with TBS-Tween, and detection was carried out with enhanced chemiluminescence (GE Healthcare). Densitometry analyses of the Western blots were done with GeneTools software (version 3.07.03, Syngene).

Fractionation. Fractionation was realized with a Proteoextract subcellular proteome extraction kit (MERCK) as recommended by the manufacturer.

Luminescence assay. After transient transfection of Super8× TOPFlash(M50) or Super8×FOPFlash(M51) with β-galactosidase (determination of transfection efficiencies) and β-catenin2×Flag vectors in six-well plates, HEK293 cells were lysed using 200 μl of passive lysis buffer (Promega). Luciferase reporter activity was measured in triplicate in a 96-well plate following back-addition of 100 μl of Luciferase assay buffer (Promega). In parallel, 70 μl of Galacton Substrate Mix (1:200 Tropix Galacton; 99:200 Tropix Galacto reaction buffer diluent, 100:200 18 MΩ water; Applied Biosystems) was incubated for 20 min out of light, and 100 μl of Tropix Accelerator-II (Applied Biosystems) was back-added before β-galactosidase activity was measured.

Proliferation assay. MCF7 cells (2×10^3) were cultured in 96-well plates using DMEM over 5 days. Each day, cell growth was determined using the MTS reagent method (Promega) according to the manufacturer's directions.

In vivo assay. Eight- to twelve-wk-old male C57BL6/CR mice were purchased from the provider Charles River Elevage (Saint-Germain sur l'Arbresle, France). Procedures were carried out according to French guidelines and as described (12) for the care of experimental animals. Mice were adapted to the environment for 1 wk prior to study and maintained in a 12:12-h light-dark cycle with water and regular diet (65% carbohydrate, 11% fat, and 24% protein). For the experiment, mice were fasted for 24 h or refed a regular diet for 18 h (glucose was added to the drinking water at a concentration of 200 g/l after the fasted period). For gavage experiments, 24-h-fasted mice received glucosamine (2.5 g/kg) or glucose (5 g/kg) orally and were killed 3 or 6 h later after intraperitoneal anesthesia (pentobarbital sodium). Colons were then collected, washed in PBS, and lysed in 2 ml of NP-40 lysis buffer {10 mM Tris·HCl, 150 mM NaCl, 1% (vol/vol) NP-40, 0.084% (wt/vol) sodium fluoride, 0.44% (wt/vol) sodium pyrophosphate, 0.018% (wt/vol) sodium orthovanadate, 1 μM PUGNAc [*O*-(2-acetamido-2-deoxy-D-gluco-pyranosylidene) amino-*N*-phenylcarbamate] and proteases inhibitors, pH 7.4}. Tissues were broken using Ultraturax, incubated with gentle agitation for 2 h, and centrifuged at 20,000 *g* for 30 min at 4°C to take over the soluble fraction. Glycemia was measured using an Accu-Chek Performa apparatus (Roche).

RESULTS

Glucose or glucosamine force-feeding modulates β-catenin expression and O-GlcNAcylation level in vivo. To gain insight into the underlying mechanism of how feeding reverberates on the expression of β-catenin, C57BL6 mice were fasted for 24 h and then refed a regular diet for 18 h or force-fed with glucose or glucosamine for 3 or 6 h. The mice's blood glucose concentrations were measured (Fig. 1*A*), and then colons were collected, homogenized, and analyzed by Western blotting (Fig. 1*B*). In refed and especially in force-fed mice, we observed increased β-catenin expression and elevation of the *O*-GlcNAcylation contents. Interestingly, it must be noted that the use of glucosamine impacted the β-catenin and the *O*-GlcNAcylation levels (Fig. 1*B*) without affecting mice glycemia (Fig. 1*A*).

We next questioned whether the expression and activity of β-catenin depended on the nutritional status and, more particularly, glucose concentration. To check this hypothesis, in vitro experiments were performed in MCF7 cells, which express a high amount of β-catenin without exhibiting any mutation in the Wnt/β-catenin pathway. The expression of β-catenin was determined according to the cell glucose status in vitro. MCF7 cells were grown in media containing increasing amounts of glucose for 24 h, and the expression of β-catenin was determined (Fig. 1*C*). β-Catenin and *O*-GlcNAcylation levels were maximal when glucose was used at 25 mM vs. 0 and 5 mM (Fig. 1*C*), correlating with the observation made in vivo (Fig. 1*B*). Interestingly, but not surprisingly, we noted that the proliferation rate of cells (MTS assays for 96 h) was accelerated according to the glucose status (Fig. 1*D*). These data indicate that the expression of β-catenin correlates with the status of glucose both in vivo and in vitro.

Expression of β-catenin and its transcriptional activity are dependent on HBP flux. After entering the cell, glucose follows different metabolic pathways: glycogen synthesis, pentoses shunt, glycolysis, or the HBP, which provides UDP-GlcNAc, the substrate for the *O*-GlcNAcylation processes (Fig. 2*A*). Owing to the quicker expression of β-catenin after administration of glucose and glucosamine to fasted mice, we thought that glucose/glucosamine influenced the expression of the protooncogene by being harnessed through the HBP. This pathway was explored using azaserine, an inhibitor of its rate-limiting enzyme GFAT (glutamine:fructose-6-phosphate amidotransferase) (Fig. 2*A*). MCF7 cells were maintained in a culture medium containing three different concentrations of glucose with or without azaserine. We observed an increase of β-catenin expression (Fig. 2*B*) and transcriptional activity (TOP/FOP-Flash assays; Fig. 2*C*) following high-glucose

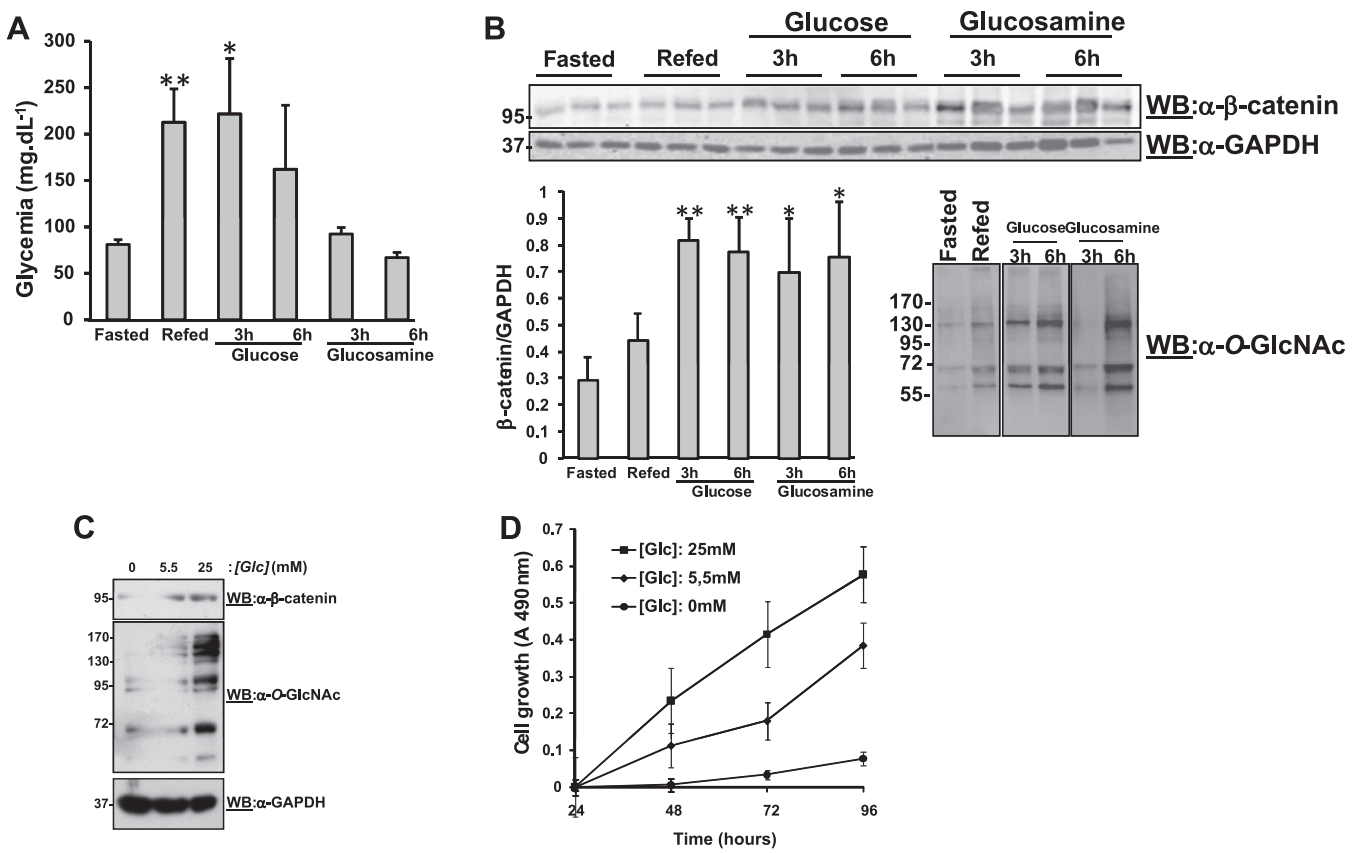

Fig. 1. β-Catenin and *O*-GlcNAcylation levels are elevated in refed and force-fed mice compared with fasted mice. C57BL6 mice were fasted for 24 h and refed a regular diet for 18 h or force-fed with glucose or glucosamine for 3 or 6 h. *A*: glycemia of each mice used in this study was measured, and values were represented as a histogram. *B*: colon homogenates were analyzed by immunoblot according to their β-catenin, GAPDH, and *O*-GlcNAcylation contents. The ratios of β-catenin/GAPDH expressions for the different conditions are represented as a histogram. *C*: MCF7 cells were cultured with 0, 5.5, or 25 mM glucose for 24 h, and expression of β-catenin and level of *O*-GlcNAcylation were determined by Western blot. *D*: proliferation rate of cells was determined for each glucose concentration condition by using the MTS method at 490 nm ($n = 6$). WB, Western Blot. Molecular mass markers are indicated at the *left* (in kDa). $^{*}P < 0.05$, $^{**}P < 0.01$.

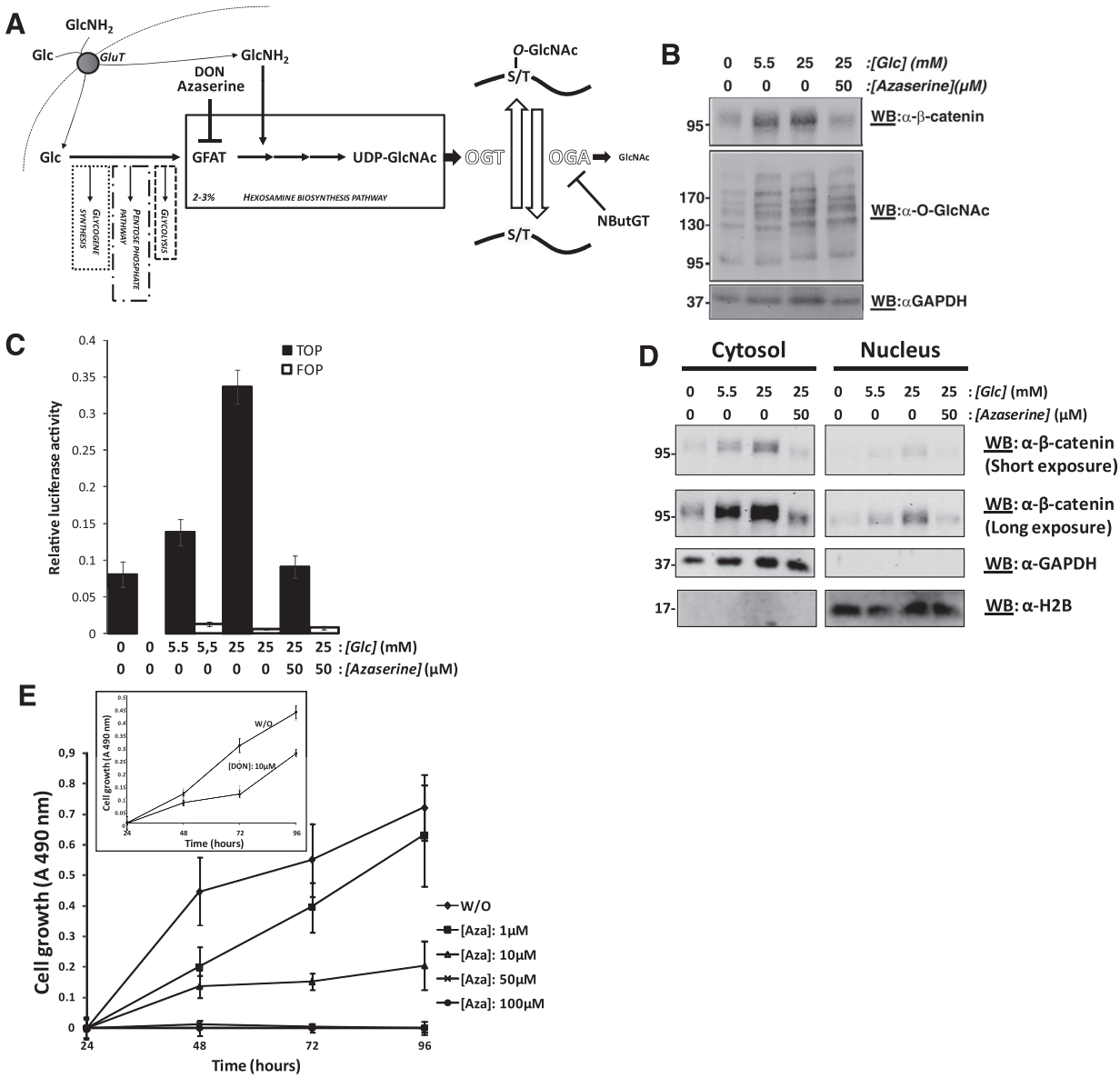

Fig. 2. β-Catenin expression, transcriptional activity, and cell proliferation are controlled by the hexosamine biosynthesis pathway (HBP). *A*: The cell circumvents 2–3% of the glucose toward the HBP for providing for UDP-GlcNAc, the substrate of many *N*-acetylglucosaminyltransferases including the *O*-GlcNAc transferase (OGT) responsible for *O*-GlcNAcylation processes. Hydrolysis of the GlcNAc moiety is catalyzed by *O*-GlcNAcase (OGA). The HBP is blocked by azaserine or DON (5-oxo-6-diazo-norleucine), two inhibitors of the rate-limiting enzyme GFAT (glutamine:fructose-6-phosphate amidotransferase), and activated by glucosamine, which shortcuts this enzyme. NButGT specifically inhibits the OGA. *B*: MCF7 cells were cultured in the absence of glucose, with 5.5 mM or 25 mM glucose with or without azaserine (for 18 h). Cell homogenates were immunoblotted with anti-β-catenin or anti-*O*-GlcNAc antibodies. Equal loading was assessed using GAPDH staining. *C*: HEK293 cells were transfected with TOP or FOP flash reporter plasmid and then incubated for 24 h in DMEM using variant conditions of glucose concentrations (0, 5.5, or 25 mM). Azaserine (Aza) was added 18 h before measurement of luciferase activity. This experiment is representative of 3 independent observations. *D*: subcellular fractionation was performed with MCF7 cells maintained in 0, 5.5, or 25 mM glucose with or without azaserine. Antibodies specifically directed against GAPDH (cytosol) and H2B (nucleus) were used to ensure the purity of each fraction. *E*: MCF7 cells were grown in DMEM supplemented with 25 mM glucose and with increasing concentrations of azaserine for 4 days. The proliferation rate of the cells was determined for each day using the MTS method at 490 nm ($n = 6$). The same experiment was done with 10 μM DON ($n = 6$) (*inset*). Molecular mass markers are indicated at the *left* (in kDa).

treatment, and, as expected, azaserine reversed the glucose effect at both the expression and the activity levels of β-catenin. This result demonstrates that the glucose-mediated β-catenin expression is conducted by the HBP. Since the functions of β-catenin depend on its subcellular localization, subcellular fractionation was performed on MCF7 cells following azaserine treatment (Fig. 2*D*). The glucose concentration upregulated the expression of the protooncogene in the cytosol and the nucleus (the same results were obtained by culturing cells with glucosamine or the OGA inhibitor PUGNAc; data not shown) without affecting the distribution, and azaserine reversed this enhancement in the two fractions. From this set of experiments, it can be deduced that the HBP flux increases the expression

and activity of β-catenin without modifying its subcellular distribution. It has been extensively reported that stabilization of β-catenin affects cell proliferation via transcription of its target genes (15, 29). Finally, after we observed that cell proliferation was dependent on the concentration of glucose (Fig. 1*D*), we tested increasing concentrations of azaserine on the proliferation capacities of MCF7 cells (Fig. 2*E*). These experiments show that the proliferation rate of the cells is blocked by the use of azaserine in a dose-dependent manner. DON was also used in proliferation rate assays and showed that, similar to azaserine, it reduced cell proliferation (Fig. 2*E, inset*). Through these findings, we demonstrated that glucose enhances the expression of β-catenin and its transcriptional capability through the HBP without modifying its subcellular distribution. We also demonstrated that MCF7 cell proliferation is controlled by the HBP flux.

HBP enhances β-catenin expression and accelerates cell proliferation by elevating O-GlcNAcylation levels. As depicted in Fig. 2*A*, UDP-GlcNAc is the main end product of the HBP. This nucleotide sugar is the donor of the GlcNAc group for all glycosylation processes, including the *O*-GlcNAcylation. It was demonstrated that plakoglobin, which follows a process of degradation similar to that of β-catenin, is protected by *O*-GlcNAcylation (14). Therefore, we suggested that the fate of β-catenin was linked to the same PTM. To check this hypothesis, MCF7 cells were incubated with increasing amounts of glucose and then incubated with NButGT, a specific inhibitor of OGA, azaserine, or glucosamine that short-cuts the HBP by bypassing GFAT (Fig. 2*A*). Interestingly, both NButGT and glucosamine elevated the expression of β-catenin (Fig. 3*A*). As we observed with glucose (Fig. 1*D*) and azaserine and DON (Fig. 2*E*), we tested the effect of NButGT on the proliferation rate of MCF7 cells (Fig. 3*B*). As expected, and contrary to

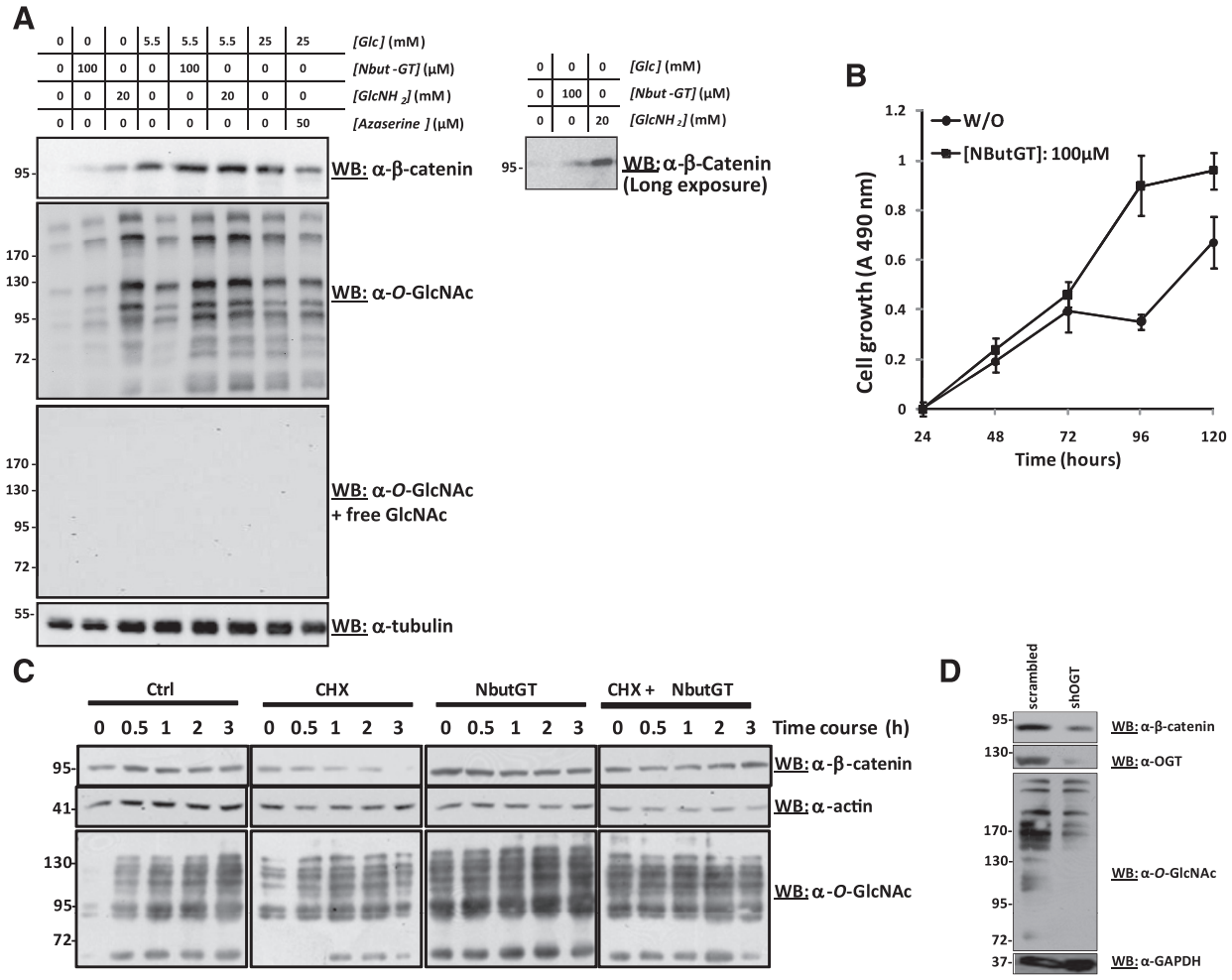

Fig. 3. Expression of β-catenin and proliferation rate of MCF7 cells are dependent on *O*-GlcNAcylation status. *A*: MCF7 cells were cultured in DMEM using various concentrations of glucose. The OGA inhibitor NButGT, the HBP activator glucosamine, and the GFAT inhibitor were tested at the indicated concentration for 18 h. Cell homogenates were immunoblotted with anti-β-catenin, anti-*O*-GlcNAc (with and without free *N*-acetylglucosamine), and anti-tubulin antibodies. *B*: MCF7 cells were grown in DMEM supplemented with 25 mM glucose with or without NButGT for 5 days. The proliferation rate of the cells was determined for each day by using the MTS method at 490 nm ($n = 6$). *C*: MCF7 cells were incubated with the protein synthesis inhibitor cycloheximide (CHX), NButGT, or both for indicated time periods. Expression of β-catenin and actin were analyzed by Western blot. Efficiency of NButGT was confirmed using an anti-*O*-GlcNAc antibody. *D*: MCF7 cells were transfected with a shRNA plasmid for 24 h to specifically decrease expression of OGT. A scrambled construction was used as a negative control. Expressions of OGT, *O*-GlcNAcylation, β-catenin, and GAPDH were thus determined by Western blot. W/O, without NButGT (vehicle). Molecular mass markers are indicated at the *left* (in kDa).

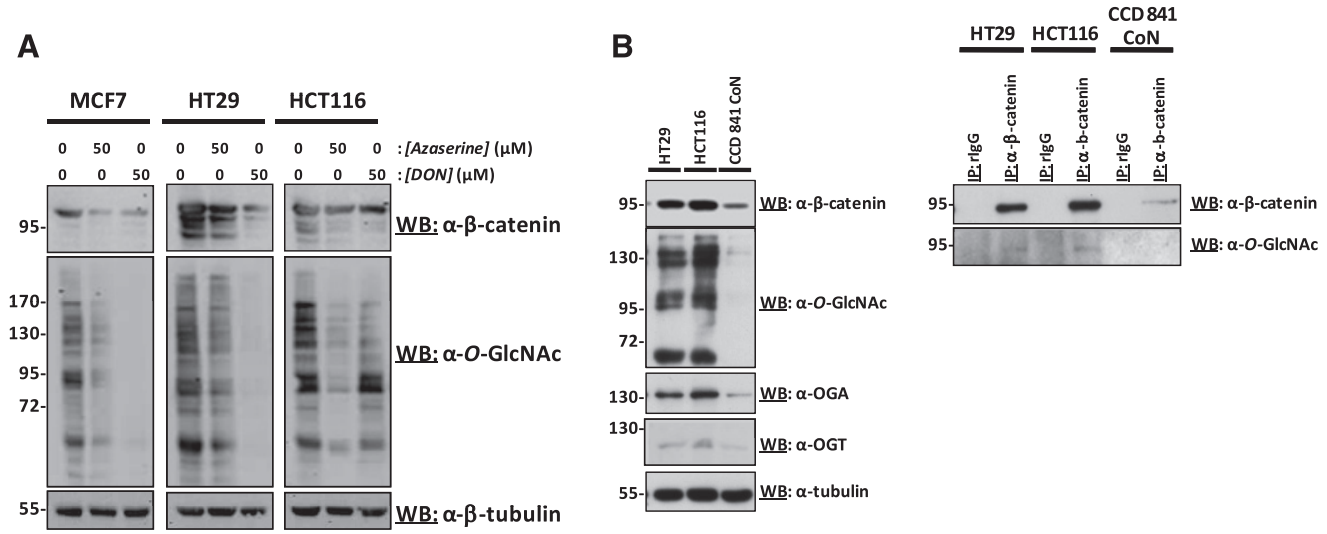

Fig. 4. β-Catenin, *O*-GlcNAc cycling enzymes, and *O*-GlcNAcylation levels are elevated in colorectal cancer cell lines. *A*: efficiency of HBP inhibitors azaserine and DON was determined in HT29 and HCT116 compared with MCF7 cells. *B*: HT29, HCT116, and CCD841CoN cells were analyzed according to their β-catenin, *O*-GlcNAcylation, OGA, and OGT contents by immunoblot (*left*). The *O*-GlcNAcylation status of each cell line's β-catenin was checked by analysis of protooncogene immunoprecipitates with anti-*O*-GlcNAc antibody (*right*). Controls of immunoprecipitation (IP) were performed using nonrelevant rabbit IgG antibodies. Molecular mass markers are indicated at the *left* (in kDa).

azaserine and DON, the OGA inhibitor accelerated the proliferation of the cells. In another set of experiments, MCF7 cells were incubated with cycloheximide (CHX) with or without NButGT. The β-catenin content was then assessed for increasing time periods (Fig. 3*C*). We observed that the inhibition of OGA compensates for the effect of CHX on the expression of β-catenin. These results demonstrated that *O*-GlcNAcylation prevents the degradation of β-catenin. Last, we used a shRNA strategy to knock down the OGT (Fig. 3*D*). We observed that the downexpression of the glycosyltransferase also downregulated the level of β-catenin.

β-catenin and O-GlcNAcylation levels are elevated in colorectal cancer cell lines compared with normal cells. Azaserine and DON were both used in MCF7 as well as HT29 (adenocarcinoma) and HCT116 (carcinoma), two colon cancer cell lines expressing high amounts of β-catenin (Fig. 4*A*) (33). The two inhibitors were efficient in MCF7 and HT29 and to a lesser extent in HCT116 cells. These differences may be explained by the fact that HCT116 cells express a β-catenin mutated in the D-box (ΔS45) that dramatically decreases its phosphorylation and impedes its correct targeting to the proteasome. The last point of the study compared the expression of β-catenin, the *O*-GlcNAc cycling enzymes, and the level of *O*-GlcNAcylation in HT29 and HCT116 cells with a normal colon cell line, CCD841CoN (21 wk gestation fetus) (Fig. 4*B*). β-Catenin, OGT, OGA, and the PTM are heavily expressed in the cancer cell lines. To measure the level of *O*-GlcNAcylation of the β-catenin in the three cell lines, the protooncogene was immunoprecipitated and analyzed according to its *O*-GlcNAcylation content (Fig. 4*B*). Interestingly, we showed a direct correlation between the modification and the expression of

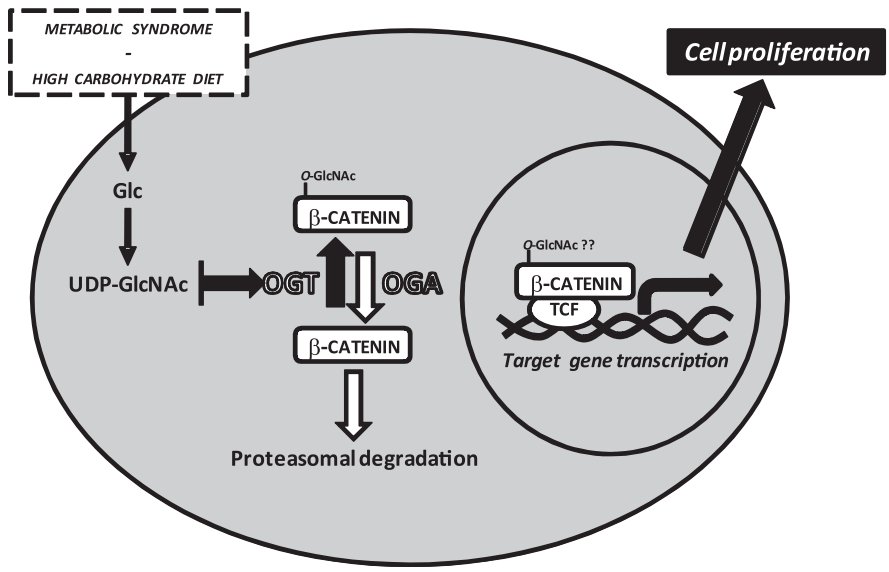

Fig. 5. Hypothetical mechanism by which diet and uncontrolled cell proliferation are linked. An excess of nutrients including carbohydrates elevates the production of UDP-GlcNAc, the end product of the HBP and substrate of OGT. Among the plethora of targets of OGT, β-catenin acquires an aberrant stability (it escapes proteasomal degradation) and therefore increased transcriptional activity, the cell gains an uncontrolled proliferation that may lead to cancer.

β-catenin. This last observation strengthens our hypothesis, according to which *O*-GlcNAcylation level β-catenin expression and emergence of cancer could be linked.

DISCUSSION

β-Catenin mutations are found in a plethora of human tumors: desmoid, endometrial, gastric, hepatic, ovarian, pancreatic, and colorectal. A defect in Wnt signaling is found in various human diseases, including osteoarthritis, Dupuytren's disease, polycystic kidney disease, and cardiovascular and neurodegenerative diseases. During their lifetimes, nearly 5% of individuals will develop a CRC, which, among the 200 forms of cancer, ranks first in terms of morbidity and mortality. The progression of CRC is a long process that is strictly organized spatially and temporally according to Vogelstein's sequence (9). During the succession of events, crucial components, such as oncogenes and tumor suppressors belonging to diverse signaling pathways, accumulate mutations (18). In 90% of CRC cases, mutations occur in the Wnt/β-catenin pathway, constituting early steps in the cancerization process of the colic and rectal mucosa (18, 27). In this way, inactivating mutations of the tumor suppressor APC are found in 85% of the sporadic forms of CRC, whereas 10% of these cancers possess activating mutations in β-catenin itself (23). Beyond the hereditary character of CRC and of the first importance played by environmental factors, it appears that the sedentary, overfed Western lifestyle enhances the risk of CRC emergence (2, 3, 5). Indeed, over time, the metabolic syndrome has been described as promoting CRC (6, 10, 17, 24). It has been established that an imbalance in food uptake and energy expenditure is associated with the development of CRC; this problem is more remarkable for obese and type 2 diabetes individuals (4–6). It is also noticeable that nutrients like carbohydrates are more influential for the progression of cancer (10, 16), but what links carbohydrate excess to CRC remains to be deciphered, even though the setting up of insulin resistance may be part of the explanation (24). Accordingly, nutritional metabolic troubles could have repercussions on the deregulation of the HBP and thus *O*-GlcNAcylation processes (Fig. 5), resulting in cell signaling failures, with Wnt signaling being directly affected in CRC.

Understanding and apprehending the underlying mechanisms causing CRC in response to a metabolic disorder should help reduce the progression of this serious health issue. β-Catenin and its associated signaling pathway are early actors in the development of CRC. The functions of β-catenin are intimately linked to its short half-life, its synthesis, and its degradation being rigorously controlled (33). Unfortunately, exposed mutations in the machinery regulating its expression can cause dramatic effects (18, 25, 27). β-Catenin is modified by numerous PTMs (30), including phosphorylation and ubiquitination (20), that control targeting of the oncoprotein to the 26S proteasome, and *O*-GlcNAcylation, whose function has been poorly studied (19, 26, 37). Owing to the homology between β-catenin and plakoglobin (14), and according to our observations, we propose that β-catenin acquires an aberrant stability, i.e., an enhanced half-life, after it is modified by the OGT (Fig. 5). This lifestyle-dependent stabilization may precede or aggravate the subsequent mutations affecting the Wnt/β-catenin pathway. Since a part of glucose participates in the production of UDP-GlcNAc, the substrate of OGT, we suggest that diet and CRC are linked through the HBP. Stabilization of β-catenin by activation or mutation of the Wnt pathway promotes the acceleration or loss of control of cell proliferation. The second case may lead to CRC. Previous studies already pointed out the crucial role of glucose in the expression of β-catenin (1, 28) and are in perfect agreement with our findings. Those authors showed that, in macrophages, glucose increases the expression of β-catenin in an HBP-dependent manner. Here, we found that, in addition to upregulating Wnt signaling by autocrine activation as demonstrated by Anagnostou and Sheperd (1), the HBP also stabilizes β-catenin by favoring its *O*-GlcNAcylation; these two consequences may be exerting a synergistic effect. We therefore propose that an elevated flux through the HBP, and accordingly an elevated *O*-GlcNAcylation status, constitutes a new process in the cancerization of the colic and rectal mucosa and explain why metabolic disorders and overfeeding enhance the risk of CRC.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.O., C.G., and T.L. conception and design of research; S.O., C.G., A.-M.M., Y.P.-C., and C.L. performed experiments; S.O., C.G., C.L., J.-C.M., and T.L. analyzed data; S.O., C.G., C.L., J.-C.M., and T.L. interpreted results of experiments; S.O. and T.L. prepared figures; S.O. and T.L. drafted manuscript; S.O., C.G., A.-M.M., Y.P.-C., C.L., J.-C.M., and T.L. approved final version of manuscript; T.L. edited and revised manuscript.

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SHORT COMMUNICATION

Serum-stimulated cell cycle entry promotes ncOGT synthesis required for cyclin D expression

S Olivier-Van Stichelen, L Drougat, V Dehennaut, I El Yazidi-Belkoura, C Guinez, A-M Mir, J-C Michalski, A-S Vercoutter-Edouart and T Lefebvre

Nuclear and cytoplasmic *O*-GlcNAc transferase (OGT) is a unique and universally expressed enzyme catalyzing *O*-GlcNAcylation of thousands of proteins. Although OGT interferes with many crucial intracellular processes, including cell cycle, only few studies have focused on elucidating the precise role of the glycosyltransferase during cell cycle entry. We first demonstrated that starved MCF7 cells reincubated with serum quickly induced a significant OGT increase concomitantly to activation of PI3K and MAPK pathways. Co-immunoprecipitation experiments performed upon serum stimulation showed a progressive interaction between OGT and β-catenin, a major factor in the regulation of cell cycle. OGT expression was also observed in starved HeLa cells reincubated with serum. In these cells, the *O*-GlcNAcylation status of the β-catenin-2XFLAG was increased following stimulation. Moreover, β-catenin-2XFLAG was heavily *O*-GlcNAcylated in exponentially proliferating HeLa cells when compared to confluent cells. Furthermore, blocking OGT activity using the potent inhibitor Ac-5SGlcNAc prevented serum-stimulated cyclin D1 synthesis and slightly delayed cell proliferation. At last, interfering with OGT expression (siOGT) blocked cyclin D1 expression and decreased PI3K and MAPK activation. Together, our data indicate that expression and catalytic activity of OGT are necessary and essential for G0/G1 transition.

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Subject Categories: cell cycle and growth regulation

Keywords: serum-stimulated cell cycle entry; *O*-GlcNAc transferase; cyclin D1; β-catenin

INTRODUCTION

O-GlcNAcylation (*O*-linked β-*N*-acetylglucosaminylation) is a post-translational modification that reversibly modifies proteins present in the cytosol, the nucleus and the mitochondria.[1–3] Two enzymes control the *O*-GlcNAcylation cycle. First, the *O*-GlcNAc transferase (OGT, uridine diphospho-*N*-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyltransferase or *O*-GlcNAc transferase) transfers the *N*-acetylglucosamine residue onto target proteins. Second, the *O*-GlcNAcase (OGA, *N*-acetyl β-glucosaminidase or *O*-GlcNAcase) removes the GlcNAc residue.[4] OGT (EC. 2.4.1.255) is assigned to the GT41 family in the CAZY (carbohydrate-active enzyme) database[5] and is found in all living beings (animals, plants, protists, bacteria, viruses) except in yeasts, in which its expression remains controversial. OGT participates in many fundamental cellular processes including cell cycle.[2] Our group previously showed that hormonal stimulation of physiologically G2-blocked *Xenopus laevis* oocytes triggered a quick increase in *O*-GlcNAcylation levels and that inhibition of OGT impaired G2/M transition.[6,7] In parallel, Slawson *et al.*[8] observed that OGT localized to the mitotic spindle and midbody during mitosis and that its overexpression resulted in supernumerary chromosomes. Later, the same authors showed that both OGT and OGA physically interact with Aurora B and PP1 to regulate the stability of the midbody and the phosphorylation and/or *O*-GlcNAcylation of vimentin at M-phase.[9,10] Finally, it was observed that OGT expression and *O*-GlcNAcylation reached a maximum level at the M-phase of the cell cycle.[11] Taken together, these studies show that OGT and *O*-GlcNAcylation in general are needed for germ cells meiosis and somatic cell mitosis. However, to our knowledge, there is no report focusing on the expression and activity of OGT during cell cycle entry (G0/G1). Here, we describe that following serum stimulation, OGT is significantly overexpressed. Blockade of OGT delays serum-stimulated cyclin D1 synthesis and cell proliferation. OGT silencing also prevents cyclin D1 expression and diminishes PI3K and MAPK activation. These are the first results demonstrating that OGT is indispensable for G0/G1 transition.

RESULTS AND DISCUSSION

OGT and OGA is a couple of non-redundant enzymes that controls *O*-GlcNAc cycling. Although much attention has been paid to OGT and although it has been described that this enzyme interferes with many crucial intracellular processes including cell cycle,[2,6–11] no study has focused on its expression and function during cell cycle entry.

OGT is upregulated upon stimulation of MCF7 cells

As described previously,[11] G0/G1 arrested MCF7 cells were stimulated by addition of fetal calf serum (FCS) and samples were collected at different times to assess OGT expression using western blot (Figures 1a and b). We observed a significant OGT increase (2.5-fold) 30 min after stimulation. FCS stimulation activated PI3K and MAPK pathways as attested by the use of antiphospho-Akt and antiphospho-Erk1/2 antibodies (Figure 1a).

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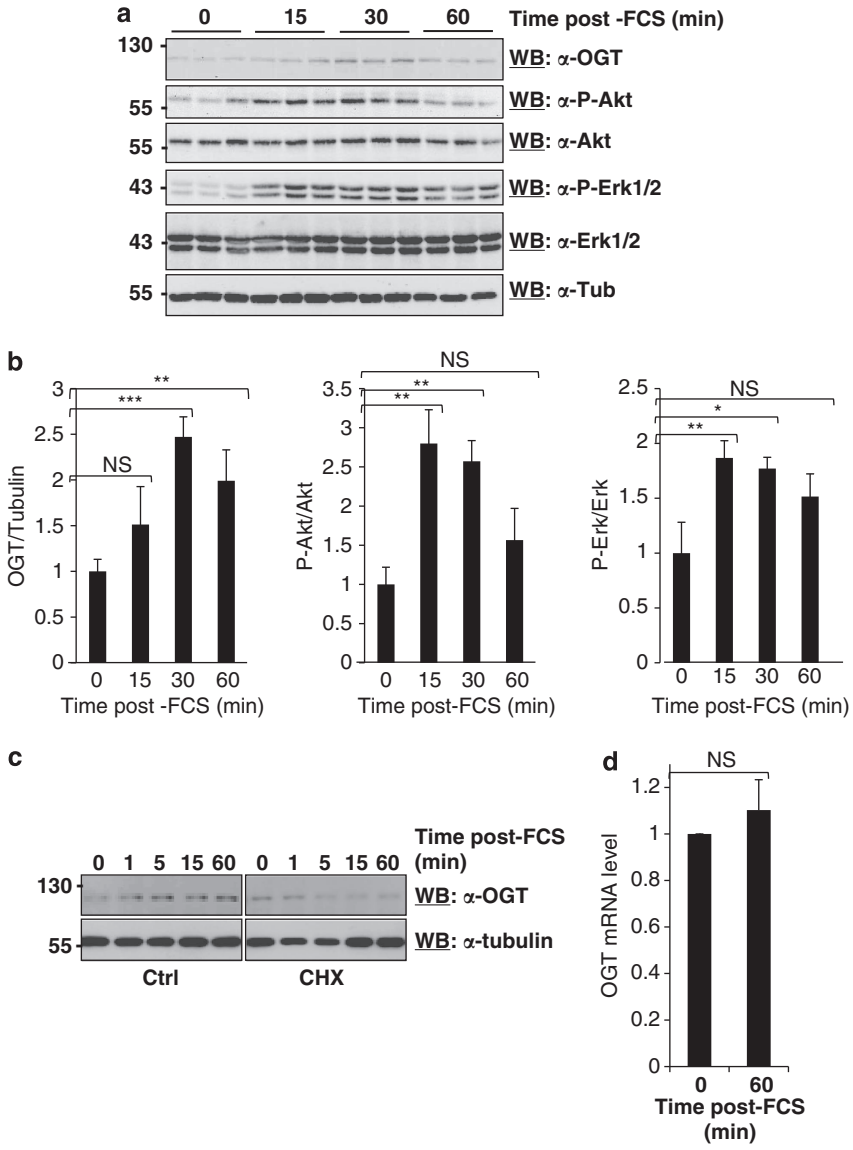

Figure 1. Stimulation of starved MCF7 cells with FCS increases OGT level. (**a**) MCF7 cells were maintained in a Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 5 IU/ml penicillin and 50 μg/ml streptomycin at 37 °C in a 5% (v/v) CO_2-enriched humidified atmosphere. Cells were stopped at G0/G1 using the FCS-starvation method.[11] Cells were FCS-starved for 48 h and then cell cycle was released by FCS addition. FCS-induced cells were collected at the indicated times after FCS addition. Cells were washed with 10 ml of cold phosphate-buffered saline and lysed directly on ice with lysis buffer (10 mM Tris/HCl, 150 mM NaCl, 1% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% sodium dodecyl sulfate (w/v) and proteases inhibitors, pH 7.4). Cell lysates were centrifuged (20 000 *g*, 10 min, 4 °C), pellets were discarded and supernatants boiled for 10 min in Laemmli buffer. Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and electroblotted on a nitrocellulose sheet (GE Healthcare, Orsay, France). Equal loading was verified using Ponceau red staining. Membranes were saturated for 45 min with 5% non-fatty acid milk in (TBS)-Tween buffer (15 mM Tris/HCl, 140 mM NaCl and 0.05% Tween20 (v/v), pH 8.0). Proteins were immunodetected using the following primary antibodies; OGT: rabbit polyclonal TI14, 1/2000 (Sigma-Aldrich, Saint-Quentin Fallavier, France); α-tubulin: mouse monoclonal B-5-1-2, 1/5000 (Santa Cruz Biotechnology, Heidelberg, Germany); Erk2: D-2, 1/5000 (Santa Cruz Biotechnology); phospho-Erk1/2: rabbit polyclonal, 1/1000 (Cell Signaling, Danvers, MA, USA); phospho-AKT: rabbit polyclonal, 1/1000 (Cell Signaling) and AKT: mouse monoclonal, 1/2000 (Cell Signaling). Membranes were incubated with primary antibodies overnight at 4 °C, washed three times (TBS–Tween, 10 min) and incubated with appropriate horseradish peroxidase-labelled secondary antibodies at a dilution of 1/10 000 for 1 h. After three more washes, detection was performed with enhanced chemiluminescence (GE Healthcare). (**b**) Histograms represent densitometric analyses of western blots (WBs). Results correspond to the mean value ± s.d. of three experiments (*$P<0.05$, **$P<0.01$, ***$P<0.001$ respectively, NS not significant). (**c**) Starved MCF7 cells were stimulated with FCS with or without the protein synthesis inhibitor cycloheximide (CHX) at a concentration of 15 μg/ml. OGT expression was analyzed by WB and protein loading was verified using alpha-tubulin. (**d**) FCS-starved and 1 h FCS-stimulated MCF7 cells OGT mRNA levels were determined by real-time PCR. Quantitative reverse transcriptase–PCR: Total RNA was reverse transcribed using random primers and MultiScribe reverse transcriptase (Applied Biosystems, Villebon sur Yvette, France). Real-time PCR analysis was performed by Power SYBR Green (Applied Biosystems) in a MX3005P fluorescence temperature cycler (Stratagene, Paris, France) according to the manufacturer's instructions. Results were normalized with respect to *RPLP0* RNAs used as internal control. The primers used are as follows: *OGT* sense 5′-TGGCTTCAGGAAGGCTATTG-3′ and antisense 5′-CAAGTCTTTTGGATGTTCATATG-3′, and *RPLP0* sense 5′-GTGATGTGCAGCTGATCAAGA-3′ and antisense 5′-GATGACCAGCCC AAAGGAGA-3′. Results correspond to the mean value ± s.d. of three experiments (NS not significant). Molecular mass markers are indicated at the left.

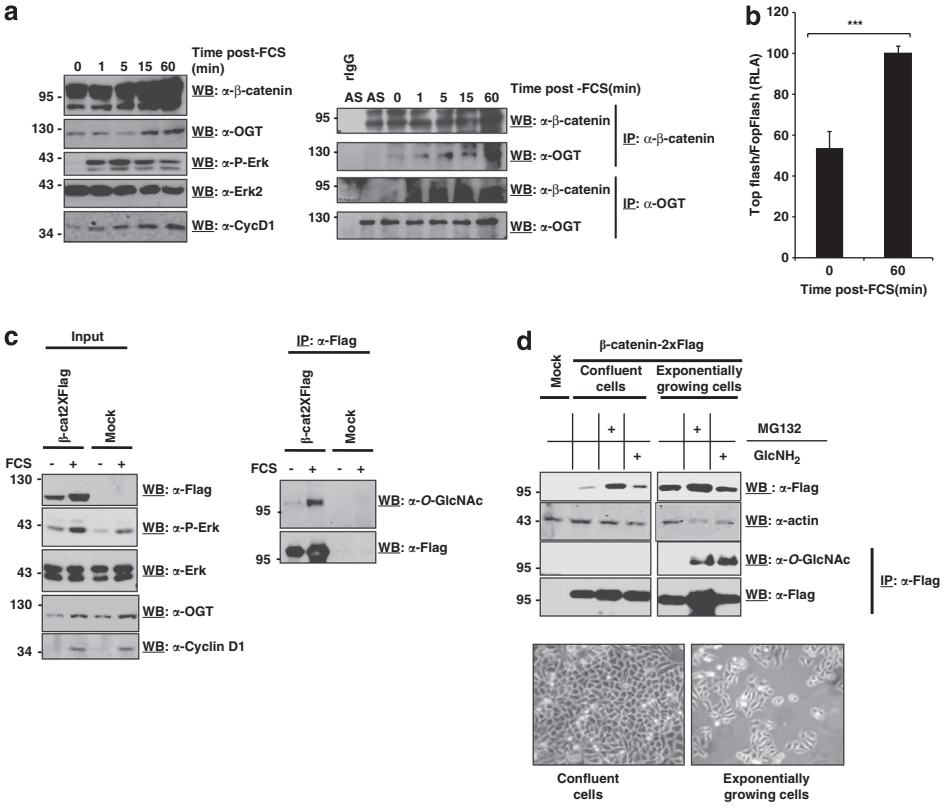

Figure 2. Upon cell cycle entry, as OGT content increases, the glycosyltransferase interacts with β-catenin (**a**). MCF7 cells were starved for 48 h and then re-supplemented with FCS for the indicated time periods. Levels of β-catenin, OGT and Erk2 were determined by western blot (WB). Activation of the MAPK pathway was probed using an antiphospho-Erk and cell cycle entry was checked using a mouse monoclonal anticyclin D1 (Santa Cruz Biotechnology) at a final dilution of 1/1000 (left panel). Co-immunoprecipitations using β-catenin or OGT-directed antibodies were performed during the time course experiment to evaluate the interaction between the two partners (right panel). MCF7 cells were washed with 10 ml of cold phosphate-buffered saline and lysed on ice in a lysis buffer containing 20 mM Tris/HCl, 150 mM NaCl, 0.5% NP-40 (v/v) and a cocktail of proteases inhibitors, pH 8.0. Whole cell extracts were centrifuged at 20 000 *g* for 10 min at 4 °C and supernatants were collected and first precleared with Sepharose-labelled protein A (GE Healthcare) for 1 h. Beads were then discarded and supernatants were incubated with rabbit anti-OGT (DM17 from Sigma) or rabbit anti-β-catenin (H-102 from Santa Cruz Biotechnology) antibodies overnight at 4 °C and then with Sepharose-labelled protein A for 1 h. Beads were gently centrifuged for 1 min and washed four times for 5 min each with the lysis buffer. Controls for immunoprecipitation specificities were performed with non-immune rabbit IgG (Santa Cruz Biotechnology). FCS stimulation of starved cells increases β-catenin transcriptional activity (**b**). Starved MCF7 cells were transfected with β-galactosidase, TOP Flash containing three optimal copies of the TCF/LEF-binding site, FOP Flash containing mutated copies of the TCF/LEF-binding site and β-catenin-2XFlag vector by the Lipofectamine2000 (Invitrogen, Saint-Aubin, France) reagent (2 μl) in six-wells plates with 0.2 μg of DNA for 24 h. Cells were stimulated by FCS for 1 h and TOP/FOP Flash reporter assays were performed. Histogram represents the relative luciferase activity. Results correspond to the mean value ± s.d. of three experiments (***$P < 0.001$). Addition of FCS to starved cells increases OGT and β-catenin levels and β-catenin O-GlcNAcylation (**c**). HeLa cells were maintained in the same conditions as described for MCF7 cells (Figure 1). Cells were transfected with pCS2 + β-catenin-2XFlag (or the empty vector) in 2.5 ml of optiMEM by the polyethylenimine (PEI, Euromedex) method (10 μl) in 100 mm diameter dishes with 2.5 μg of DNA for 6 h and then incubated for 48 h in 10 ml of fresh complete medium. One day later, cells were FCS-starved and reincubated with FCS for 1 h. The activity of the MAPK pathway was checked by evaluating phosphorylation of Erk. β-catenin was probed using the mouse monoclonal anti-Flag (M2 from Sigma) at a dilution of 1/5000. (left panel). Expression of cyclin D1 was also evaluated. β-catenin was immunopurified using the anti-FLAG antibody and the immunoprecipates were stained either with a mouse monoclonal anti-*O*-GlcNAc antibody (RL2, Ozyme, Saint-Quentin en Yvelines, France) at a dilution of 1/1000 or with the anti-FLAG antibody (right panel). β-catenin is heavily O-GlcNAcylated in exponentially HeLa growing cells (**d**). After transfection with pCS2 + β-catenin-2XFlag, HeLa cells were cultured and collected either when in the exponential growth phase or when cell confluence was reached. Cells were cultured either without any drug or with 1 μM MG132 (*N*-carbobenzoxyl-Leu-Leu-leucinal, Sigma) or 20 mM glucosamine. β-catenin (anti-Flag) expression was measured by WB. Rabbit polyclonal anti-actin (I–19 from Santa Cruz Biotechnology) was used at a dilution of 1/10 000. β-catenin was also immunopurified using the anti-FLAG and immunoprecipates were stained either with anti-*O*-GlcNAc antibody (RL2) or with anti-FLAG antibody (M2). Light microscopy pictures (right) were taken just before cell collection. IP, immunoprecipitation; rIgG, rabbit non-immune immunoglobulin G; AS, asynchronous cells. Molecular mass markers are indicated at the left.

Cells treated with the protein synthesis inhibitor cycloheximide before stimulation did not show OGT increase in response to FCS (Figure 1c), indicating that protein translation is required to enhance OGT level. In parallel, OGT mRNA level, assessed by real-time PCR, remained unchanged 60 min post FCS treatment (Figure 1d). Thus, we conclude that OGT is regulated at the translational level shortly after addition of FCS. This is the first time that OGT induction is reported so rapidly after FCS treatment. Yang *et al.*[11] recently showed that, upon FCS stimulation, OGT levels increased during all the cell cycle, suggesting that OGT might have some oncogenic properties. This hypothesis was first proposed by Caldwell *et al.*[12] who reported higher hexosamine biosynthetic pathway activity and OGT levels in breast cancer cells, and demonstrated that interfering with the glycosyltransferase

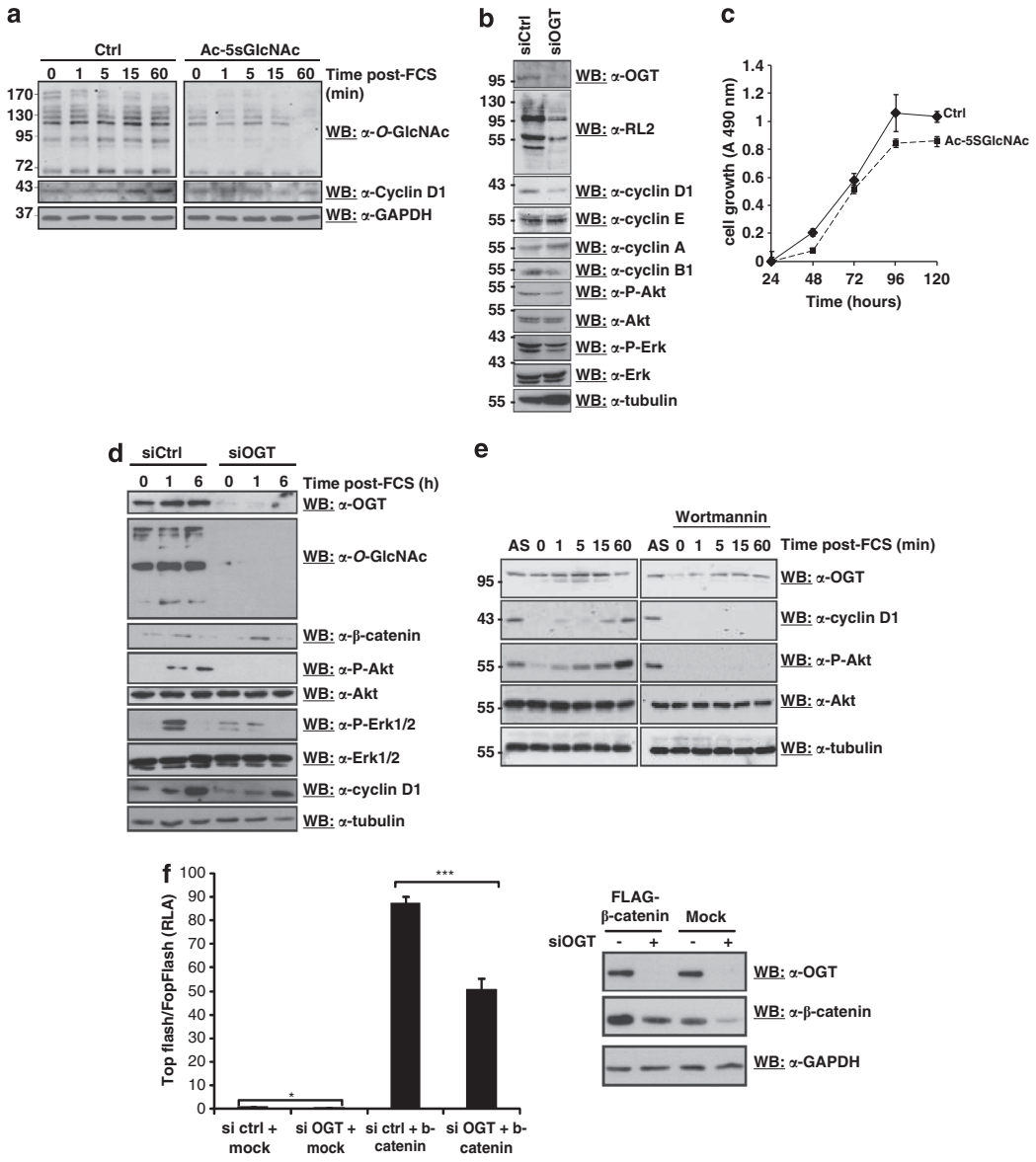

Figure 3. Inhibiting OGT catalytic activity or interfering with its expression hinders FCS-stimulated cell cycle entry. (**a**) Following FCS starvation, MCF7 cells were incubated with FCS for the indicated time periods with or without the OGT inhibitor Ac-5SGlcNAc at a final concentration of 100 μM. Cell lysates were then analyzed by western blot (WB) according to their *O*-GlcNAc and cyclin D1 content. Equal loading was checked by using a rabbit polyclonal anti-GAPDH (Abcam, Paris, France) at a dilution of 1/5000. (**b**) Asynchronous MCF7 cells were reverse-transfected with Lipofectamine RNAiMax (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions using 10 nM small interfering RNA targeting OGT[25] or a control siRNA (MISSION siRNA universal negative control #1, Sigma). Cell lysates were analyzed by WB according to their cyclin D1, cyclin E, cyclin A, cyclin B1, phospho-Akt, Akt, phospho-Erk1/2, Erk1/2 and tubulin contents. (**c**) MCF7 cells (2×10^3) were cultured in 96-wells plates using Dulbecco's modified Eagle's medium with or without 100 μM Ac-5SGlcNAc over 5 days. Each day, cell growth was determined using the MTS reagent method (Promega, Madison, WI, USA) according to the manufacturer's directions at 490 nm ($n = 6$). (**d**) Starved MCF7 cells were reverse-transfected with Lipofectamine using siRNA targeting OGT or a control siRNA as described in **b**. 24 h later, cells were FCS-deprived for 48 h and then stimulated for the indicated time periods. Cell lysates were analyzed by WB using anti-OGT, anti-*O*-GlcNAc, anti-β-catenin, anti-phospho-Akt, anti-Akt, anti-phospho-Erk1/2, anti-Erk1/2, anti-cyclin D1 and anti-tubulin antibodies. (**e**) Starved MCF7 cells were stimulated with FCS for the indicated time periods in conjunction with 10 nM wortmannin, an inhibitor of the PI3K pathway. Cell lysates were analyzed by WB according to their OGT and cyclin D1 content. Activation or inhibition of the PI3K pathway was assessed using an anti-P-Akt antibody. Equal loading was checked by using an anti-tubulin antibody. (**f**) HEK293T cells were cultured under the same conditions as described for MCF7 and HeLa cells (Figure 1). Following OGT silencing (see above for details), HEK293T cells were transfected with β-galactosidase, TOP Flash, FOP Flash and β-catenin2-XFlag vector or an empty vector by the Lipofectamine2000 reagent (Figure 2b) for 24 h to perform TOP/FOP Flash reporter assay. Histogram represents the relative luciferase activity (RLA). Results correspond to the mean value ± s.d. of three experiments (*$P < 0.05$, ***$P < 0.001$, respectively; NS not significant). OGT and β-catenin expression corresponding to the luciferase activity assay were measured by WB. GAPDH was used to attest equal loading.

expression reduced tumor growth. Thus, OGT synthesis and activity may regulate fundamental factors involved in the control of the cell cycle.

β-catenin and OGT interact rapidly upon G1 phase entry

We and others have previously reported that the oncoprotein β-catenin, a major cell cycle factor, can be *O*-GlcNAcylated by

OGT.[13–16] However, the role of this modification remained poorly understood. Studies performed on plakoglobin,[17] a member of the catenin family, showed that mutation of Thr14, an *O*-GlcNAcylated site close to the D-box that controls plakoglobin degradation, into alanine slightly increased plakoglobin stability. We also reported that β-catenin stability is regulated by the glucose status, the hexosamine biosynthetic pathway (which provides UDP-GlcNAc, the substrate for *O*-GlcNAcylation processes) flux and *O*-GlcNAcylation.[14,18,19] At last, RNA interference of OGA in the colorectal cancer metastatic cell line SW620 resulted in β-catenin overexpression.[20] Our hypothesis is that *O*-GlcNAcylation may serve as a protective signal for short half-life proteins such as β-catenin. Upon cell cycle entry, the expression of β-catenin continuously increases during G1 phase until it reaches a maximal level at G2/M transition.[21–23] To establish that this increase is due to *O*-GlcNAcylation, we investigated to measure the OGT interaction with β-catenin after addition of FCS to quiescent cells. First, we observed a concomitant increase of OGT and β-catenin as soon as 15 min after stimulation with FCS (Figure 2a, left panel). Co-immunoprecipitations performed under the same conditions showed that OGT and β-catenin interacted within minutes (Figure 2a, right panel) as soon as G0/G1 transition was triggered, as confirmed by phosphorylation of Erk and according to the β-catenin target gene cyclin D1 profile (Figure 2a, left panel). In this way, note that β-catenin transcriptional activity doubled upon FCS stimulation (Figure 2b) as attested by reporter gene analysis using TOP/FOP Flash constructs.

β-catenin is highly expressed and *O*-GlcNAcylated in response to FCS

Total levels of exogenous β-catenin and its *O*-GlcNAcylated fraction were evaluated by western blotting in β-catenin-2XFLAG overexpressing HeLa cells that were either FCS-starved or starved and reincubated with FCS for 1 h (Figure 2c, left panel). As expected, the total amount of β-catenin was found higher in FCS-stimulated cells. Moreover, and similar to MCF7 cells (Figure 1), an increased OGT expression was observed in response to FCS. In accordance, we also showed that modification of β-catenin by *O*-GlcNAc was remarkably enhanced concomitantly to OGT induction (Figure 2c, right panel). This is consistent with our previous report demonstrating that endogenous β-catenin was stabilized by *O*-GlcNAcylation in a model of oocyte maturation (*X. laevis*).[13]

Furthermore, β-catenin expression and *O*-GlcNAcylation levels were also compared in confluent and exponentially growing HeLa cells. Figure 2d shows that β-catenin protein was more abundant in proliferating cells than in quiescent cells. Strikingly, β-catenin was found *O*-GlcNAcylated in proliferating cells but not in quiescent one, suggesting that high level of β-catenin expression is dependent on *O*-GlcNAcylation.

Inhibition or downexpression of OGT impairs cell cycle entry

Because FCS stimulation induced OGT expression, we then tested the effect of OGT deficiency on cyclin D1 expression. To this end, we inhibited OGT either at the transferase activity level, using the recently described compound Ac-5SGlcNAc,[24] or at the expression level, using RNA interference (siOGT). Treatment with Ac-5SGlcNAc resulted in a marked decrease of overall *O*-GlcNAcylation in response to FCS (Figure 3a). Furthermore, cyclin D1 did not accumulated following FCS addition as in control condition (Figure 3a). To know whether OGT controls the expression of other cyclins, and consequently is crucial for all steps of the cell cycle, we depleted asynchronous MCF7 of OGT through the use of siRNA (Figure 3b). We observed lower amounts of cyclin D1 and cyclin B1, corroborating previous findings,[6,7] in MCF7 transfected with siOGT when compared with siCtrl (universal negative control), whereas expression of cyclin E and cyclin A were unaffected. The same result was achieved by inhibiting OGT at the catalytic level (Supplementary Figure S1). Moreover, OGT silencing decreased the phosphorylated forms (active) of Akt and Erk1/2, the downstream effectors of the PI3K and MAPK pathways respectively, in asynchronous cells. In this way, longer time-course experiments showed that Ac-5SGlcNAc slightly delayed cell proliferation (Figure 3c). RNA interference of OGT also resulted in inhibition of FCS-induced cyclin D1 expression and in inhibition of PI3K/MAPK activation as observed above for asynchronous cells (Figure 3d). β-catenin expression remained similar in control and siOGT-treated cells. We next tested wortmannin, a PI3K inhibitor in FCS-stimulated cells (Figure 3e). We observed that reciprocally, inhibiting the PI3K pathway decreased FCS-induced OGT, and cyclin D1, expression, supposing a feedback loop between PI3K and OGT. To evaluate the impact of OGT silencing on the transactivation of genes responsive to the β-catenin/TCF/LEF complex, we performed a reporter gene analysis using the TOP/FOP Flash system in HEK293T cells (Figure 3f). Silencing OGT expression decreased by 50% the transcription activity of the β-catenin/TCF/LEF complex in this model. Analysis of β-catenin expression by western blot indicates that the transcriptional activity decrease correlated with a loss of β-catenin expression in FCS-induced HEK293T cells.

All together, our results indicate that OGT is a potent regulator of the cell cycle entry essential for FCS-stimulated cells to activate mitogenic pathways and to express cyclin D1. Thus, we propose that abnormal increase of OGT activity, which may occur in a context of nutrient excess that directly modulates the levels of the OGT substrate UDP-GlcNAc, would affect the cell cycle, as recently discussed by others.[12] In such a context, OGT could arguably exert some oncogenic effect, by allowing the cell cycle of the affected cells to bolt off.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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O-GlcNAcylation stabilizes β-catenin through direct competition with phosphorylation at threonine 41

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ABSTRACT **Dysfunctions in Wnt signaling increase β-catenin stability and are associated with cancers, including colorectal cancer. In addition, β-catenin degradation is decreased by nutrient-dependent *O*-GlcNAcylation. Human colon tumors and colons from mice fed high-carbohydrate diets exhibited higher amounts of β-catenin and *O*-GlcNAc relative to healthy tissues and mice fed a standard diet, respectively. Administration of the *O*-GlcNAcase inhibitor thiamet G to mice also increased colonic expression of β-catenin. By ETD-MS/MS, we identified 4 *O*-GlcNAcylation sites at the N terminus of β-catenin (S23/T40/T41/T112). Furthermore, mutation of serine and threonine residues within the D box of β-catenin reduced *O*-GlcNAcylation by 75%. Interestingly, elevating *O*-GlcNAcylation in human colon cell lines drastically reduced phosphorylation at T41, a key residue of the D box responsible for β-catenin stability. Analyses of β-catenin *O*-GlcNAcylation mutants reinforced T41 as the most crucial residue that controls the β-catenin degradation rate. Finally, inhibiting *O*-GlcNAcylation decreased the β-catenin/α-catenin interaction necessary for mucosa integrity, whereas *O*-GlcNAcase silencing improved this interaction. These results suggest that *O*-GlcNAcylation regulates not only the stability of β-catenin, but also affects its localization at the level of adherens junctions. Accordingly, we propose that *O*-GlcNAcylation of β-catenin is a missing link between the glucose metabolism deregulation observed in metabolic disorders and the development of cancer.—Olivier-Van Stichelen, S., Dehennaut, V., Buzy, A., Zachayus, J.-L., Guinez, C., Mir, A.-M., El Yazidi-Belkoura, I., Copin, M.-C., Boureme, D., Loyaux, D., Ferrara, P. Lefebvre, T. *O*-GlcNAcylation stabilizes β-catenin through direct competition with phosphorylation at threonine 41. *FASEB J.* 28, 000–000 (2014). www.fasebj.org**

Key Words: Wnt signaling · cancer · ETD-MS/MS

WNT SIGNALING IS AN EVOLUTIONARILY conserved pathway involved in biological processes, including embryogenesis and intestinal tissue renewal (1). Cancer development is closely associated with deregulation of Wnt signaling and its key player β-catenin (2). β-Catenin is primarily found at adherens junctions in complex with E-cadherin and α-catenin and ensures epithelial integrity (3). Excess β-catenin is targeted to the proteasome by an efficient phosphorylation/ubiquitinylation-mediated degradation system (4, 5). First, a destruction complex including the tumor suppressors adenomatous polyposis coli (APC) and axin, as well as the kinases GSK3β and CK1α, sequestrates β-catenin (6). β-Catenin sequestration leads to the phosphorylation of the destruction box (D box) at S33, S37, T41, and S45; to the ubiquitination of β-catenin at K19 and K49; and to its proteasomal degradation. Intact β-catenin migrates into the nucleus and drives the expression of genes crucial for cell cycle progression (7–10).

Genetic alterations in Wnt signaling are frequently found in leukemia, lymphoma, and medulloblastoma and in gastric, hepatocellular, breast and colorectal cancers (CRCs) and include mutations in the APC,

Abbreviations: APC, adenomatous polyposis coli; CRC, colorectal cancer; D box, destruction box; DMEM, Dulbecco's modified Eagle's medium; EDT-MS/MS, electron-transfer dissociation–tandem mass spectrometry; GFAT, glutamine:fructose-6-phosphate amidotransferase; HBP, hexosamine biosynthesis pathway; HCD, high-carbohydrate diet, LC-MS/MS, liquid chromatography–tandem mass spectrometry; OGA; *O*-linked *N*-acetylglucosaminase; *O*-GlcNAc, *O*-linked *N*-acetylglucosamine; OGT, *O*-linked *N*-acetylglucosamine transferase; OGTT, oral glucose tolerance test; PBS, phosphate-buffered saline; PPP, pentose phosphate pathway; PTM, posttranslational modification; SD, standard diet; siCtrl, scrambled control; siRNA, small interfering RNA; TM, tetramutant; WT, wild type

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axin, TCF4, LRP5, and β-catenin genes (8). In addition, Wnt signaling is hyperactivated by a miR-301a-dependent PTEN-targeting mechanism in breast cancer (11). Direct mutations of β-catenin are found in hepatocellular carcinoma and CRC at 33 and 10%, respectively, increasing the protein's stability and therefore its oncogenic properties (12, 13). Diet, lifestyle, and metabolic pathologies also increase the risk of cancer, with a 2-fold increase of CRC in patients with type 2 diabetes (14–16). To understand the links between nutrition and CRC, we focus on β-catenin and its dynamic posttranslational modification (PTM) by *O*-linked *N*-acetylglucosamine (*O*-GlcNAc; ref. 17).

O-GlcNAc transferase (OGT) adds a single *N*-acetylglucosamine group to target proteins using the activated donor UDP-GlcNAc, the end product of the hexosamine biosynthesis pathway (HBP; ref. 18). A second enzyme, *O*-GlcNAcase (OGA), dynamically removes the GlcNAc residue. The UDP-GlcNAc concentration, and consequently *O*-GlcNAcylation, correlates closely with the nutrient status of the cell, and the nucleotide-sugar is therefore regarded as a nutrient sensor. We previously demonstrated that colons from unfed-state mice force-fed glucose or glucosamine have higher levels of both β-catenin expression and *O*-GlcNAcylation (19). Moreover, we observed higher levels of β-catenin, *O*-GlcNAcylation, and OGT in 2 CRC cell lines in comparison with a fetal colon cell line. These results led us to hypothesize that diet and metabolic disorders predispose for CRC by promoting an increase in β-catenin expression through up-regulation of HBP and *O*-GlcNAcylation. We demonstrated that modulation of the HBP flux by glucosamine stimulation yields increased β-catenin level in MCF7 cells, whereas its level is decreased on inhibition of the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). In addition, simply manipulating the *O*-GlcNAcylation status of the cell directly correlates with the stability of β-catenin, suggesting that glycosylation protects it from degradation (19). Here, we describe one of the mechanisms by which *O*-GlcNAcylation may reduce β-catenin proteasomal susceptibility.

MATERIALS AND METHODS

Human tumor tissues

Colon tumor tissues and matching tumor-adjacent normal tissues from the same patient were obtained from the Tumor Bank, Regional Reference Center in Cancer [Centre Hospitalier Régional Universitaire (CHRU) of Lille, Lille, France; agreement CSTMT076]. Patient data are presented in **Table 1**.

Cell culture

HEK293T, MCF7, HT29, and HCT116 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), and CCD841CoN cells were maintained in Eagle's minimum essential medium (EMEM). All the cell lines were maintained in medium supplemented with 10% (v/v) fetal calf serum (heat inactivated for HT29 and HCT116 cells), 2 mM L-glutamine, 5 IU/ml penicillin, and 50 μg/ml streptomycin at 37°C, in a 5% (v/v) CO_2-enriched, humidified atmosphere.

Plasmids and transfection

Wild-type (WT) and tetramutant (TM; S33A, S37A, T41A, S45A) pCS2$^+$-β-catenin-2XFLAG (provided by Dr. C. Liu, University of Kentucky, Lexington, KY, USA) are described elsewhere (4, 5). To generate the β-catenin *O*-GlcNAcylation mutants, we used the following primer sequences: S23A-β-catenin, sense oligonucleotide 5′-CGAAAGGCAGCAGTGGCCCACTGGCAGCAGCAG-3′/antisense oligonucleotide 5′-CTGCTGCTGCCAGTGGGCCACTGCTGCCTTTCG-3′; T40A-β-catenin, sense oligonucleotide 5′-ATTCATTCTGGAGCAGCCACCACAGCACCA-3′/antisense oligonucleotide 5′-TGGTGCTGTGGTGGCTGCTCCAGAATGAAT-3′; T41A-β-catenin, sense oligonucleotide 5′-ATTCTGGAGCAACCGCCACAGCACCATCTTT-3′/antisense oligonucleotide 5′-AAAGATGGTGCTGTGGCGGTTGCTCCAGAAT-3′; and T112A-β-catenin, sense oligonucleotide 5′-CAGATTCCATCCGCACAATTTGACTCT-3′/antisense oligonucleotide 5′-AGAGTCAAATTGTGCGGATGGAATCTG-3′. To generate the TM 4M β-catenin (S23A/T40A/T41A/T112A), we first generated the T40A/T41-β-catenin mutant using sense oligonucleotide 5′-ATTCTGGAGCAGCCGCCACAGCACCATCTTT-3′/antisense oligonucleotide 5′-AAAGATGGTGCTGTGGCGGCTGCTCCAGAAT-3′. Then, the S23A/T40/T41-β-catenin and the S23A/T40A/T41A/T112A-β-catenin (4M) were

TABLE 1. *Information on tissues used in the study and medical history of the related patients*

Patient and ref. no.	Tissue	Tumor tissue (%)	Healthy tissue (%)	Necrosis (%)	Cells (%)	Stroma (%)	pT	pN	Nodes removed	Nodes affected	Medical history
1									20	0	Indeterminate (1.75 m; 85 kg); slight overweight
15417	Tumor	100	0	1	50	50	3	0			
15419	Healthy	0	100				3	0			
2									10	0	Diabetes, hypertension
9269	Tumor	100	0	<5	70	30	3	0			
9271	Healthy	0	100				3	0			
3									12	0	Hypertension, diabetes, temporary ischemic attack
8453	Tumor	90	10	0	60	40	3	0			
8458	Healthy	0	100				3	0			
4									29	0	Hypertension, dyslipidemia
14998	Tumor	90	10	<5	80	20	4	0			
15001	Healthy	0	100				4	0			

pT, primary tumor; pN, regional lymph nodes.

simultaneously generated by using the oligonucleotides mentioned above.

The HA-ubiquitin expression vector was provided by Dr. C. Couturier (Biology Institute of Lille, Lille, France).

MCF7 or HEK293T cells were transfected with 2.5 μg of DNA and 10 μl of Lipofectamine (Lipo2000; Invitrogen, Cergy Pontoise, France) in 10 ml of DMEM in 100 mm diameter dishes.

Small interfering RNA (siRNA)

With the exception of CCD841CoN cells, which were reverse transfected for 72 h, all cells were reverse transfected for 48 h with RNAiMax (Invitrogen), according to the manufacturer's instructions, using 10 nM of a pool of 4 siRNAs targeting OGA (siGenome Smart Pool, Human MGEA5, M-012805-01; Dharmacon, Lafayette, CO, USA), 10 nM siRNA targeting OGT (20), or a scrambled control (siCtrl) sequence (19).

Small molecule supplements

Proteasome inhibitor MG132 (Sigma-Aldrich, Saint-Quentin Fallavier, France) was used at a concentration of 1 μM. Glucosamine was used at a concentration of 5 mM. The OGT and OGA inhibitors, Ac5S-GlcNAc and NButGT, respectively (provided by Pr. D. Vocadlo, Simon Fraser University, Burnaby, BC, Canada), were used at a concentration of 100 μM. For thiamet G use, see *In vivo* Experiments below.

Immunoprecipitation and coimmunoprecipitation assays

Cells were washed with 10 ml cold phosphate-buffered saline (PBS), then lysed on ice with either IP lysis buffer [10 mM Tris/HCl, 150 mM NaCl, 1% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% sodium dodecyl sulfate (w/v), and protease inhibitors; pH 7.4] or octyl lysis buffer [25 mM Tris HCl, 150 mM NaCl, 1.7% (w/v) octyl glucoside, 0.084% (w/v) sodium fluoride, 0.44% (w/v) sodium pyrophosphate, 0.018% (w/v) sodium orthovanadate, 1 μM PUGNAc, and protease inhibitors; pH 7.5] for immunoprecipitation or co-IP lysis buffer [20 mM Tris/HCl, 150 mM NaCl, 0.5% Nonidet P-40 (v/v) and protease inhibitors; pH 8.0] for coimmunoprecipitation. The cell extracts were then centrifuged at 20,000 *g* for 10 min at 4°C. The supernatants were precleared with Sepharose-labeled protein A/G for 1 h. The beads were discarded after a 1 min centrifugation at 2500 *g*, and the supernatants were incubated with 2 μg of either mouse monoclonal anti-FLAG antibody (M2; Sigma-Aldrich) or rabbit polyclonal anti-β-catenin (H102; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rocked at 4°C overnight. Controls for immunoprecipitation specificities were performed with rabbit IgG (Santa Cruz Biotechnology). Antibody-bound proteins were recovered by rocking samples with 20 μl of Sepharose-labeled protein A/G (GE Healthcare, Buc, France) for 1 h at 4°C. For immunoprecipitation, the beads were gently centrifuged for 1 min at 2500 *g* and washed as follows: IP lysis buffer; IP lysis buffer supplemented with 500 mM NaCl; IP lysis buffer/TNE (10 mM Tris/HCl, 150 mM NaCl, and 1 mM EDTA; pH 7.4; v/v); and finally TNE alone. For coimmunoprecipitation, the beads were washed 4 times with co-IP lysis buffer. Bound proteins were eluted by boiling in Laemmli buffer.

Western blot analysis

Proteins were resolved on 8 or 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose (GE Healthcare). Equal loading and transfer efficiency were confirmed by Ponceau red staining. The membranes were blocked for 45 min with 5% (w/v) non-fatty-acid milk in Tris-buffered saline (TBS)-Tween buffer [15 mM Tris/HCl, 140 mM NaCl, and 0.05% Tween20 (v/v); pH 8.0]. The membranes were incubated overnight at 4°C with the appropriate antibodies (described below). The membranes were then washed 3 times with TBS-Tween for 10 min and incubated with either an anti-rabbit or an anti-mouse horseradish peroxidase–labeled secondary antibody at a dilution of 1:10,000 for 1 h. Finally, the blots were washed with TBS-Tween 3 times for 10 min each, and signal was detected by enhanced chemiluminescence (GE Healthcare). Mouse monoclonal anti-*O*-GlcNAc (RL2; VWR International, Fontenay-sous-Bois, France), mouse monoclonal anti-phosphoT41-β-catenin (C8616; Sigma-Aldrich), rabbit polyclonal anti-phospho-T41/S45-β-catenin (9565; Cell Signaling Technology, Leiden, The Netherlands), rabbit polyclonal anti-phospho-S33/S37/T41-β-catenin (9561; Cell Sig-

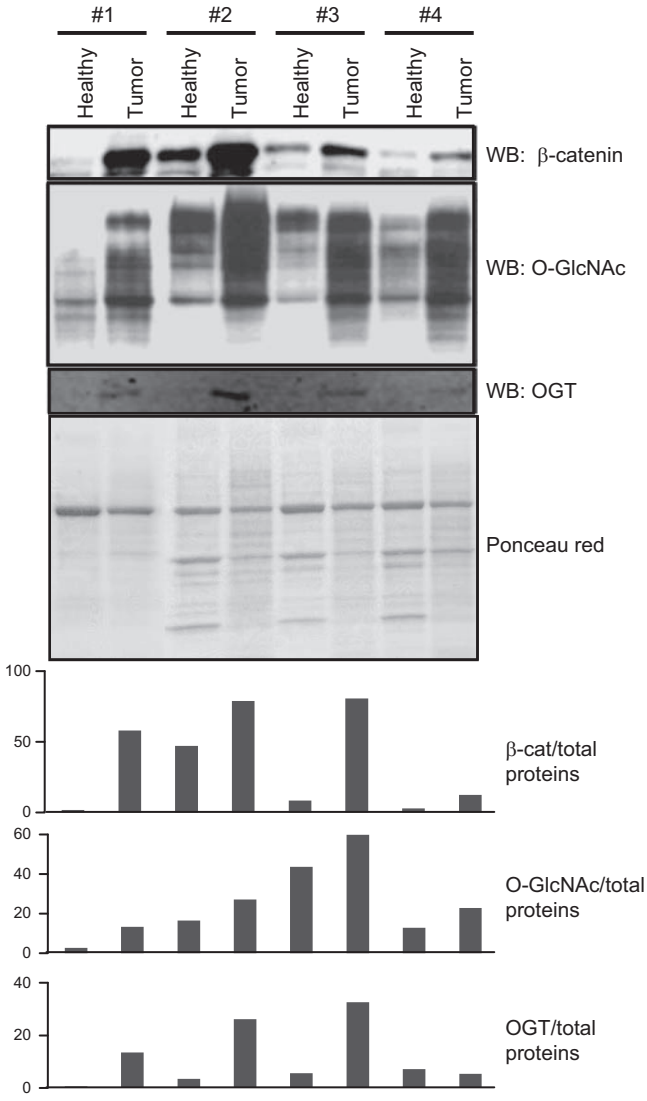

Figure 1. Human colorectal tumor tissue exhibits higher levels of β-catenin, *O*-GlcNAcylation, and OGT than does healthy tissue. Colon tumor and matching tumor-adjacent normal tissues from the same patient were analyzed by Western blot for levels of β-catenin, *O*-GlcNAcylation, and OGT contents. Ponceau red staining of the whole extracts confirmed equal loading (40 μg proteins/lane).

naling Technology), and rabbit polyclonal anti-HA (Santa Cruz Biotechnology) were used at a final dilution of 1:1000. Rabbit polyclonal anti-OGT (AL28) and chicken anti-OGA (345) (both provided by Prof. G. W. Hart, Johns Hopkins University, Baltimore, MD, USA), rabbit polyclonal anti α-catenin (H297; Santa Cruz Biotechnology), and rabbit polyclonal anti-β-catenin (H102; Santa Cruz Biotechnology) were used at a dilution of 1:2000. Mouse monoclonal anti-FLAG (M2; Sigma-Aldrich) and rabbit polyclonal anti-GAPDH (Abcam, Cambridge, UK) were used at a dilution of 1:5000.

Cell fractionation

The Proteoextract subcellular proteome extraction kit (Merck, Darmstadt, Germany) was used for cellular fractionation, as recommended by the manufacturer.

In vivo experiments

Eight-week-old male C57BL/6J mice were purchased from Charles River (Saint-Germain sur l'Arbresle, France) and adapted to the environment for 1 wk before the experiments. The mice were maintained in a 12 h light-dark cycle with water and a standard diet (SD; 65% carbohydrate, 11% fat, and 24% protein; SAFE, Augy, France) or a high-carbohydrate diet (HCD; 75% carbohydrate, 3% fat, and 22% protein; SAFE) for 9 wk. Procedures were performed according to the French guidelines for the care of experimental animals. Colons were collected, washed in PBS, and lysed in 2 ml of Nonidet P-40 lysis buffer [10 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40 (v/v), 0.018% Na_3VO_4, 1 μM PUGNAc, and protease inhibitors; pH 7.4]. An UltraTurrax disperser (Sigma-Aldrich) was used for tissue disruption. Samples were incubated with gentle agitation for 2 h at 4°C, after which the soluble fraction was obtained after centrifugation at 20,000 *g* for 30 min at 4°C.

For chronic thiamet G (Sigma-Aldrich) treatment, mice were intraperitoneally injected daily with thiamet G (20 mg/kg/d in PBS) or with PBS for 15 d. Before euthanasia, mice were denied access to food for 24 h.

Oral glucose tolerance test (OGTT)

To test glucose tolerance, mice were force-fed glucose (1 g glucose/kg) after overnight food withdrawal. Glycemia was measured every 15 min for 2 h with an Accu-Chek Performa apparatus (Roche, Basel Switzerland) before the animals were euthanized.

Preparation of β-catenin for mass spectrometry

HT29 cells and samples from 4 patients with CRC were lysed in octyl lysis buffer. The lysate was first precleared with protein-A-Sepharose, and β-catenin was isolated by immunoprecipitation. The Coomassie-stained band corresponding to β-catenin was excised, reduced with DTT, alkylated with iodoacetamide, and in-gel digested with trypsin (Promega

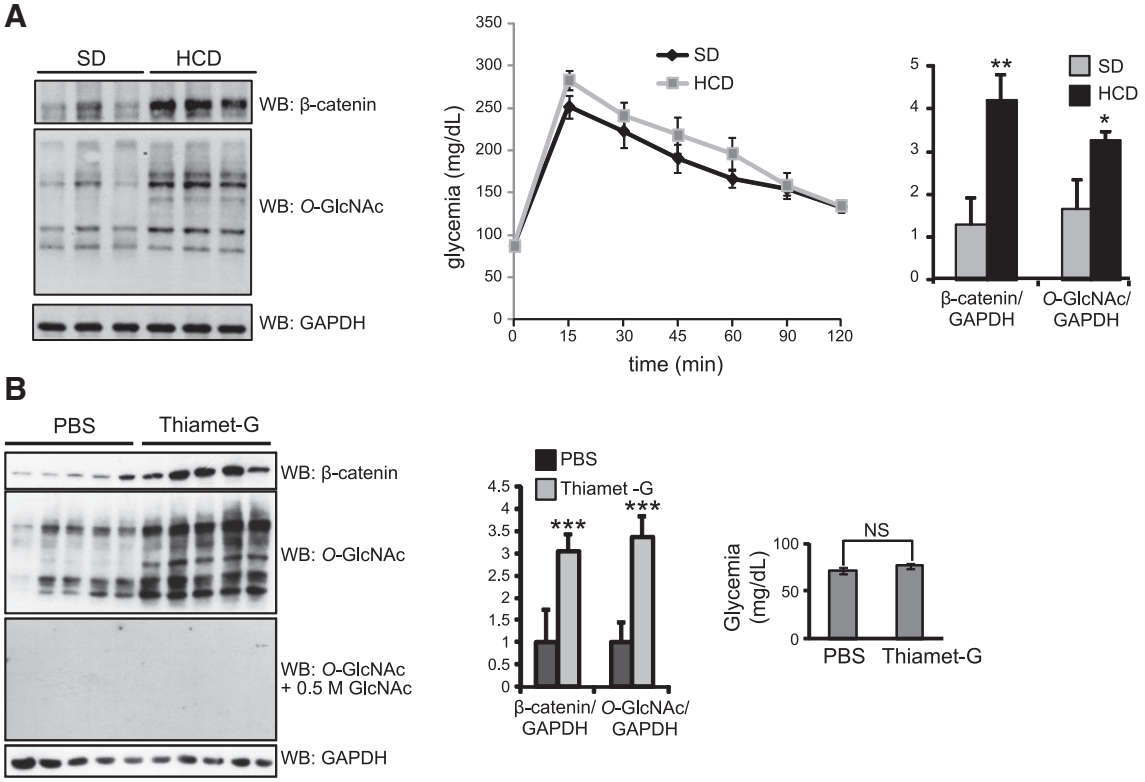

Figure 2. Mice fed an HCD or injected with thiamet G exhibit increased β-catenin and *O*-GlcNAc levels. *A*) Colon homogenates from mice fed an HCD or an SD for 9 wk were analyzed by Western blot for β-catenin, *O*-GlcNAc, and GAPDH (loading control) levels. OGTTs were performed, and blood glucose levels were determined for the HCD- and SD-fed mice by overnight food withdrawal after glucose force-feeding (differences are statistically nonsignificant). *B*) Colon homogenates from mice treated with the OGA inhibitor thiamet G for 2 wk were analyzed as in panel *A*. Anti-*O*-GlcNAc antibody condition was treated with free *N*-acetylglucosamine to show specificity. Glycemic levels were measured for the same animals with no significant differences observed. *A, B*) Average ± SD ratios of β-catenin/GAPDH and *O*-GlcNAc/GAPDH are shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

France, Charbonnieres, France; ref. 21). Peptides were extracted with 50 mM NH_4HCO_3 and 50% CH_3CN in 0.2% HCOOH and analyzed by nano-liquid chromatography–tandem mass spectrometry (LC-MS/MS) after partial evaporation in a Speed-vac concentrator (Thermo Fisher Scientific, Villebon-sur-Yvette, France), to remove acetonitrile.

Nano-LC-MS/MS analysis

LC-MS/MS experiments were performed on a nanoAcquity UPLC system (Waters, Milford, Ma, USA), connected to an LTQ Orbitrap Velos ETD mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray source. β-Catenin trypsin digest was loaded onto a precolumn (20 mm × 180 μm i.d.; Symmetry C18; Waters) and washed with solvent A (0.2% HCOOH) at 20 μl/min for 5 min. The peptides were then eluted on a C18 reverse-phase nanoflow column (200 mm × 75 μm i.d.; BEH130 C18; Waters) with a linear gradient of 7–40% solvent B [H_2O/CH_3CN/HCOOH, 10/90/0.2 (v/v/v)] for 120 min, 30–90% solvent B for 20 min, and 90% solvent B for 5 min, at a flow rate of 200 nl/min. The mass spectrometer was first operated in the data-dependent mode, to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 300–1700) were acquired in the Orbitrap with a resolution of 60,000 at m/z 400. The LTQ Orbitrap Velos ETD performed a full MS scan followed by 5 data-dependent electron-transfer dissociation–tandem mass spectrometry (ETD-MS/MS) scans with detection of the ETD fragment ions in the linear ion trap. Target values were $1e^6$ for full FTMS scans and $1e^4$ for ion trap MSn scans, with a maximum injection time of 200 ms. The anion target value was $3e^5$. ETD activation time was 100 ms. Supplemental activation was used for all ETD MSn scans. Dynamic exclusion was also enabled with an exclusion time of 180 s, with 1 repeat count and 180 s repeat duration. Additional targeted ETD-MS/MS experiments were performed, but only on precursors identified as *O*-GlcNAc-modified during the previous data-dependent experiments. ETD-MS/MS scans were acquired for the total duration of the peptide chromatographic peak. These MS/MS scans were then averaged, to obtain the final ETD-MS/MS spectrum.

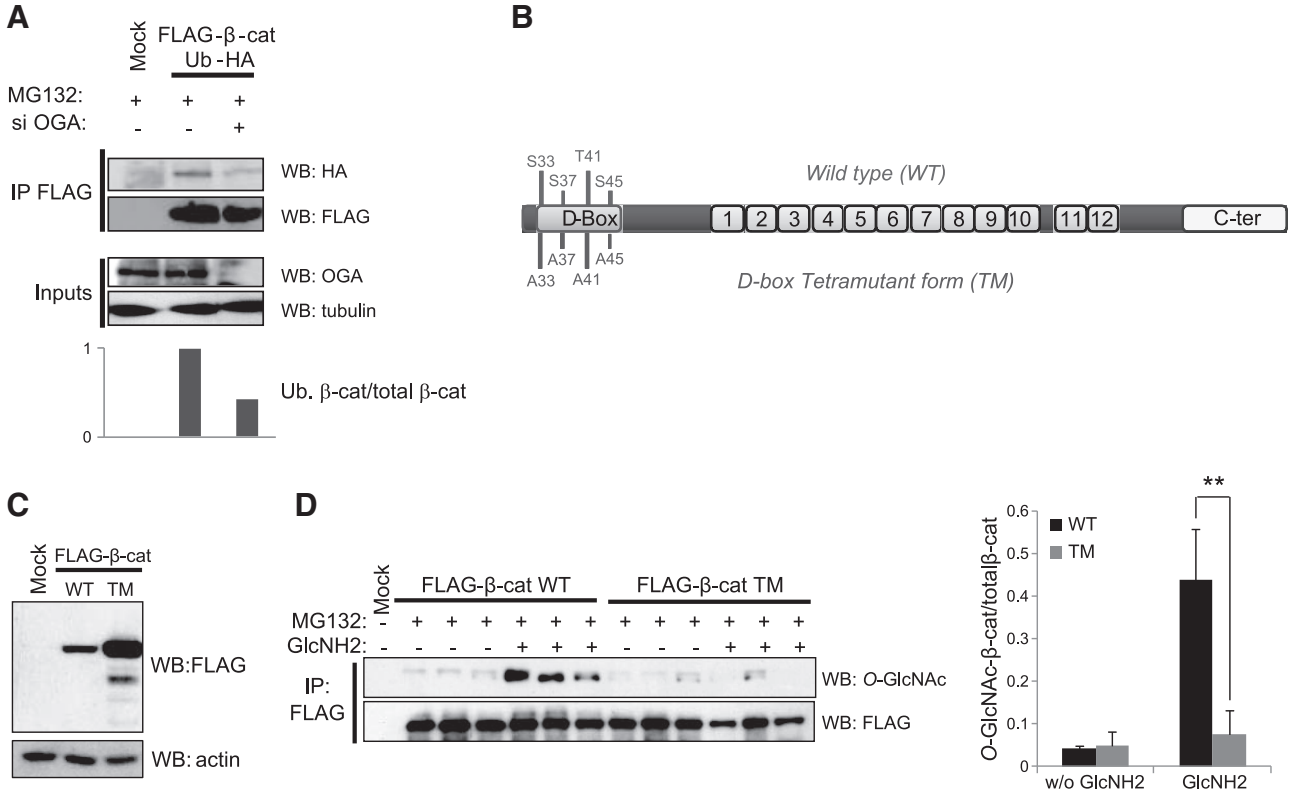

Figure 3. *O*-GlcNAcylation occurs in the D box of β-catenin and reduces its ubiquitinylation. *A*) MCF7 cells were cotransfected with vectors encoding FLAG-β-catenin and ubiquitin-HA and treated with siOGA and MG132. β-Catenin immunoprecipitates were analyzed by Western blot with anti-FLAG and anti-HA antibodies. *B*) Schematic representation of the β-catenin structure. The N-terminal region contains a D box, in which phosphorylation of 4 specific residues drives the ubiquitinylation and the subsequent proteasomal degradation of β-catenin. CK1α first phosphorylates S45, and then GSK3β successively modifies T41, S37, and S33. To investigate the *O*-GlcNAcylation of the D box, we used a mutant form of β-catenin in which these 4 phosphorylated residues were mutated into alanine (TM). The central region of β-catenin contains 12 imperfect sequence repeats of 42 aa (the armadillo repeats) that are essential in the interaction with many partners, including cadherins, APC, and TCF/LEF. The C-terminal region displays the transactivator function required for activation of target genes. *C*) MCF7 cells were transfected with FLAG-β-catenin (WT), TM-FLAG-β-catenin (TM), or an empty vector (Mock). At 48 h after transfection, the cells were lysed, and the lysates were analyzed by Western blot with anti-FLAG and -actin (loading control) antibodies. *D*) MCF7 cells were transfected with the vectors described above. At 36 h after transfection, the cells were treated with or without 5 mM glucosamine for 16 h and lysed, and β-catenin was immunoprecipitated with an anti-FLAG antibody. Immunoprecipitates were analyzed by Western blot with anti-*O*-GlcNAc and anti-FLAG antibodies. Average ± SD ratios of *O*-GlcNAcylated-β-catenin/total β-catenin are shown. $^{**}P < 0.01$.

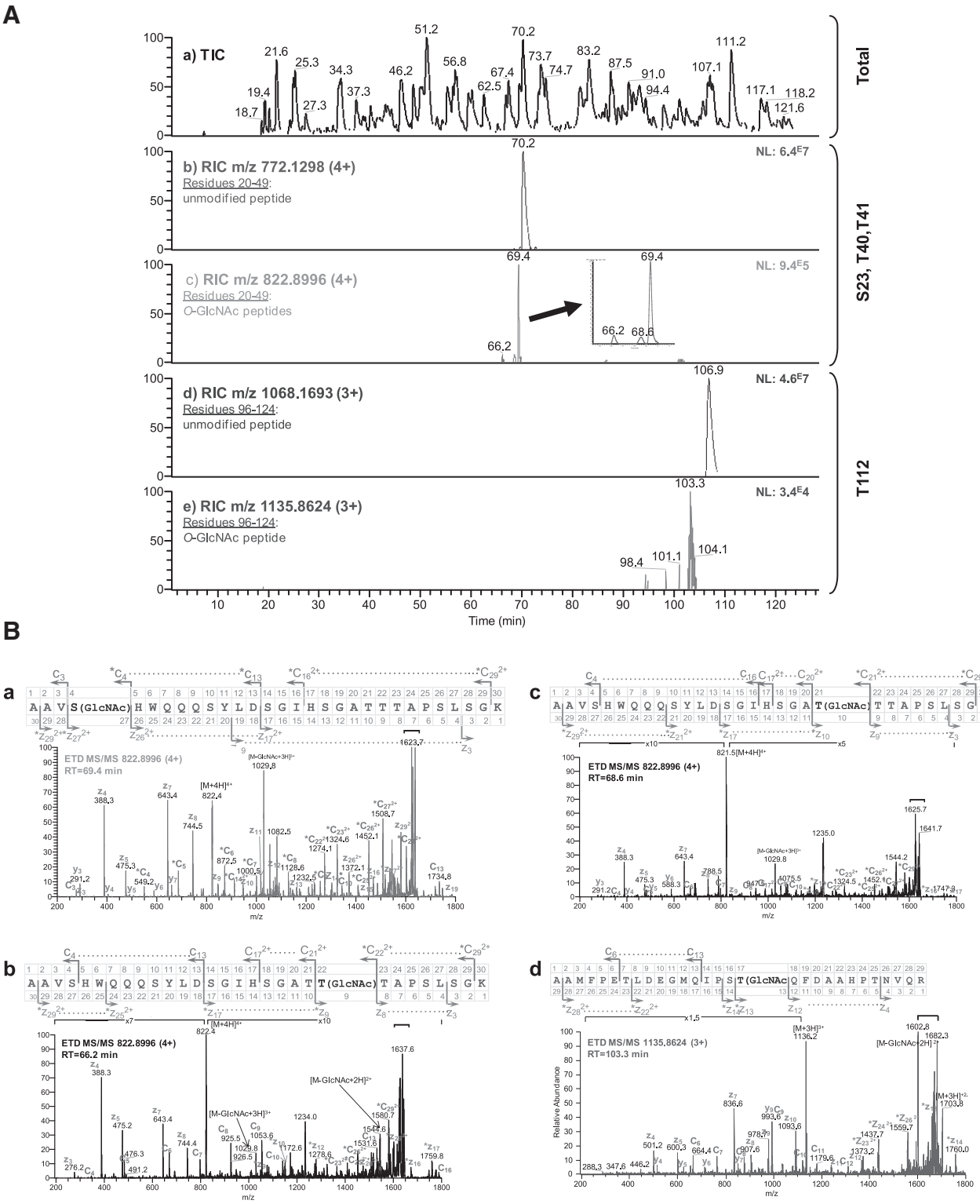

Figure 4. β-Catenin is *O*-GlcNAcylated at S23, T40, T41, and T112 in HT29 cells. *A*) Nano-LC-ETD MS/MS analysis of an in-gel β-catenin trypsin digest. *a*) Total ion current (TIC) chromatogram, *m/z* 300–1700. *b*) Reconstructed ion chromatogram (RIC) of the $[M+4H]^{4+}$ ion, *m/z* 772.1298, corresponding to the unmodified peptide, residues 20–49 of β-catenin. *c*) RIC of the $[M+4H]^{4+}$ ion, *m/z* 822.8996, corresponding to 3 differentially *O*-GlcNAc-modified peptides, residues 20–49 of β-catenin. *d*) RIC of the $[M+3H]^{3+}$ ion, *m/z* 1068.1693, corresponding to the unmodified peptide, residues 96–124 of β-catenin. *e*) RIC of the $[M+3H]^{3+}$ ion, *m/z* 1135.8624, corresponding to the *O*-GlcNAc modified peptide, residues 96–124 of β-catenin. NL, normalized level. *B*) Sequencing by ETD-MS/MS of precursor ions corresponding to the *O*-GlcNAc residues S23, T40, T41, and T112 in β-catenin. *a*) ETD-MS/MS spectrum of a tryptic *O*-GlcNAc-modified peptide precursor ion at *m/z* 822.8996 $[M+4H]^{4+}$ from β-catenin. MS/MS scans were acquired for the total duration of the chromatographic peak at a retention time of 69.4 min,

(*continued on next page*)

LC-MS/MS data processing

LC-MS/MS data, acquired with Xcalibur 2.1.0.1140 software (Thermo Fisher Scientific), were processed with Visual Basic software (Microsoft, Redmond, WA, USA) developed with XRawfile libraries (Thermo Fisher Scientific). The program generates an MS/MS peak list (MGF file) that is used for database searching. This MGF file contains the exact parent mass and the retention time associated with each LTQ-MS/MS spectrum.

Database searching

An internal MASCOT 2.1 server (Matrix Science, Boston, MA, USA; http://www.matrixscience.com/) using the Swiss-Prot database was used to identify peptides and their PTMs.

The PTM search parameters used were an increase of +57.02146 Da on cysteine residues (fixed carboxyamidomethylation), +15.99491 Da addition on methionine residues (dynamic oxidation), +42.010565 on protein N-terminal residues (dynamic N-terminal acetylation), +79.966331 on serine and threonine residues (dynamic phosphorylation), and +203.079373 on serine/threonine residues (dynamic *O*-GlcNAcylation). The precursor mass tolerance was set to 5 ppm, and the fragment ion tolerance was set to 0.5 Da. The number of missed cleavage sites for trypsin was set to 3. Identified *O*-GlcNAc-modified peptide sequences and the exact site of modification were confirmed by manual interpretation of the targeted ETD-MS/MS spectra.

RESULTS

β-Catenin, OGT, and *O*-GlcNAcylation levels are higher in colon tumor tissues than in tumor-adjacent normal tissues

Human colon tumor tissues and tumor-adjacent normal tissues from 4 patients (see Table 1 for information about the tumor samples) were isolated, and levels of β-catenin, *O*-GlcNAc, and OGT were analyzed by Western blot (**Fig. 1**). Levels of β-catenin, *O*-GlcNAc, and OGT are elevated in tumor tissues compared to levels in tumor-adjacent normal tissue, and no mutation in β-catenin sequence was detected in the patient samples (data not shown). In addition, our previous work suggested that metabolic disorders, characterized by an up-regulation of HBP, high *O*-GlcNAcylation, and changes in β-catenin expression, correlate with an increase in occurrence of CRC (19). With these data, we hypothesized that, using cellular and mouse models, we could better characterize the mechanism by which OGT, *O*-GlcNAcylation, and β-catenin levels correlate in CRC.

HCD or OGA inhibition enhance *O*-GlcNAcylation and β-catenin expression *in vivo*

To further characterize the relationship between *O*-GlcNAc and β-catenin in CRC, we profiled C57BL6 mice fed an HCD (**Fig. 2*A***). At 9 wk after the beginning of the HCD, the mice were denied access to food overnight, and an OGTT was performed. Compared to the mice fed an SD, those fed an HCD had a slight (nonsignificant) increase in blood glucose. Colons of the unfed mice were collected and homogenized, and lysates were analyzed by Western blot. In the mice fed an HCD, we observed higher levels of β-catenin expression and *O*-GlcNAc in comparison to those fed an SD. In addition, when Thiamet-G, a potent OGA inhibitor, was injected into the peritoneum daily for 2 wk, colonic β-catenin levels increased (Fig. 2*B*), whereas blood glucose levels were not affected. These observations reinforce the hypothesis that perturbation of *O*-GlcNAc levels through an HCD or enzyme inhibition correlates with an elevation of β-catenin expression.

T41 *O*-GlcNAcylation and phosphorylation influence degradation of β-catenin

In MCF7 cells, which are free of Wnt signaling mutations, *O*-GlcNAcylation interferes with β-catenin protea-

as shown in panel *Ac*. Thirty MS/MS spectra were averaged to obtain this spectrum. The peptide AAVS(GlcNAc)HWQQQSYLDSGIHSGATTTAPSLSGK (residues 20–49 of β-catenin) contained 1 site of *O*-GlcNAc modification at S23. The *c* ion series, c_4 to c_{13} (c_4 being modified), and the z^{2+} ion series, ${z_{17}}^{2+}$ to ${z_{29}}^{2+}$ (${z_{27}}^{2+}$ being modified), demonstrate that S23 is *O*-GlcNAcylated. Fragments carrying the *O*-GlcNAc moiety are indicated by an asterisk. *b*) ETD-MS/MS spectrum of a tryptic *O*-GlcNAc modified peptide precursor ion at m/z 822.900 $[M+4H]^{4+}$ from β-catenin. MS/MS scans were acquired for the total duration of the chromatographic peak at a retention time of 66.2 min, as shown in panel *Ac*. Thirty MS/MS spectra were averaged to obtain this spectrum. The peptide AAVSHWQQQSYLDSGIHSGATT(GlcNAc)TAPSLSGK (residues 20–49 of β-catenin) contained 1 site of *O*-GlcNAc modification at T41. The *z* ion series, z_3 to z_{17} (z_9 being modified); the *y* ion series, y_3 to y_{10} (y_9 being modified); and the ${c}^{2+}$ ion series, ${c_{17}}^{2+}$ to ${c_{29}}^{2+}$ (${c_{22}}^{2+}$ being modified) demonstrate that T41 is *O*-GlcNAcylated. Fragments carrying the *O*-GlcNAc moiety are indicated by an asterisk. *c*) ETD-MS/MS spectrum of a tryptic *O*-GlcNAc modified peptide precursor ion at m/z 822.900 $[M+4H]^{4+}$ from β-catenin. MS/MS scans were acquired for the total duration of the chromatographic peak at a retention time of 68.6 min, as shown in panel *Ac*. Thirty MS/MS spectra were averaged to obtain this spectrum. The peptide AAVSHWQQQSYLDSGIHSGAT(GlcNAc)TTAPSLSGK (residues 20–49 of β-catenin) contained 1 site of *O*-GlcNAc modification at T40. The *z* ion series, z_4 to z_{17} (z_{10} being modified), and the ${c}^{2+}$ ion series, ${c_{17}}^{2+}$ to ${c_{29}}^{2+}$ (${c_{21}}^{2+}$ being modified), demonstrates that T40 is *O*-GlcNAcylated. Fragments carrying the *O*-GlcNAc moiety are indicated by an asterisk. *d*) ETD MS/MS spectrum of a tryptic *O*-GlcNAc modified peptide precursor ion at m/z 1135.8624 $[M+3H]^{3+}$ from β-catenin. MS/MS scans were acquired for the total duration of the chromatographic peak at a retention time of 103.3 min, as shown in panel *Ae*. Thirty MS/MS spectra were averaged to obtain this spectrum. The peptide AAMFPETLDEGMQIPST(GlcNAc)QFDAAHPTNVQR (residues 96–124 of β-catenin) contained 1 site of *O*-GlcNAc modification at T112. The *z* ion series, z_4 to z_{14} (z_{13} being modified), demonstrates that T112 is *O*-GlcNAcylated. Fragments carrying the *O*-GlcNAc moiety are indicated by an asterisk. Horizontal brackets: charge reduced ions and fragment.

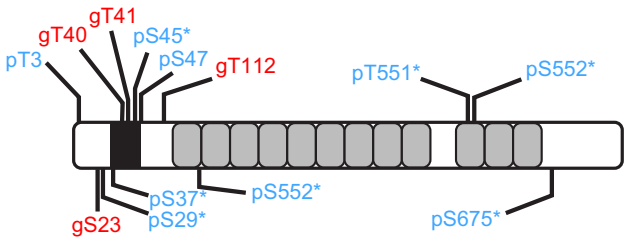

Figure 5. *O*-GlcNAcylation (red) and phosphorylation (blue) sites mapped in the study. Asterisks indicate that the corresponding phosphorylation sites are described in the literature. Black box: D box; gray boxes: armadillo repeats.

somal degradation (19). To better understand the mechanism responsible for β-catenin stabilization by *O*-GlcNAcylation, we transfected MCF7 cells with a FLAG-tagged β-catenin, an HA-tagged ubiquitin, and siRNA was used to down-regulate OGA expression (**Fig. 3*A***). After the cells were treated with the proteasome inhibitor MG132 to stabilize the ubiquitinated forms of β-catenin, the β-catenin was immunoprecipitated. Western blots were probed for ubiquitin *via* the HA tag. Results showed that siOGA (or NButGT, data not shown) reduced the level of ubiquitinated β-catenin.

Ubiquitination and subsequent targeting of β-catenin to the proteasome is controlled by phosphorylation of key residues (S33/S37/T41/S45) located within its D box (Fig. 3*B*). The priming site of phosphorylation resides at S45 and is driven by CK1α. This phosphorylation is followed by the phosphorylation of T41 and finally S37 and S33 by GSK3β. Then, the E3 ubiquitin ligase β-TrCp binds to phospho-S33 and phospho-S37 (5), leading to the ubiquitination of the K19 and K49 moieties (22), after which β-catenin is targeted for degradation (23). To determine whether *O*-GlcNAcylation competes with phosphorylation to modify the β-catenin D box, we examined the level of O-GlcNAcylation on a TM form of β-catenin in which S33, S37, T41, and S45 were replaced by alanine (Fig. 3*B*). As previously demonstrated, this mutant is more stable than the WT β-catenin (ref. 4 and Fig. 3*C*). After transfection with WT or TM FLAG β-catenin, MCF7 cells were treated with MG132, to freeze β-catenin degradation, and glucosamine, to increase UDP-GlcNAc production while bypassing the rate-limiting enzyme of the HBP, GFAT. Equal amounts of β-catenin were immunopurified, and *O*-GlcNAc levels were analyzed by Western blot (Fig. 3*D*). Addition of glucosamine enhanced the *O*-GlcNAcylation of WT β-catenin but not the glycosylation of TM, suggesting that β-catenin is *O*-GlcNAcylated at one or several of the 4 phosphorylation sites that drive the ubiquitination of β-catenin.

To determine the site at which β-catenin is *O*-GlcNAcylated in a biologically relevant cancer cell line, HT29, we used ETD-MS/MS. Along with nano-LC/MS/MS, which profiles different peptide retention times, ETD-MS/MS allowed the production of specific *c*- and *z*-type fragment ions without the loss of labile PTM (**Fig. 4*A***). ETD-MS/MS analyses of peaks corresponding to modified peptides revealed 4 *O*-GlcNAcylation sites located at S23, T40, T41, and T112 (Fig. 4*B* and Supplemental Figs. S1–S4). In addition, 2 novel and 7 previously described phosphorylation sites were mapped. The *O*-GlcNAcylation and phosphorylation sites identified in this study are depicted in **Fig. 5** and listed in **Table 2**.

We next investigated whether the *O*-GlcNAcylation of β-catenin influences its phosphorylation status (**Fig. 6**). First, we tested increasing glucose amounts and a condition in which glucosamine was used on 3 colon cell lines: CCD841CoN, a normal fetal cell line, and HT29 and HCT116, 2 colon cancer cell lines (Fig. 6*A*). Equal amounts of endogenous β-catenin were immunoprecipitated and analyzed according to their *O*-GlcNAc content and their phosphorylation status at T41/S45. We observed that these conditions increased the *O*-GlcNAcylation of β-catenin in the HT29 and

TABLE 2. *List of the* O*-GlcNAcylation and phosphorylation sites mapped in the study*

Residues	Sequence	Modified residue	Mascot ion score
2-19	AT(Pho)QADLMELDMAMEPDRK	3	46
20-49	AAVS(GlcNAc)HWQQQSYLDSGIHSGATTTAPSLSGK	23	36
	AAVSHWQQQSYLDSGIHSGAT(GlcNAc)TTAPSLSGK	40	M
	AAVSHWQQQSYLDSGIHSGATT(GlcNAc)TAPSLSGK	41	M
	AAVSHWQQQS(Pho)YLDSGIHSGATTTAPSLSGK	29	M
	AAVSHWQQQYLDSGIHS(Pho)GATTTAPSLSGK	37	M
	AAVSHWQQQSYLDSGIHSGATTTAPS(Pho)LSGK	45	76
	AAVSHWQQQSYLDSGIHSGATTTAPSLS(Pho)GK	47	M
96-124	AAMFPETLDEGMQIPST(GlcNAc)QFDAAHPTNVQR	112	38
186-200	HAIMRS(Pho)PQMVSAIVR(T)	191	39
191-200	S(Pho)PQMVSAIVR(T)	191	48
550-565	RT(Pho)SMGGTQQQFVEGVR(M)	551	82
550-565	RTS(Pho)MGGTQQQFVEGVR(M)	552	107
551-565	T(Pho)SMGGTQQQFVEGVR(M)	551	98
551-565	TS(Pho)MGGTQQQFVEGVR(M)	552	93
672-684	KRLS(Pho)VELTSSLFR(T)	675	75
673-684	RLS(Pho)VELTSSLFR(T)	675	73

Phosphorylation (Pho) and *O*-GlcNAcylation (GlcNAc) sites are underscored.

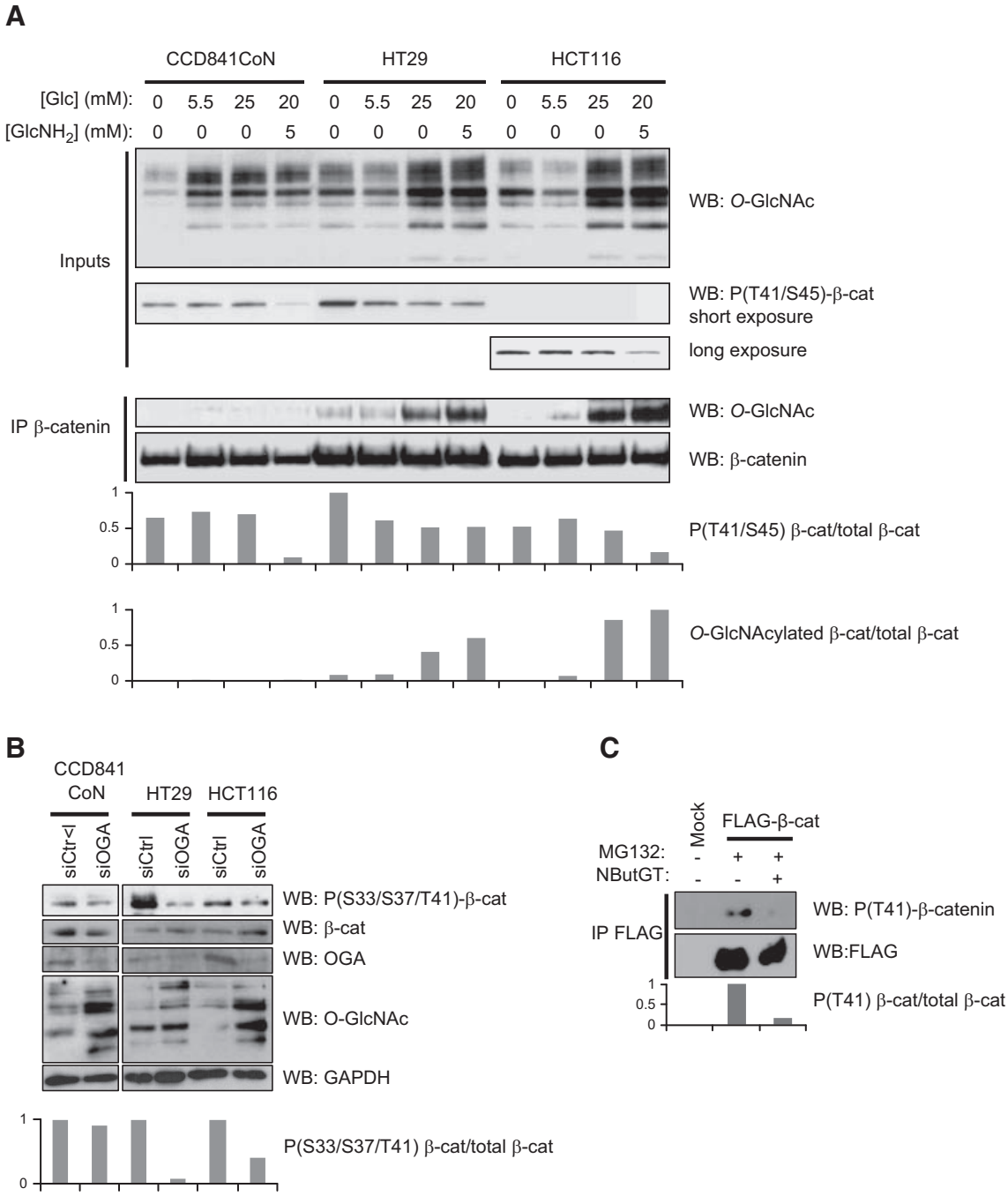

Figure 6. *O*-GlcNAcylation of β-catenin D box competes with its phosphorylation. *A*) CD841CoN, HT29, and HCT116 cells were cultured in the presence of increasing concentrations of glucose (Glc) or with glucosamine ($GlcNH_2$). Whole-cell lysates were probed for *O*-GlcNAc level and phosphorylation of β-catenin at T41 and S45. Equal amounts of immunopurified β-catenin were loaded and analyzed according to their *O*-GlcNAc levels by immunoblot. *B*) CD841CoN, HT29, and HCT116 cells were transfected with either siOGA or siCtrl and incubated with MG132. β-Catenin, phospho-β-catenin, *O*-GlcNAcylation, OGA, and GAPDH (loading control) levels were determined by immunoblot. *C*) HEK293 cells were transfected with WT FLAG-β-catenin and incubated with MG132, with or without NButGT. Cell lysates were immunopurified with an anti-FLAG antibody, and the immunoprecipitates were analyzed with a phospho-T41-dependent antibody.

HCT116 cells and that glucosamine (and glucose for HT29) treatment correlated with a decrease in phosphorylation at T41/S45. A longer exposure was necessary to detect the T41 phosphorylation of β-catenin in the HCT116 cells, as S45 is deleted in this cell line. Whereas the CCD841CoN cells showed a decreased in their T41/S45 phosphorylation status, the *O*-GlcNAcylation of β-catenin was below the detection limit, which is not surprising, as normal cells have lower levels of *O*-GlcNAc than cancer cells have (19, 24–26). Knockdown of OGA interfered with the phosphorylation of β-catenin in the HT29 and HCT116 cells, whereas no effect was observed in the CCD841CoN cells (Fig. 6*B*), consistent with the lack of detection of β-catenin *O*-

GlcNAcylation in this cell line in contrast with HT29 and HCT116 (Fig. 6*A*). Last, a decrease in pT41-β-catenin was observed in HEK293 cells transfected with WT FLAG-β-catenin and treated with NButGT (Fig. 6*C*). Together, our results support a reciprocal relationship between phosphorylation and O-GlcNAcylation at T41 of β-catenin.

O-GlcNAcylation at S23, T40, and T112 is not involved in β-catenin ubiquitinylation and stability

We generated single-point mutants for all *O*-GlcNAc-modified sites of β-catenin, as well as another TM (4M: S23A/T40A/T41A/T112A; **Fig. 7*A***). We investigated the protein's expression (Fig. 7*B*), subcellular localization (Fig. 7*C*), and ubiquitinylation (Fig. 7*D*) in HEK293T cells. We observed a higher expression of the T41A mutant (and consequently of the mutant 4M) compared with WT β-catenin (Fig. 7*B*). However, we found no difference in S23A and a slight increase in T40A and T112A. Subcellular fractionation showed that the T41A single-point mutant and 4M accumulate in the cytoplasm, suggesting stabilization of the protein (Fig. 7*C*, F1). Cotransfection of the different β-catenin mutants with HA-ubiquitin demonstrated that T41A and 4M were less ubiquitinated than WT β-catenin, whereas we observed a faint decrease in ubiquitinylation of the S23A, T40A, and T112A mutants in comparison with WT β-catenin (Fig. 7*D*). Overall, these results support that *O*-GlcNAcylation and phosphorylation at T41 rather than modification at S23, T40, or T112 regulates β-catenin ubiquitinylation and stability.

O-GlcNAcylation interferes in the α-catenin–β-catenin interaction

Among the 4 *O*-GlcNAcylation sites that we identified, only T41 (known also to be a phospho-site) appeared to be involved in β-catenin stabilization, suggesting that *O*-GlcNAcylation regulates other functions of β-catenin. T112 is located in the β-catenin–α-catenin interaction domain, and it has been proposed that this residue is phosphorylated by CK2 to increase the affinity between β- and α-catenins (27). We hypothesized that *O*-GlcNAcylation would affect the β-catenin interaction at the adherens junctions by changing this interaction. To answer this question, we inhibited *O*-GlcNAcylation by treatment of MCF7 cells with Ac-5SGlcNAc, an inhibitor of OGT (28), and tested the interaction of β-catenin with α-catenin and E-cadherin by coimmunoprecipitation (**Fig. 8*A***). We observed that Ac-5SGlcNAc had no effect on the interaction between β-catenin and E-cadherin. On the other hand, decreased *O*-GlcNAcylation clearly diminished the interaction between the α- and β-catenins. Such a decrease was also observed when OGT was silenced with siRNA (Fig. 8*B*), whereas knockdown of the expression of OGA enhanced the interaction between the 2 catenins (Fig. 8*C*). However, coimmunoprecipitation experiments between the different mutants of *O*-GlcNAcylation of β-catenin (described in Fig. 7*A*) and α-catenin revealed no significant difference in comparison with the WT protein (**Fig. 9**).

DISCUSSION

Cancer is the second leading cause of death in Western societies, next to cardiovascular disease. Modern lifestyles, characterized by junk-food diets, low energy expenditure, and metabolic disorders, increase the risk of cancer (29). Intriguingly, several groups reported that *O*-GlcNAcylation and OGT are more elevated in cancer-derived cells (breast, ref. 24; pancreas, ref. 25; prostate, ref. 26; and colon, ref. 19; see also refs. 30, 31 for reviews), when compared to normal cells.

In this study, *O*-GlcNAcylation and OGT were highly expressed in tumors from patients with CRC, each displaying at least 1 problem related to metabolic disorder: diabetes, hypertension, overweight, or dyslipidemia (Table 1). *O*-GlcNAc levels are closely tied to those of the donor UDP-GlcNAc, which is implicated as a key player in many metabolic pathways (31, 32). We proposed that there is a key relationship between metabolic disorders and cancers that is governed by nutrient flux.

Cancer cells change their metabolism and increase glucose utilization through the Warburg effect (33). Indeed, an initial observation noted that the wing of a hen with Rous sarcoma produced more lactic acid than the normal wing (34, 35). It was therefore originally suggested that a deficiency in glucose metabolism occurs at the origin of cancer (36). More recently, the metabolism shift found in tumor cells was found to be a consequence rather than a cause of cancer, and oncogenic signaling pathways controlling HIF1 are responsible for this change (37). Interestingly, the use of both glucose and glutamine are up-regulated in cancer cells (38), and both nutrients are used to generate UDP-GlcNAc, the donor for the *O*-GlcNAcylation processes (31, 32). In cancer cells, increased glucose affects glycolysis, the HBP, and the pentose phosphate pathway (PPP). Indeed, regarding the PPP, it has been recently demonstrated that *O*-GlcNAcylation of the glycolysis rate-limiting enzyme PFK1 at S529 interferes with the binding of the metabolic sensor fru-2,6-bis-phosphate (39). Glucose is then diverted to generate 6-phospho-gluconate, a precursor of pentoses needed for nucleic acid synthesis during active cell division. Thus, cancer cells consume their glucose differently; nonoxidative consumption of glucose is sufficient to generate enough ATP to proliferate, and above all, it allows the production of nucleic acids, amino acids, glycans, and lipids, to increase the biomass. We suggest that HBP activation contributes to hexosamine synthesis for cellular structure construction as well as regulation of metabolic flux, signaling pathways, and cell homeostasis processes. β-Catenin, the central component of Wnt signaling and an *O*-GlcNAcylated protein, perfectly fits this hypothesis.

Three of the 4 *O*-GlcNAc sites identified on β-catenin are located within the protein flexible domain (S23/

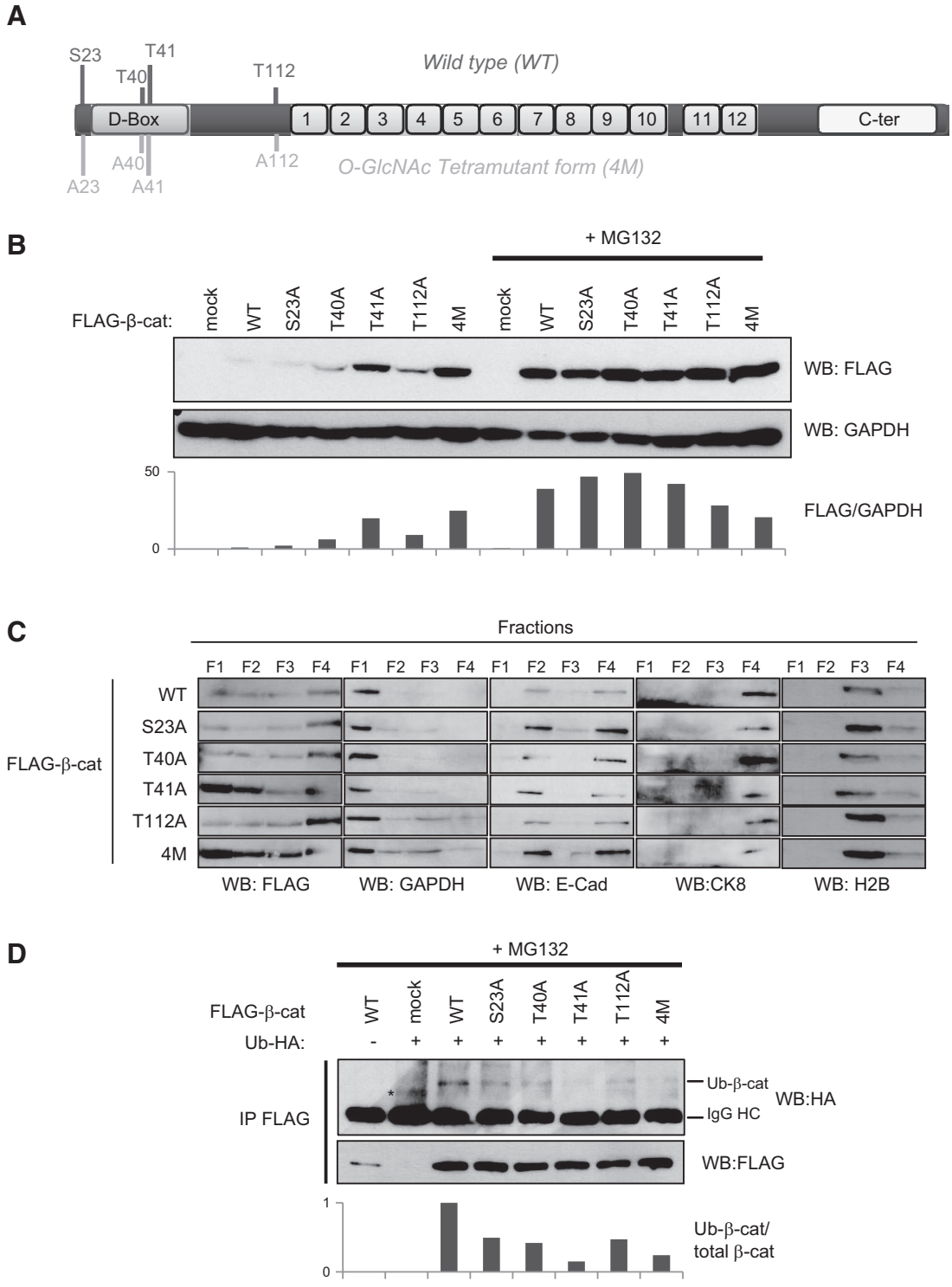

Figure 7. *O*-GlcNAcylation at T41, but not at S23, T40, or T112, regulates β-catenin ubiquitinylation and stability. *A*) Schematic representation of β-catenin showing the *O*-GlcNAcylation sites mapped in the study. Single-point mutants and a tetramutant of β-catenin (4M: S23A/T40A/T41A/T112A) were generated. *B*) HEK293T cells were transfected either with a WT or mutant FLAG-β-catenin or with an empty vector (mock). At 36 h after transfection, the cells were treated with or without 1 μM MG132 for 16 h. Cell lysates were analyzed by Western blot for β-catenin using the anti-FLAG tag and anti-GAPDH antiboides (loading control). *C*) Cells were transfected as described in panel *B*. At 48 h after transfection, a subcellular fractionation was performed, and distribution of FLAG-β-catenin in each fraction (F1, cytosol; F2, membranes; F3, nucleus; and F4, cytoskeleton) was investigated by Western blot analysis. Antibodies directed against GAPDH (F1), E-cadherin (F2 and F4), H2B (F3), and CK8 (F4) were used to ensure the purity of each fraction. *D*) Cells were cotransfected with the different β-catenin constructs and with a ubiquitin-HA-expressing vector. At 36 h after transfection, the cells were treated with 1 μM MG132 for 16 h and lysed, and immunoprecipitation was performed with an anti-FLAG antibody. β-Catenin immunoprecipitates were analyzed by Western blot for β-catenin (anti-FLAG) and ubiquitin (anti-HA). Asterisk indicates a nonspecific band. IgG HC, immunoglobulin G heavy chain; Ub-β-cat, ubiquitinated β-catenin.

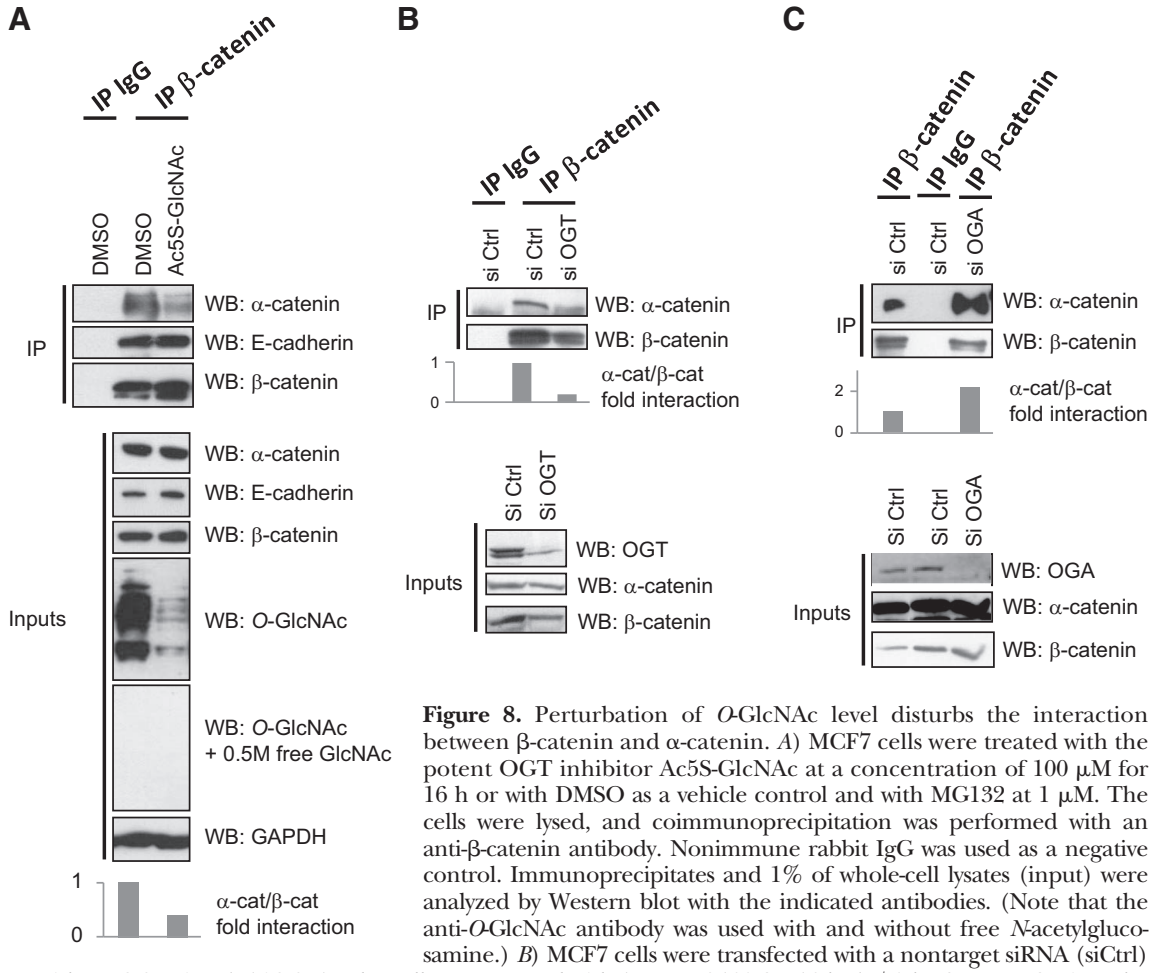

Figure 8. Perturbation of *O*-GlcNAc level disturbs the interaction between β-catenin and α-catenin. *A*) MCF7 cells were treated with the potent OGT inhibitor Ac5S-GlcNAc at a concentration of 100 μM for 16 h or with DMSO as a vehicle control and with MG132 at 1 μM. The cells were lysed, and coimmunoprecipitation was performed with an anti-β-catenin antibody. Nonimmune rabbit IgG was used as a negative control. Immunoprecipitates and 1% of whole-cell lysates (input) were analyzed by Western blot with the indicated antibodies. (Note that the anti-*O*-GlcNAc antibody was used with and without free *N*-acetylglucosamine.) *B*) MCF7 cells were transfected with a nontarget siRNA (siCtrl) or with an OGT siRNA (siOGT). The cells were treated with 1 μM MG132 for 16 h. At 72 h after transfection, the cells were lysed, and coimmunoprecipitation and Western blot analysis were performed as in panel *A, C*) The same procedure as in panel *B* was performed, except that an OGA siRNA (siOGA) was used.

T40/T41; Fig. 7*A*). This domain concentrates the phosphorylation and ubiquitination sites that regulate the fate of β-catenin. First, CK1α modifies S45 and subsequently T41, S37, and S33 are modified by GSK3β. APC then prevents the dephosphorylation of the D box (40). The E3 ubiquitin ligase β-TrCp binds to phospho-S33 and phospho-S37 (5) and ubiquitinates of K29 and K49 (22), thus targeting β-catenin to the proteasomal pathway (23). S23 is mutated in hepatocellular cancers (41) and phosphorylated by GSK3β (42), but its function is

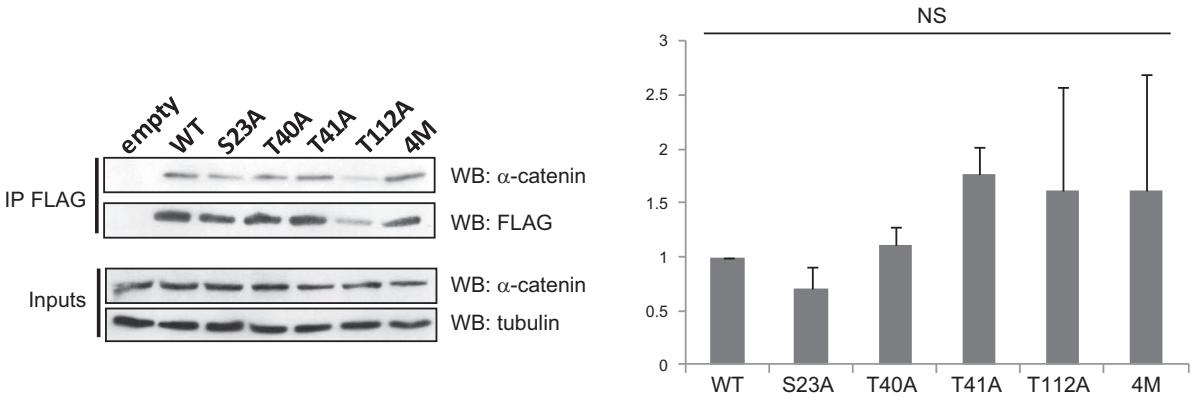

Figure 9. *O*-GlcNAcylation of β-catenin at S23, T40, T41, and T112 does not affect its interaction with α-catenin. HEK293T cells were transfected with WT or mutant FLAG-β-catenin or an empty vector (Mock). At 36 h after transfection, the cells were treated with 1 μM MG132 for 16 h. The cells were lysed, and β-catenin was immunoprecipitated with the FLAG Tag. Immunoprecipitated β-catenin and 1% of the whole-cell lysate (inputs) were analyzed by Western blot with the indicated antibodies. Densitometric values of 3 distinct experiments are represented. NS, not significant.

not well understood, although it has been suggested that it takes part in embryogenesis and carcinogenesis. We hypothesized that the proximity of S23 with K19 suggests that the S23 *O*-GlcNAcylation interferes with the K19 ubiquitination, but we found only a slight decrease in the ubiquitination of S23A-β-catenin when compared to WT (Fig. 7*D*). Last, it has been demonstrated that the β-catenin protein homologue plakoglobin is *O*-GlcNAcylated at T14, equivalent to β-catenin S23 (43). The *O*-GlcNAcylation of plakoglobin at T14 prevents its proteasomal degradation emphasizing the importance of β-catenin S23 *O*-GlcNAcylation (Fig. 7).

The fourth *O*-GlcNAcylation site was identified as T112 in the interaction domain with α-catenin. It has been proposed that phosphorylated T112 yields an increase in affinity between β- and α-catenins (27). Although perturbing the *O*-GlcNAcylation processes interfered with the α-/β-catenin interaction (Fig. 8), none of the β-catenin mutants exhibited a drastic change in the α-catenin interaction shown by immunoprecipitation (Fig. 9).

When mutated, the GSK3β substrate T41, but not T40, causes the most changes in β-catenin ubiquitination, expression, and cytosolic accumulation (Fig. 7). Phospho-modified T41 is a checkpoint allowing the subsequent phosphorylation of S33 and S37. The proteasome machinery recognizes phospho-modified S33 and S37, as required for β-catenin degradation. Logically, S33, S37, T41, and S45 are frequently mutated in cancers, leading to an uncontrolled stabilization of β-catenin and an increase in its oncogenic features. By competing with phosphorylation on T41, *O*-GlcNAcylation stabilizes β-catenin in the same manner and may increase its oncogenic properties as well. Interestingly, metabolic disorders, such as type 2 diabetes, correlate with elevated levels of CRC (14–16). We propose that increased levels of glucose (by nutrient excess, unhealthy diet, or metabolic disorders) cause or exacerbate cancer emergence, especially CRC. Indeed, elevation of CRC incidence may occur due to *O*-GlcNAcylation of WT nonmutated β-catenin. Our study provides a mechanistic explanation of how nutrition and cancer are linked and support that T41 is a critical residue for β-catenin regulation. FJ

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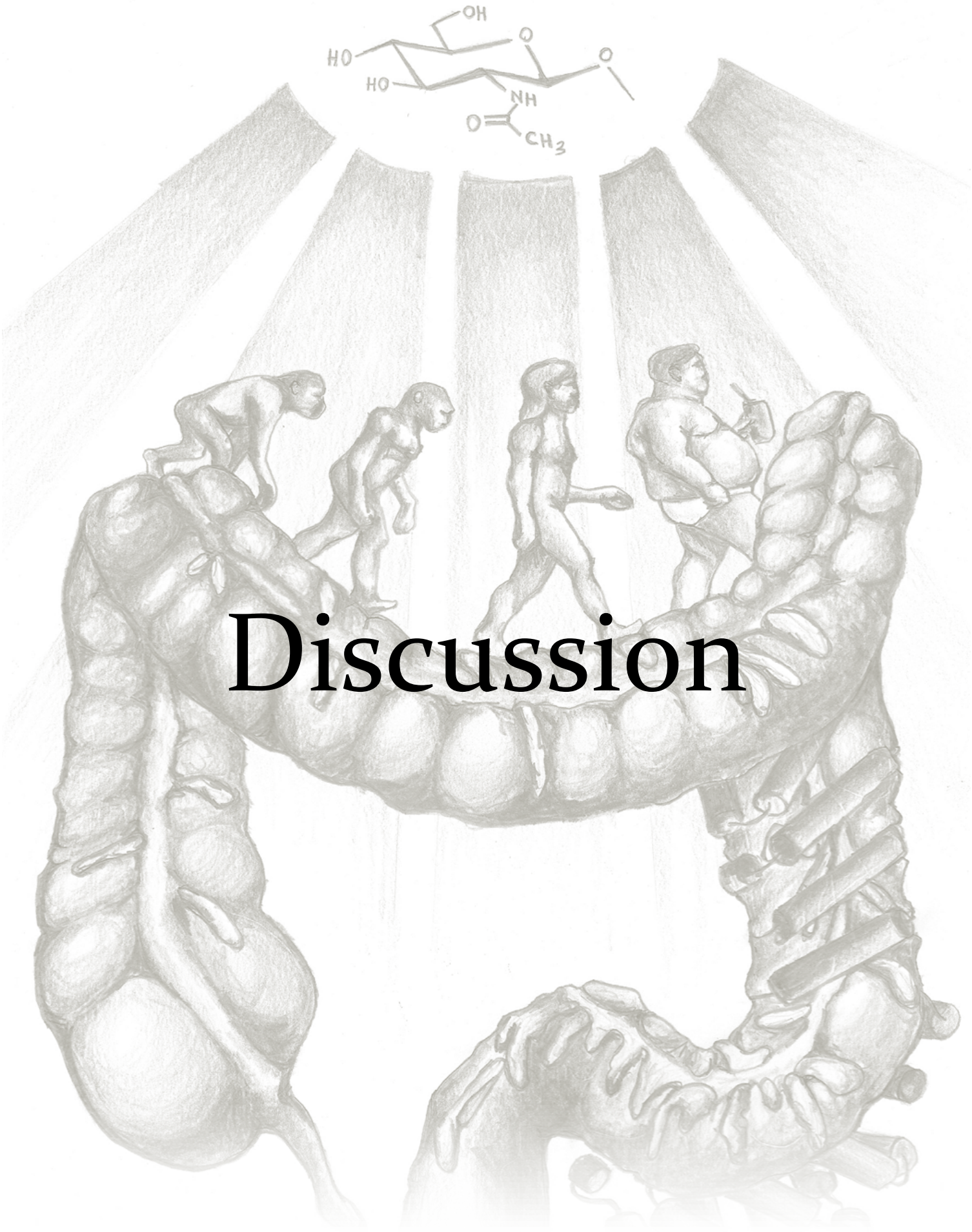


Discussion

La voie Wnt/β-caténine est une voie de signalisation impliquée dans des processus physiologiques, tels que l'embryogénèse ou le renouvellement tissulaire, mais également dans des processus pathologiques parmi lesquels la cancérogénèse. Bien que de nombreux travaux aient été publiés à ce sujet (6809 entrées sur PubMed au 15 octobre 2012 dont 921 pour l'année en cours ; mots clés: Wnt β-catenin), certains mécanismes de régulation de la voie restent à découvrir. En effet, les questions les plus fréquemment posées concernant la cancérogénèse colique et rectale sont : quels sont les facteurs de risque influençant la cancérisation et quels mécanismes moléculaires cela implique-t-il ?

Les désordres nutritionnels et métaboliques influencent l'émergence de plusieurs catégories de cancers dont le cancer colorectal. Le risque de développer ce type de cancer est par exemple doublé chez un sujet diabétique en comparaison à un individu sain (Ahmed et al., 2006 ; Khaw et al., 2004 ; Pais et al., 2009). De plus, la majorité des patients atteints d'un cancer colorectal montrent une intolérance au glucose ou une résistance à l'insuline (Yam et al., 1996 ; Pais et al., 2009). Pourtant, bien que quelques hypothèses impliquant la résistance à l'insuline ont été avancées (Publication n°1 : Olivier et al., 2011), aucun mécanisme précis ne permet à l'heure actuelle de lier les désordres métaboliques et nutritionnels au cancer colorectal. Une meilleure compréhension des mécanismes situés en amont ou complétant les aspects génétiques du cancer constituerait une avancée notable dans la recherche sur le cancer.

La découverte de la *O*-GlcNAcylation par Torres et Hart en 1984 a mis au jour un nouveau mode de de régulation des voies de signalisation (Torres et Hart, 1984). Cette modification post-traductionnelle était pour l'époque originale puisque concurrençant la phosphorylation des protéines intracellulaires. Cette modification permet de relier le statut nutritionnel de l'organisme à l'activité des protéines cytosoliques et nucléaires. En effet, la synthèse, du nucléotide sucre donneur par la voie de biosynthèse des hexosamines est dépendante de nombreux métabolismes (acides aminés, nucléotides et acides gras) mais, c'est surtout la concentration en glucose qui détermine l'abondance de l'UDP-GlcNAc. Cette caractéristique fait de la *O*-GlcNAcylation un senseur nutritionnel de l'organisme et, plus particulièrement elle reflète au niveau cellulaire, les variations de glycémie. C'est cette propriété et la découverte de la *O*-GlcNAcylation de la β-caténine qui a inspiré cette étude (Zhu et al., 2001 ; Lefebvre et al., 2004a).

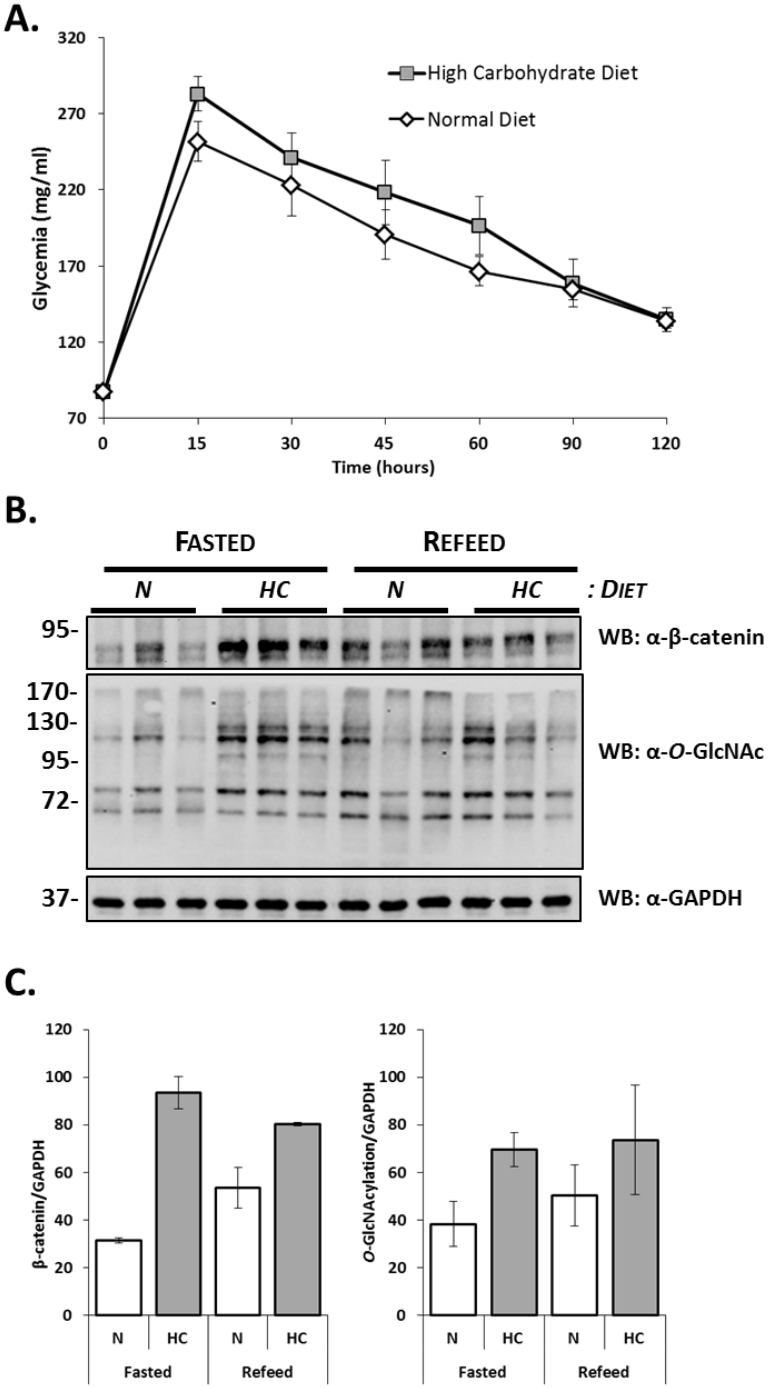


Figure 32 : Un régime long riche en glucides entraine l'intolérance au glucose ainsi que la stabilisation permanente de la β-caténine dans le colon des souris C57Bl6. Les souris ont été nourries pendant 3 mois avec un régime normal **(N)** (65% glucide, 11% lipide, and 24% protéine) ou riche en glucides **(HC)** (75% glucide, 22% lipide, and 3% protéine). Elles ont ensuite été mises à jeun pendant 24h **(Fasted)** et renourries sur le régime approprié pendant 18h **(Refeed)**. (A) Un test de tolérance au glucose a été effectué et a mis en évidence l'apparition du désordre métabolique. (B)(C) L'observation d'extrait cellulaire de colon a mis en évidence l'augmentation à jeun des niveaux de β-caténine corrélée avec l'augmentation des protéines totales *O*-GlcNAcylées.

La question que nous nous sommes posée au tout début de cette étude est la suivante: **"La β-caténine *O*-GlcNAcylée est-elle un des éléments qui lient les désordres nutritionnels et métaboliques à la cancérisation colique et rectale ?"**

Afin d'étudier ce problème, nous nous sommes dans un premier temps demandés si la demi-vie de la β-caténine était modifiée par les conditions métaboliques et nutritionnelles. En effet, une demi-vie aberrante de la β-caténine est un des effecteurs principaux dans l'initiation du cancer colique et rectal. Cette caractéristique émerge en réponse à des mutations du complexe de destruction (85 %) ou de la β-caténine elle-même (10 %), réduisant sa dégradation protéasomale. Nous avons aussi observé que le régime alimentaire à court terme chez la souris influence l'expression de la β-caténine de l'épithélium colique (Publication n°2 : Olivier-Van Stichelen et al., 2012). De même, l'intolérance au glucose induit par un régime prolongé riche en glucides, augmente l'expression de la β-caténine (Données non publiées) (Figure 32). Nous avons démontré que ce phénomène résultait plutôt d'une stabilisation post-traductionnelle de la β-caténine que d'une néosynthèse de la protéine. Par ailleurs, des études *in vitro* ont permis de montrer que cette stabilisation était due, d'une part à l'activité de la voie de biosynthèse des hexosamines et d'autre part à la *O*-GlcNAcylation en elle-même (Publication n°2 : Olivier-Van Stichelen et al., 2012). Des variations des niveaux de glucose extracellulaire modulent par ailleurs la stabilité de la β-caténine, renforçant un peu plus le rôle de senseur nutritionnel de la *O*-GlcNAcylation. Cette observation est en accord avec l'étude d'Anagnostou et Shepherd montrant l'influence de la voie de biosynthèse des hexosamines sur l'expression de la β-caténine (Anagnostou et Shepherd, 2008). Pour aller plus loin et pour comprendre comment la *O*-GlcNAcylation influence la demi-vie de la β-caténine, nous avons entrepris la cartographie des sites de *O*-GlcNAcylation de cette dernière en collaboration avec Sanofi-Aventis (Toulouse) (Publication n°4 : Olivier-Van Stichelen et al., en préparation). La localisation des sites nous a particulièrement aidés dans la compréhension du mécanisme moléculaire précis de cette régulation. Nous nous sommes concentrés sur les sites de *O*-GlcNAcylation localisés dans la partie N-terminale de la β-caténine, domaine flexible nécessaire à sa dégradation protéasomale. Cette extrémité possède la destruction box, dont la phosphorylation détermine le devenir de la β-caténine, le site d'interaction avec β-TrCp (une E3-ubiquitine ligase) et les sites d'ubiquitinylation (cf partie II.E).

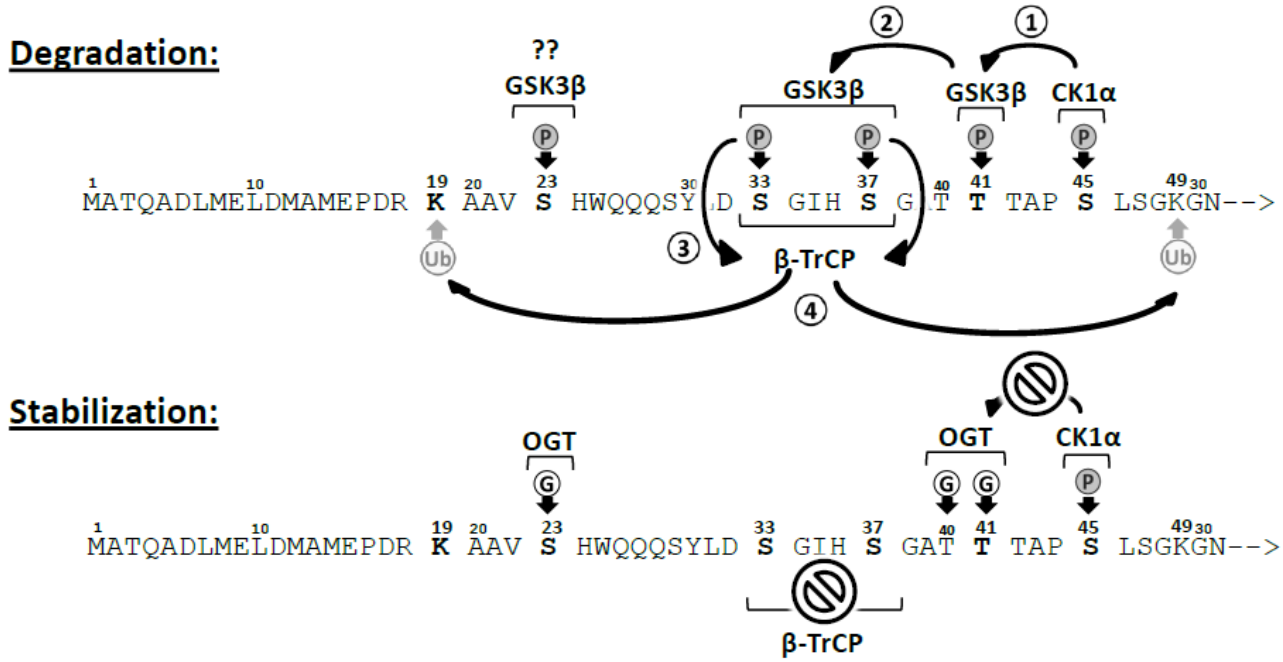
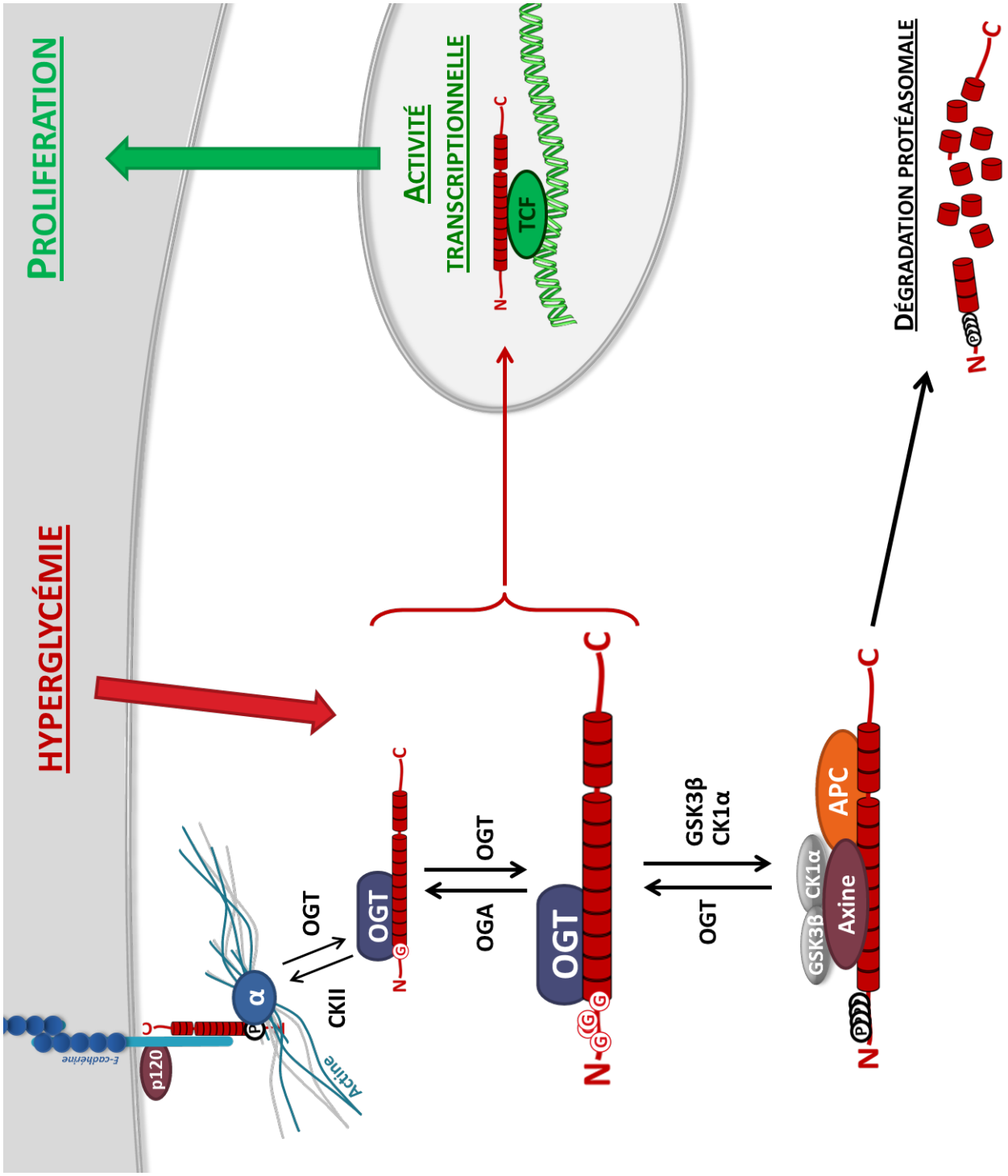


Figure 33 : *O*-GlcNAcylation *N*-terminale de la β-caténine et proposition de mécanisme de stabilisation. Après recrutement dans son complexe de destruction, la β-caténine est phosphorylée par CK1α sur la sérine 45 (1), puis la GSK3β sur la thréonine 41 puis sur les sérines 33 et 37 (2). L'E3 ubiquitine ligase β-TrCp reconnait les résidus 33 et 37 phosphorylés (3) et entraine l'ubiquitinylation de la β-caténine sur les lysines 19 et 49 (4). La β-caténine est ensuite envoyée au protéasome pour dégradation.

L'interaction avec le suppresseur de tumeur APC est indispensable pour empêcher la déphosphorylation de la D-Box par PP2A (Su et al., 2008) (Figure 5). Les sites de *O*-GlcNAcylation en positions 23, 40 et 41 pourrait interférer directement avec les processus de phosphorylation/ubiquitinylation/dégradation, due à la proximité avec les sites de modifications post-traductionnelles ou les domaines d'interaction avec des protéines clés (APC, β-TrCp) (Publication n°4 : Olivier-Van Stichelen et al., en préparation) (Figure 33). Par exemple, la thréonine 41 montre une compétition directe entre phosphorylation et *O*-GlcNAcylation. Ainsi la phosphorylation promeut la dégradation de la β-caténine, tandis que la *O*-GlcNAcylation, en empêchant la phosphorylation, la préviendrait. La thréonine 40 *O*-GlcNAcylée pourrait également interférer avec les processus de phosphorylation en bloquant, par exemple, la fixation des kinases. Ceci reste néanmoins à démontrer avec l'utilisation d'un mutant ponctuel sur ce résidu. La sérine 23, quant à elle, est située à proximité du site d'ubiquitinylation K19 et pourrait interférer avec l'ajout de cette autre modification post-traductionnelle. De même l'utilisation d'un mutant ponctuel de cette sérine permettrait de vérifier cette hypothèse. Cette sérine est également modifiée par GSK3β mais le rôle de cette phosphorylation reste à définir (Van Noort et al., 2002). Néanmoins, les mutations de ce résidu décrits dans certains cancers hépatocellulaires démontrent son caractère fondamental (Legoix et al., 1999) (Figure 33).

La stabilisation aberrante de la β-caténine est suffisante pour induire une activité transcriptionnelle activatrice de la prolifération, comme c'est le cas au cours de la phase d'initiation du cancer colorectal (Morin et al., 1997). En effet, en condition physiologique, les cellules progénitrices à la base des cryptes fournissent les cellules de l'épithélium intestinal qui migrent vers le sommet de ces cryptes en se différenciant. Pour cela, il est nécessaire d'inactiver l'activité transcriptionnelle de la β-caténine dès le début de la migration cellulaire. Une stabilisation anormale de la β-caténine au niveau de ces cellules empêche leur différenciation et leur confère une stabilité anormale jusqu'à la former une masse tumorale qui envahit les tissus adjacents. Dans le cas de la stabilisation de la β-caténine par *O*-GlcNAcylation, nous avons démontré qu'elle stimule plus fortement la transcription de ses gènes cibles et accélère la prolifération cellulaire (Figure 34) (Publication n°2 : Olivier-Van Stichelen et al., 2012).



igure 34 : Fonctions de la *O*-GlcNAcylation de la β-caténine. L'extrémité N-terminale de la β-caténine en réponse à l'augmentation de la concentration en glucose, est modifiée par un résidu de GlcNAc. La compétition de ces résidus avec les mécanismes de phosphorylation/ubiquitinylation entraine la stabilisation de la protéine. Elle acquerrait ainsi son activité oncogénique, résultant en une augmentation de la prolifération cellulaire.

Lors de cette phase proliférative, l'OGT semble jouer un rôle particulier. En effet, suite à la reprise du cycle cellulaire en G1, une augmentation de l'expression d'OGT ainsi que son interaction avec la β-caténine est observée. Au cours de cette phase, une augmentation de la quantité de β-caténine est observée dans le but, entre autres, de stimuler la synthèse de cycline D1 et la progression dans la phase G1 du cycle cellulaire. L'interaction avec l'OGT suggère que la β-caténine serait modifiée en réponse à la stimulation du cycle cellulaire (Publication n°3 : Olivier-Van Stichelen et al., 2012, en révision). Cette hypothèse est soutenue par l'observation d'une forme *O*-GlcNAcylée de la β-caténine dans les cellules en phase exponentielle de croissance. Nous avons également observée cette même augmentation dans les cellules cancéreuses en comparaison aux cellules saines (HT29, HCT116 vs CCD841CoN), comme précédemment démontrée pour les cellules mammaires (Caldwell et al., 2010). Dans ce sens, des analyses d'échantillons issus de biopsies coliques ont montré une expression accrue des protéines *O*-GlcNAcylées, d'OGT et de β-caténine dans la partie tumorale. De même, la *O*-GlcNAcylation de la β-caténine sur la sérine 23 a été détectée dans les tissus cancéreux. Conjointement, ces résultats suggèrent un rôle important de la *O*-GlcNAcylation dans les tissus cancéreux liés à la modification de la β-caténine (Publication n°3 : Olivier-Van Stichelen et al., 2012, en révision) .

Nous avons ensuite cherché à savoir si la modulation de la *O*-GlcNAcylation était possible dans ces cellules. Nous souhaitions ainsi pouvoir augmenter la dégradation de la β-caténine et diminuer ainsi son aspect oncogénique. Une diminution de l'apport en glucose ainsi que l'inhibition de l'OGT diminuent l'expression de la β-caténine, à la fois dans les cellules ne montrant pas de mutations au sein de la voie Wnt, mais également dans les cellules cancéreuses coliques, montrant des mutations de l'APC ou de la β-caténine. Ceci suggère que ces cellules, malgré l'existence de mutations au sein de la voie Wnt/β-caténine, sont capables, mais dans une moindre mesure, de réguler l'expression de la β-caténine par sa *O*-GlcNAcylation. Ces dernières observations suggèrent que la *O*-GlcNAcylation pourrait agir, suite à des défauts métaboliques, en amont des premières mutations, mais également en parallèle d'altérations génétiques héréditaire ou sporadique associées aux cancers colorectaux.

Figure35 : Diagramme de Venn décrivant les différentes altérations génétiques observées dans les patients atteints de cancer colorectaux (adapté de Walther et al., 2009).

D'après l'hypothèse de Knudson, il faut plusieurs mutations pour enclencher le processus de cancérisation et nous pouvons supposer que la *O*-GlcNAcylation aberrante s'ajoute et renforce l'effet délétère des mutations pour promouvoir la cancérisation (Knudson, 1971).De plus, on sait que certains cas de cancers colorectaux, appelés « triple négatif », ne montrent aucune mutation d'oncogènes ou suppresseurs de tumeurs décrite à ce jour (Figure 35). La cause de la cancérisation pour ces patients (27%) constitue donc "une énigme " pour la science. La *O*-GlcNAcylation pourrait ici fournir une partie de l'explication.

Le but de cette étude était donc d'essayer d'établir un lien, et de fournir une tentative d'explication, concernant l'influence des désordres métaboliques sur le cancer colorectal. Nous avons avancé l'hypothèse selon laquelle la β-caténine *O*-GlcNAcylée constituerait un des éléments reliant ces groupes de pathologies. Cette relation nous permet d'ouvrir des perspectives pour l'avenir de ce sujet d'étude. En effet, l'essentiel ici est que la *O*-GlcNAcylation constituerait une dérégulation précédant ou s'ajoutant à l'apparition des mutations génétiques. Cette modification pourrait, si les études qui suivront renforcent nos données, être diminuée efficacement par des inhibiteurs pharmacologiques.

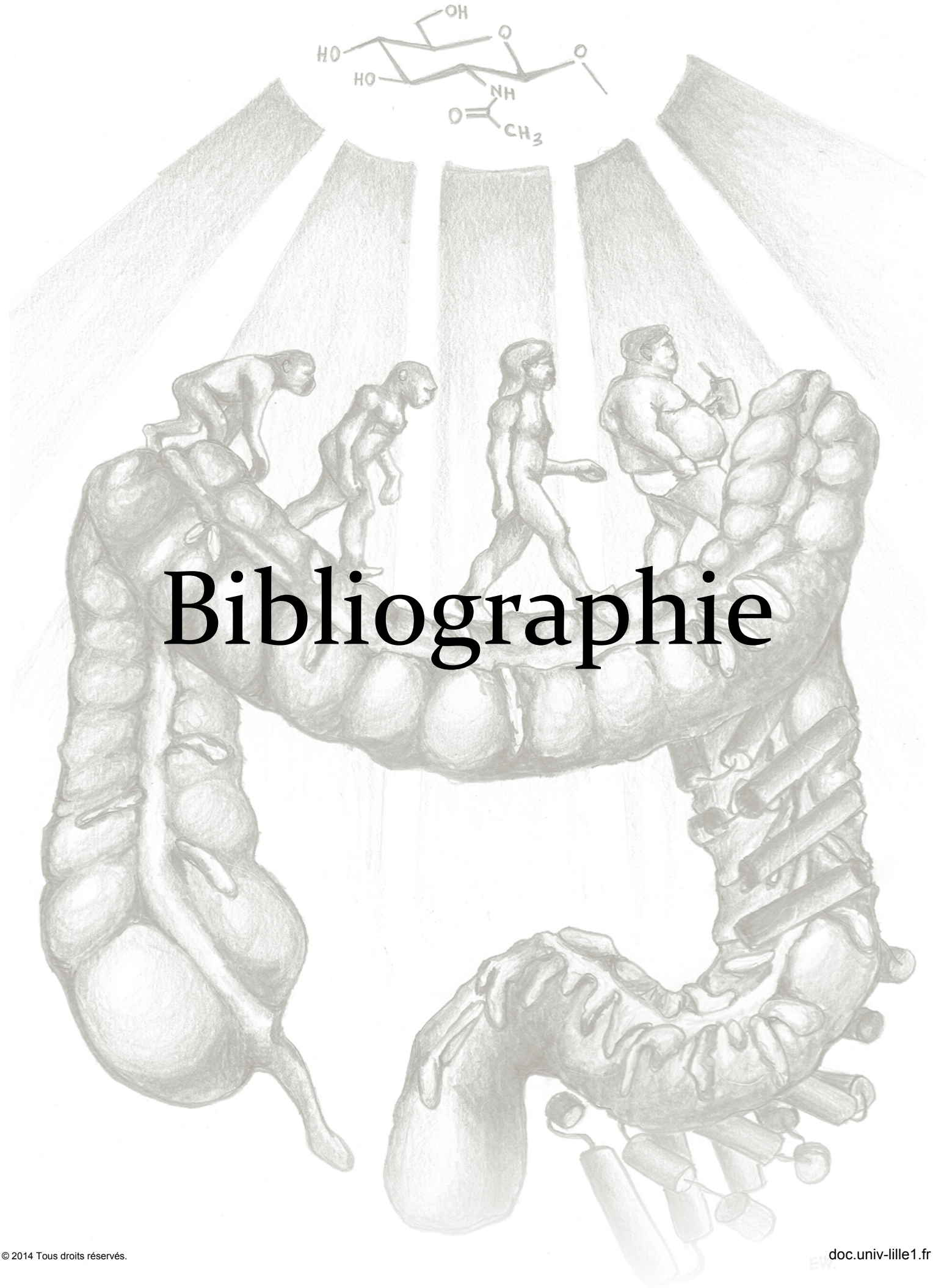
A l'heure actuelle, la détection du cancer colorectal reste un problème de santé publique. La mise en place d'une campagne de dépistage grâce au test Hemoccult a permis le dépistage de masse. Malheureusement, il implique la présence de sang dans les selles, observée uniquement dans le cas d'une lésion à un stade avancé. La coloscopie reste donc encore à l'heure actuelle le moyen le plus fiable de détecter une masse cancéreuse. Néanmoins, les tumeurs doivent être de taille relativement importante afin d'être repérées. Ceci implique une masse tumorale de près d'un centimètre de diamètre, composée d'un milliard de cellules. Pour ces raisons, la recherche de nouveaux outils de diagnostic précoce reste un challenge pour les scientifiques. La biopsie des tissus lésés reste le seul moyen de donner un diagnostic définitif du cancer. A ce stade, on peut imaginer que la β-caténine *O*-GlcNAcylée puisse faire office de marqueur cancéreux précoce, donnant une information sur l'état des cellules de l'épithélium, et cela indépendamment des mutations du cancer. Pour cela, nous avons entrepris la synthèse d'anticorps ciblant à la fois la β-caténine S23-*O*-GlcNAc et T41-*O*-GlcNAc, les deux sites majeurs de modifications de la protéine.

Cette partie du travail n'est pour le moment pas terminée et fera l'objet d'une étude plus approfondie par notre laboratoire.

Durant cette étude, nous avons également observé une *O*-GlcNAcylation élevée dans les cellules cancéreuses. A l'heure actuelle, une des techniques d'imagerie utilisées, la tomographie par émissions de positons (TEP) permet de déceler les cellules possédant un métabolisme élevé du glucose (dû à l'effet Warburg) par l'injection en intraveineuse de 2-désoxy-2(18F)Fluoro-D-glucose. Cette technique est très avantageuse car peu invasive mais reste essentiellement utilisée pour la détection des récidives et métastases du cancer (Papathanassiou et al., 2009).

Inversement, dans notre hypothèse, des niveaux élevés de *O*-GlcNAcylation apparaîtraient rapidement dans la séquence de développement du cancer et pourrait être mis à profit pour une détection en test ELISA de biopsies réalisées lors de coloscopies de routine. Un autre point de notre étude est également à approfondir. Comme mentionné ci-dessus, nous avons observé que malgré l'utilisation de cellules cancéreuses portant des mutations soit sur APC (HT29), soit sur la β-caténine elle-même (HCT116), la stabilisation de la β-caténine pouvait être influencée par la présence d'inhibiteurs de l'OGT ou de l'OGA. Cette modulation est néanmoins moins efficace que sur les cellules non-mutées dans la voie Wnt mais renforce l'hypothèse selon laquelle la *O*-GlcNAcylation compléterait l'effet délétère des mutations pour atteindre le phénotype cancéreux. Cela suppose qu'une régulation de l'expression de la β-caténine, et de son activité transcriptionnelle, est possible dans les cellules cancéreuses, malgré la présence de mutations. Il serait donc envisageable de cibler les cellules cancéreuses avec des inhibiteurs de *O*-GlcNAcylation et ainsi de diminuer leur progression. Néanmoins, une telle inhibition entraînerait la dérégulation de toutes les protéines *O*-GlcNAcylées. Les défauts engendrés seraient dans ce cas beaucoup plus importants que le simple ralentissement de la prolifération cellulaire. La solution serait donc de cibler directement la *O*-GlcNAcylation de la β-caténine. Pour cela, il nous faudrait découvrir par quels moyens l'OGT acquiert sa spécificité d'action envers certaines protéines afin de bloquer directement l'interaction entre l'OGT et une protéine cible de notre choix, la β-caténine.

La science est en constante évolution. Les collaborations entre les différentes disciplines scientifiques, physique, chimie, mathématiques, médecine et biologie, permettent chaque jour d'entrevoir de nouvelles techniques de dépistages et de traitements des cancers. Ainsi, pouvons-nous espérer qu'un jour, cette étude puisse entrer dans l'élaboration d'une nouvelle technique de détection ou de traitement.



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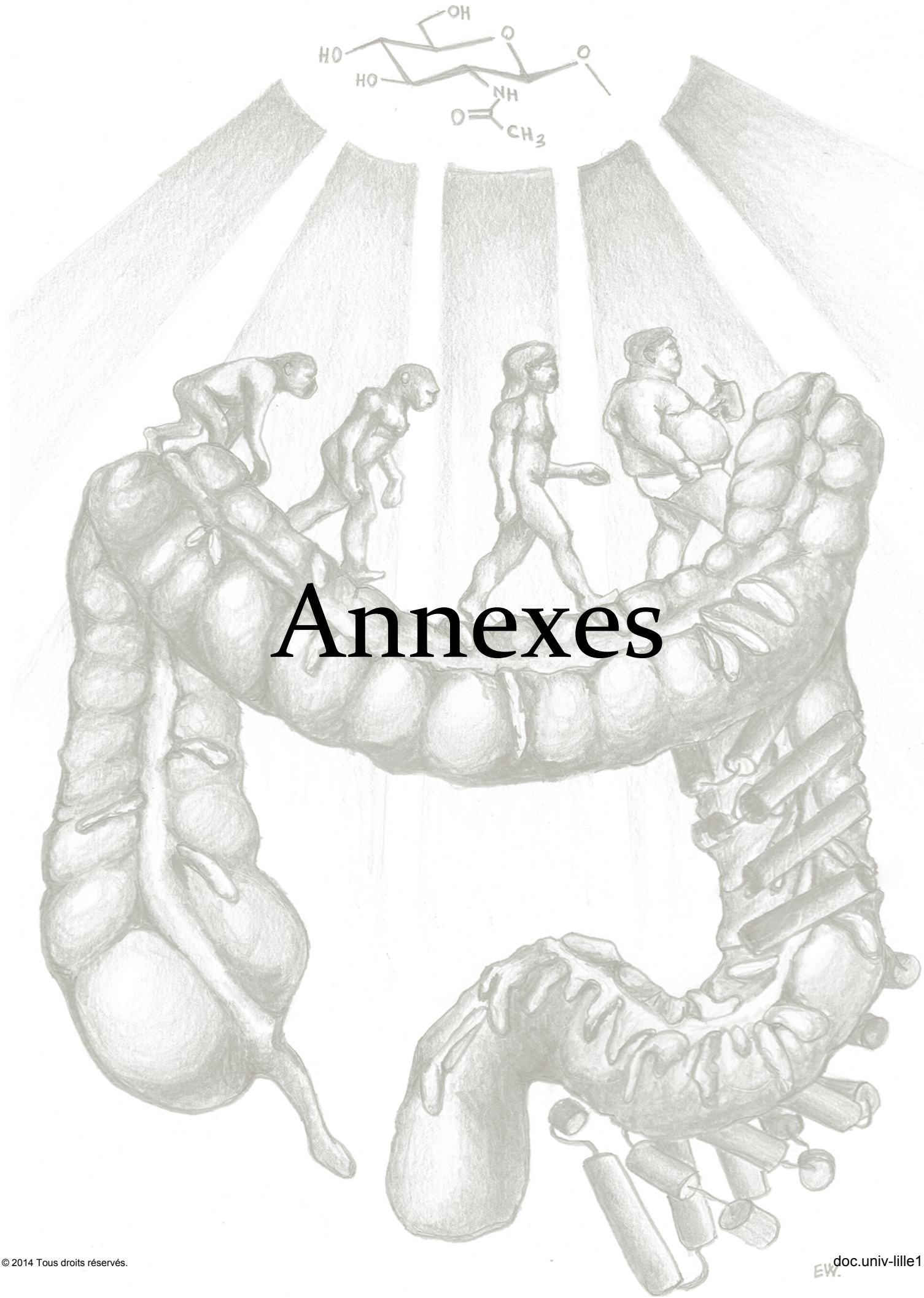

Annexes

En marge de mes travaux de thèse, j'ai également participé à la publication de deux articles scientifiques ainsi qu'à l'écriture d'une revue et d'un chapitre d'ouvrage :

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Characterization of *O*-GlcNAc cycling and proteomic identification of differentially *O*-GlcNAcylated proteins during G1/S transition

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Article publié dans FASEB J (2013)*:*

The FASEB Journal • Research Communication

Insulin signaling controls the expression of *O*-GlcNAc transferase and its interaction with lipid microdomains

Yobana Perez-Cervera,*,† Vanessa Dehennaut,* Moyira Aquino Gil,† Katia Guedri,* Carlos Josué Solórzano Mata,*,† Stéphanie Olivier-Van Stichelen,* Jean-Claude Michalski,* François Foulquier,* and Tony Lefebvre*,1

Revue publiée dans

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Review

Dysregulation of the nutrient/stress sensor *O*-GlcNAcylation is involved in the etiology of cardiovascular disorders, type-2 diabetes and Alzheimer's disease

Tony Lefebvre [a,*], Vanessa Dehennaut [b], Céline Guinez [a], Stéphanie Olivier [a], Ludivine Drougat [a], Anne-Marie Mir [a], Marlène Mortuaire [a], Anne-Sophie Vercoutter-Edouart [a], Jean-Claude Michalski [a]

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Chapitre d'ouvrage publié dans *Methods in Molecular Biology (2013) :*

Antibodies and Activity Measurements for the Detection of *O*-GlcNAc Transferase and Assay of its Substrate, UDP-GlcNAc

Tony Lefebvre, Ludivine Drougat, Stephanie Olivier-Van Stichelen, Jean-Claude Michalski, and Anne-Sophie Vercoutter-Edouart

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ABSTRACT

Background: DNA replication represents a critical step of the cell cycle which requires highly controlled and ordered regulatory mechanisms to ensure the integrity of genome duplication. Among a plethora of elements, post-translational modifications (PTMs) ensure the spatiotemporal regulation of pivotal proteins orchestrating cell division. Despite increasing evidences showing that *O*-GlcNAcylation regulates mitotic events, the impact of this PTM in the early steps of the cell cycle remains poorly understood.
Methods and results: Quiescent MCF7 cells were stimulated by serum mitogens and cell cycle progression was determined by flow cytometry. The levels of *O*-GlcNAc modified proteins, *O*-GlcNAc Transferase (OGT) and O-GlcNAcase (OGA) were examined by Western blotting and OGA activity was measured during the progression of cells towards S phase. A global decrease in *O*-GlcNAcylation was observed at S phase entry, concomitantly to an increase in the activity of OGA. A combination of two-dimensional electrophoresis, Western blotting and mass spectrometry was then used to detect and identify cell cycle-dependent putative *O*-GlcNAcylated proteins. 58 cytoplasmic and nuclear proteins differentially *O*-GlcNAcylated through G1/S transition were identified and the *O*-GlcNAc variations of Cytokeratin 8, hnRNP K, Caprin-1, Minichromosome Maintenance proteins MCM3, MCM6 and MCM7 were validated by immunoprecipitation.
Conclusions: The dynamics of *O*-GlcNAc is regulated during G1/S transition and observed on key proteins involved in the cytoskeleton networks, mRNA processing, translation, protein folding and DNA replication.
General significance: Our results led us to propose that *O*-GlcNAcylation joins the PTMs that take part in the regulation of DNA replication initiation.

1. Introduction

In response to mitogenic signals, quiescent cells enter the cell cycle and progress in G1 phase in order to prepare DNA replication occurring during S phase. In charge of maintaining the genome integrity from mother cell to daughter cells, duplication of the entire genome requires highly sequential and fine-tuned control processes, including checkpoints along the cell cycle. The G1/S transition is one of the critical checkpoints that determines whether cells are able to continue through S phase. This checkpoint is mainly governed by the phosphorylation status of the retinoblastoma protein pRb [1]. Upon mitogenic stimulation, pRb is indeed inhibited by successive phosphorylation events orchestrated by the Cyclin D/CDK4-6 and Cyclin E/CDK2 complexes respectively acting in early and late G1 phase. This induces the destabilization of the pRb-E2F complex, the release of the transcription factor E2F, and the subsequent transcriptional activation of the E2F-dependent genes further required for G1/S transition and DNA duplication. Phosphorylation of pRb at the G1/S transition hence illustrates how crucial the post-translational modifications (PTMs) are needed to fine-tune the activity of key proteins involved in the control of cell cycle progression [1,2]. In addition to phosphorylation and ubiquitination, several studies have demonstrated that the *O*-linked beta-N-acetylglucosaminylation (*O*-GlcNAcylation) of proteins takes part in the regulation of cell division and more particularly in mitosis events.

O-GlcNAcylation of proteins is known as an abundant and reversible modification governed by *O*-GlcNAc transferase (OGT) that transfers the *N*-acetylglucosamine (GlcNAc) residue onto Ser/Thr of proteins from the nucleotide-sugar donor uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), and *O*-GlcNAcase (OGA) that removes the sugar from proteins [3]. The *O*-GlcNAcylation level is responsive to various extracellular environmental changes, such as nutrients, growth factors or stress [4]. Affecting many fundamental

Abbreviations: CDK, Cyclin dependent kinase; CHX, Cycloheximide; CK, Cytokeratin; hnRNP, Heterogeneous nuclear ribonucleoprotein; HU, Hydroxyurea; MCM, Minichromosome maintenance; *O*-GlcNAc, *O*-linked beta-*N*-acetylglucosaminylation; OGA, *O*-GlcNAcase or beta-*N*-acetylglucosaminidase; OGT, *O*-GlcNAc transferase; PTM, Post-translational modification; pRb, Retinoblastoma protein; UDP-GlcNAc, Uridine diphosphate *N*-acetylglucosamine

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biological functions such as protein interactions, degradation, stability, or subcellular localization of a wide functional range of proteins [4–6], *O*-GlcNAcylation can act either in competition or in concert with phosphorylation, on the same sites or at proximal sites. While the cross-talk between *O*-GlcNAcylation and phosphorylation is obvious, its regulation is complex and depends on the target proteins and the *O*-GlcNAc sites within the modified proteins [6,7]. Previous studies performed on human somatic cell lines and in the well-established mitosis model of *Xenopus laevis* oocytes have highlighted that the dynamics of *O*-GlcNAc was regulated in a cell cycle-dependent manner. Indeed, we demonstrated that G2/M transition was accompanied with an increase in the level of *O*-GlcNAcylated proteins and required OGT activity in *Xenopus* oocytes [8,9]. In somatic cells, several studies showed that the *O*-GlcNAcylated protein level and OGT expression were higher in M phase than in G1 phase [8,10,11]. Moreover, recent studies have shown that the H2A, H2B, H3 and H4 nucleosomal histones were *O*-GlcNAcylated in a cell cycle-dependent manner [12–14]. These findings indicate that, in conjunction with the many post-translational modifications affecting the histone core, O-GlcNAc modification may participate in the regulation of chromatin compaction during cell cycle progression and mitosis. For instance, it has been proposed that the reduction of H3 *O*-GlcNAcylation during S phase might help the pre-replicative complex to reach the chromatin [13]. In addition, disruption of *O*-GlcNAc level by OGT/OGA overexpression or OGA knockdown affected cell cycle progression and induced severe defects in mitosis, indicating that *O*-GlcNAc cycling is crucial for the correct sequence of events leading to cellular division [11,15]. These observations are concordant with the transitory association of OGT and OGA with the mitotic kinase Aurora B and the phosphatase PP1C at the end of mitosis [16], highlighting the cross-talk between *O*-GlcNAcylation and phosphorylation to fine-tune the activity of proteins involved in cytokinesis control. Finally, deregulation in *O*-GlcNAc cycling by forcing cells to overexpress or silence OGT, points out indirect roles of OGT in the cell cycle machinery, including the degradation of the CDK inhibitor $p27^{kip1}$ [17] and the activity of the cyclin B/CDK1 complex [7]. Although the understanding of the role of *O*-GlcNAc modification in cellular division is growing, the current knowledge of the regulation of *O*-GlcNAc dynamics during cell cycle progression remains mostly unknown, and more particularly in the early steps of the cell cycle.

In this work, we characterized the dynamics of *O*-GlcNAcylation through G1/S transition in synchronized MCF7 human cells. We investigated the level of OGT and OGA proteins and measured OGA activity through G1/S transition. Using two-dimensional (2-D) electrophoresis and mass spectrometry, we identified nucleocytoplasmic proteins for which differences in *O*-GlcNAcylation status were observed between G1 and S phases. We showed that changes in *O*-GlcNAc dynamics were associated with S phase entry and differentially affected cytosolic and nuclear proteins, including several components of the minichromosome maintenance MCM2-7 complex that takes part in the initiation of DNA replication.

2. Materials and methods

2.1. Cell culture and synchronization

The human breast carcinoma MCF7 cell line was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum (FCS) (Lonza), 200 mM L-glutamine, 10 U/mL penicillin and 10 μg/mL streptomycin (Life Technologies, Invitrogen, St Aubin, France) at 37 °C in a humidified atmosphere enriched with 5% CO_2. Cell cycle synchronization was achieved either by serum deprivation or hydroxyurea (HU) treatment. For serum deprivation experiments, MCF7 cells were washed twice with PBS (Lonza) and cultured in serum-free medium for 48 h. To release the cell-cycle block, cells were grown in the presence of 10% FCS and harvested at the indicated time periods. For chemical synchronization, MCF7 cells were incubated in DMEM medium supplemented with 10% FCS and containing 2.5 mM HU (Sigma-Aldrich, La Verpillière, France) for 16 h, returned to normal medium for 13 h and then placed back into the medium containing HU for 13 h. Finally, cells were released into normal media for 4 h before harvesting [18]. In some experiments, cells were treated for the indicated time periods with 10 μg/mL cycloheximide (CHX) (Sigma-Aldrich) or 100 nM thiamet G [19] (a gift of Pr Jerôme Lemoine).

2.2. Cell cycle flow cytometry analysis

Cell cycle distribution was determined by staining DNA with propidium iodide. 10^6 cells treated as described above were washed in PBS and harvested using trypsin. After centrifugation at 600 ×*g* for 7 min, the cell pellet was washed with PBS and fixed in 70% (v/v) ice-cold ethanol for 2 h. Cells were centrifuged, washed with PBS, and then incubated with 50 μg/mL propidium iodure (Sigma-Aldrich) and 50 μg/mL RNAse A (Sigma-Aldrich) at 37 °C for 1 h. Flow cytometry analysis was performed on a FACScan instrument using the associated CellQuest software package (Becton Dickinson, Le Pont de Claix, France). The distribution of cells in the different phases of the cell cycle was analyzed using the Modfit software in the synchronization wizard mode (Becton Dickinson).

2.3. Cell lysis and Western blotting

Cells were lysed on ice for 10 min in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton-X100) containing the complete protease inhibitors cocktail (Roche Diagnostics, Meylan, France), 50 mM sodium fluoride (Sigma-Aldrich), 100 μM orthovanadate (Sigma-Aldrich) and 100 μM GlcNAc (Sigma-Aldrich). Cell lysate was centrifuged at 20,000 ×*g* for 10 min and supernatant was collected. Protein concentration was measured using the microBCA protein assay kit (Pierce, Fisher Scientific, Illkirch, France). Proteins (20 μg) were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Hybond ™-C EXTRA, GE Healthcare, Orsay, France). Membranes were blocked in 5% (w/v) nonfat dry milk in Tris-Buffered Saline with 0.05% (v/v) Tween 20 (TBS-T) and probed with primary antibodies directed against *O*-GlcNAc (RL2, 1:1000) (Ozyme, Montigny le Bretonneux, France), OGT (Ti14, 1:2000) (Sigma-Aldrich), OGA (345, 1:4000, a gift of Pr. G. W Hart), cyclin D1 (1:1000) (Millipore, Molsheim, France), cyclin E (clone HE12, 1:1000) (Millipore) and GAPDH (1:4000) (Abcam, Cambridge, UK) overnight at 4 °C. After 3 washes in TBS-T, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse/rabbit IgG-HRP linked, 1:10000, GE Healthcare); anti-Chicken IgY (IgG) (whole molecule)-Peroxidase antibody, 1:20000, Sigma-Aldrich) for 1 h at room temperature. After 3 washes in TBS-T, blots were developed using enhanced chemiluminescence (ECL plus Reagent, GE Healthcare). After detection, blots were stripped in the antibody stripping buffer (Gene Bio-Application LTD, Euromedex, Souffelweyersheim, France) for 15 min at room temperature, extensively washed in TBS-T and reprobed using different antibodies. Densitometry measurements were obtained using a GS800 calibrated imaging Densitometer including the Quantity One® software for image acquisition and analysis (Bio-Rad Laboratories, Marnes la Coquette, France).

2.4. Immunoprecipitation

Cells were lysed in IP buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 0.5% (v/v) NP-40) containing the various inhibitors as previously described. After centrifugation, protein concentration was determined. Protein extracts (1 mg) were first precleared and incubated with the indicated antibody (2–5 μg) for 2 h at 4 °C (MCM3, Cell Signaling, Ozyme; Cytokeratin 8 (C51), MCM6 (H-300), MCM7 (141.2), normal rabbit or mouse IgG, Santa Cruz; hnRNP K (3C2), Abcam; Caprin-1 (ProteinTech Group, Euromedex). Protein G-sepharose beads (GE

Healthcare) were resuspended in IP lysis buffer (1:1) and 50 µL were added and incubated for 1 h at 4 °C. Beads were washed three times with IP lysis buffer and boiled in Laemmli buffer before SDS-PAGE and Western blotting.

2.5. OGA activity assay

OGA activity was performed using 4-nitrophenyl N-acetyl-β-D-glucosaminide (pNP-GlcNAc) as a substrate (Sigma-Aldrich), as previously described [20]. MCF7 cell lysates were freshly prepared in 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, containing previously described inhibitors, except GlcNAc. Protein concentration was measured using the microBCA protein assay kit. The OGA activity assay was conducted in a final volume of 100 µl, containing 40 µl protein lysate, 50 mM sodium cacodylate buffer pH 6.5, 2 mM pNP-GlcNAc, and 50 mM GalNAc, in presence or not of 100 nM Thiamet G, and incubated for 1 h at 37 °C. The reaction was stopped by adding 900 µL of 500 mM sodium carbonate and the absorbance was read at 400 nm using a spectrophotometer (Epoch Spectrophotometer System, BioTek Instruments, Colmar, France). For each sample, the absorbance measured in presence of Thiamet G was subtracted from the absorbance measured without Thiamet G, giving the absorbance due to OGA hydrolase activity. The values of the apparent OGA activity were reported per mg of protein. One hundred percent of OGA activity (arbitrary unit) was given for the time point 0 (quiescent MCF-7 cells after 48 h starvation).

2.6. Confocal immunofluorescence microscopy

MCF-7 cells were grown on glass coverslip in 60-mm plate dishes. At the time of harvest, cells were washed twice in PBS and fixed in 4% (w/v) paraformaldehyde in PBS for 30 min at room temperature. Cells were washed in PBS containing 100 mM glycine (pH 7.4) and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 20 min. Cells were blocked with PBS containing 5% (v/v) normal goat serum (Life Technologies, Invitrogen), 2% (w/v) bovine serum albumin (Sigma-Aldrich) and 2% (v/v) FCS for 1 h at room temperature and incubated overnight at 4 °C with the primary antibody diluted in the blocking solution (RL2, 1:100). Cells were washed three times in PBS and incubated in the dark with Alexa Fluor 568-conjugated secondary antibody (1:600) (Molecular Probes, Life Technologies, Invitrogen) for 1 h at room temperature before mounting coverslips in Mowiol solution (Calbiochem, Merck chemicals, Nottingham, UK). Images were acquired using an inverted Leica SP5 spectral microscope (Bio Imaging Center Lille, IRI CNRS USR 3078, IFR 147, Villeneuve d'Ascq, France) and analyzed using the LAS 6000 AF software.

2.7. Subcellular fractionation, two-dimensional electrophoresis and mass spectrometry analysis

Cytosolic and nuclear fractions were prepared as previously described [21]. MCF-7 cells were harvested at the indicated times, washed twice in cold-PBS and incubated for 30 min on ice with the lysis buffer I (20 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.5% (v/v) Triton-X100, 5 mM $MgCl_2$) containing previously described inhibitors. Lysates were centrifuged at 1000 ×*g* for 10 min. The supernatant was again centrifuged at 20,000 ×*g* for 20 min and the final supernatant was considered as the cytosolic fraction. The first pellet was washed once in lysis buffer I and centrifuged again at 1000 ×*g* for 10 min. The supernatant was discarded and proteins in the remaining pellet were solubilized for 30 min on ice in the lysis buffer II (20 mM Tris–HCl pH 7.6, 400 mM NaCl, 1% Triton-X100, 50 mM KCl and 20% glycerol) containing previously described inhibitors. Finally, the supernatant obtained after centrifugation at 1300 ×*g* for 10 min was considered as the nuclear fraction. For both fractions, protein concentration was determined using the microBCA protein assay kit. Subcellular fractions were desalted and concentrated either by acetone precipitation for the cytoplasmic fraction (80% (v/v) acetone, 2 h at −20 °C) or using the 2D-Clean Up kit (GE Healthcare) for the nuclear fraction. Protein pellets were then dried under Speed Vac, solubilized in the rehydratation buffer (8 M Urea, 2 M Thiourea, 2% (w/v) CHAPS, 10 mM dithiothreitol (DTT), 1.2% (v/v) Immobilized pH Gradient (IPG) buffer (pH 4–7), bromophenol blue), and loaded onto IPG strip (18-cm or 7-cm, pH 4–7, GE Healthcare) by overnight passive rehydratation at room temperature. The first-dimensional isoelectric focusing (IEF) was carried out on a Protean IEF Cell (Bio-Rad Laboratories) for 7-cm strips using the following program: 250 V for 2 h, 1000 V for 2 h, 8000 V for 12 h and 500 V for 9 h. For the 18 cm strips, the Investigator 2D electrophoresis System was used (Perkin-Elmer, Villebon sur Yvette, France). The separation was performed for 20 h at 70,000 Volt-hours, with a maximum of 5000 V and 80 µA/strip. When IEF was complete, strips were incubated twice for 20 min for 18 cm strips or 10 min for 7 cm strips in the equilibration buffer (375 mM Tris–HCl pH 8.8, 6 M urea, 2% (w/v) SDS and 30% (v/v) glycerol) containing 1% (w/v) DTT, then 2% (w/v) iodoacetamide. The second dimensional separation was performed using 8% SDS–PAGE. After 2-D electrophoresis, gels were either silver stained or transferred onto nitrocellulose membranes. For immunoblotting, proteins were detected as described above, except that the ECL Advance™ blocking reagent (GE Healthcare) was used to block and incubate membranes with RL2 antibody. Both gels and films were scanned on GS800 calibrated imaging Densitometer using the Quantity One® software for image acquisition. After comparison of the *O*-GlcNAcylated proteins detected in the nuclear and cytoplasmic fractions obtained from cells in G0, G1 phase or S phase, 2D-Western blot and silver-stained 2D-gels (run in parallel) were carefully matched using the Image J® software. The spots of interest were excised from the gels, destained and digested by trypsin (G-Biosciences, Agro-Bio, La Ferté Saint-Aubin, France) overnight at 37 °C as previously described [21]. Protein identification was performed by Nano-LC-ESI-MS/MS on a hybrid quadrupole time-of-flight mass spectrometer (Q-Star, Applied Biosystems, Foster City, California, USA) equipped with a nano-electrospray ion source coupled with a nano HPLC system (LC Packings Dionex, Amsterdam, The Netherlands) [22]. Database searching was performed using Mascot software (MS/MS ion search module, Matrix Science London, UK) in the Swiss-Prot database (Sprot 0411 version, 525,207 sequences). Search parameters were as follows: *Homo sapiens* as taxonomy, 50 ppm tolerance for the parent ion mass and 50 mmu for the MS/MS fragment ions, one missed cleavage allowed, carbamidomethylation of cysteine and methionine oxidation as possible modifications. Only protein candidates with a significant Mascot score were taken into consideration (significance threshold for candidate <0.05 using MudPIT scoring method and an expectation value for ion peptides <0.05).

3. Results

3.1. The level of O-GlcNAcylated proteins decreases during G1/S transition

The impact of *O*-GlcNAc protein modification in the molecular events controlling mitosis begins to be understood. However the role of this modification in the early steps of the cell cycle remains to be defined. To address this point, the level of *O*-GlcNAcylated proteins was investigated during G1/S transition and S-phase progression by Western blot analysis. MCF7 cells were synchronized in G0 by serum deprivation before being stimulated to re-enter the cell cycle by addition of serum-derived growth factors. Flow cytometry analysis shows that serum starvation resulted in more than 85% of G0/G1-arrested cells (Fig. 1A). Stimulation of these cells with serum resulted in the initiation of S phase approximately 15 h after serum addition. The resulting relative proportion of cells in S phase was increased from 10% to 22% (Fig. 1A). The synchronized progression of cells through the cell cycle was confirmed by Western blot analysis

against cyclin D and cyclin E (Fig. 1B). The amount of Cyclin D peaked in mid-G1 (12 h) while the maximum level of Cyclin E coincided with the G1/S transition (15 h). In parallel, *O*-GlcNAcylation level was assessed by Western blot analysis using the RL2 anti-*O*-GlcNAc monoclonal antibody. Strong variations were observed in the steady-state level of *O*-GlcNAc modified proteins as cells progressed through the cell cycle (Fig. 1B). The level of *O*-GlcNAcylated proteins significantly increased when cells progressed in G1 phase (from 100% to 130%) and rapidly decreased when cells entered and progressed through S phase (from 130% to 60%) (Fig. 1B). Indirect immunofluorescence with RL2 antibody corroborated these observations (Fig. 1C). In quiescent cells, the *O*-GlcNAc protein staining was detected in the nuclear and cytoplasmic compartments although it was predominant in the nucleus. The nuclear staining of *O*-GlcNAcylated proteins was much more intense as the cells progressed through G1 phase (12 h) and strongly decreased once cells entered and progressed into S-phase. These results indicate that the dynamics of *O*-GlcNAc is differentially regulated in the nuclear and cytoplasmic compartments during the progression of serum-stimulated cells towards S phase.

As the steady-state level of *O*-GlcNAc modified proteins is controlled by OGT and OGA, the expression level of these enzymes was assessed by Western blot analysis. Interestingly, a general increase in the level of both OGT and OGA proteins was observed as the cells progressed through S phase (Fig. 1B). It is important to note that in S phase, the increase in OGA protein was more pronounced than the one observed for OGT (350% *versus* 250% compared with quiescent cells), likely consistent with the low level of *O*-GlcNAcylated proteins detected in S phase. We then used hydroxyurea (HU) to determine whether these results could be observed using a chemical method of synchronization. MCF7 cells were synchronized at the G1/S border by HU treatment, and release from HU promoted a marked increase of S-phase cells, as confirmed by flow cytometry analysis (Fig. 1D). Following release from the HU block, S-phase progression correlated with a 30% reduction in the steady-state of *O*-GlcNAc proteins. In contrast to the results obtained with the serum deprivation–stimulation method (Fig. 1B), the decreased of O-GlcNAc signal was observed on specific high molecular weight protein bands (Fig. 1E). This may be a result of the method of cell synchronization: While HU is a ribonucleotide reductase inhibitor that blocks the step of DNA synthesis, serum deprivation affects multiple upstream signaling and metabolic pathways that link mitogenic stimulation to cell cycle entry. Meanwhile, OGT and OGA protein levels reached respectively 125% and 172% of the level detected in G1/S-blocked cells (Fig. 1E). The rate of increase in O-GlcNAc cycling enzymes protein level was thus similar to what we observed between the time points 15 h and 18 h following serum stimulation (Fig. 1B). Altogether, these data indicate

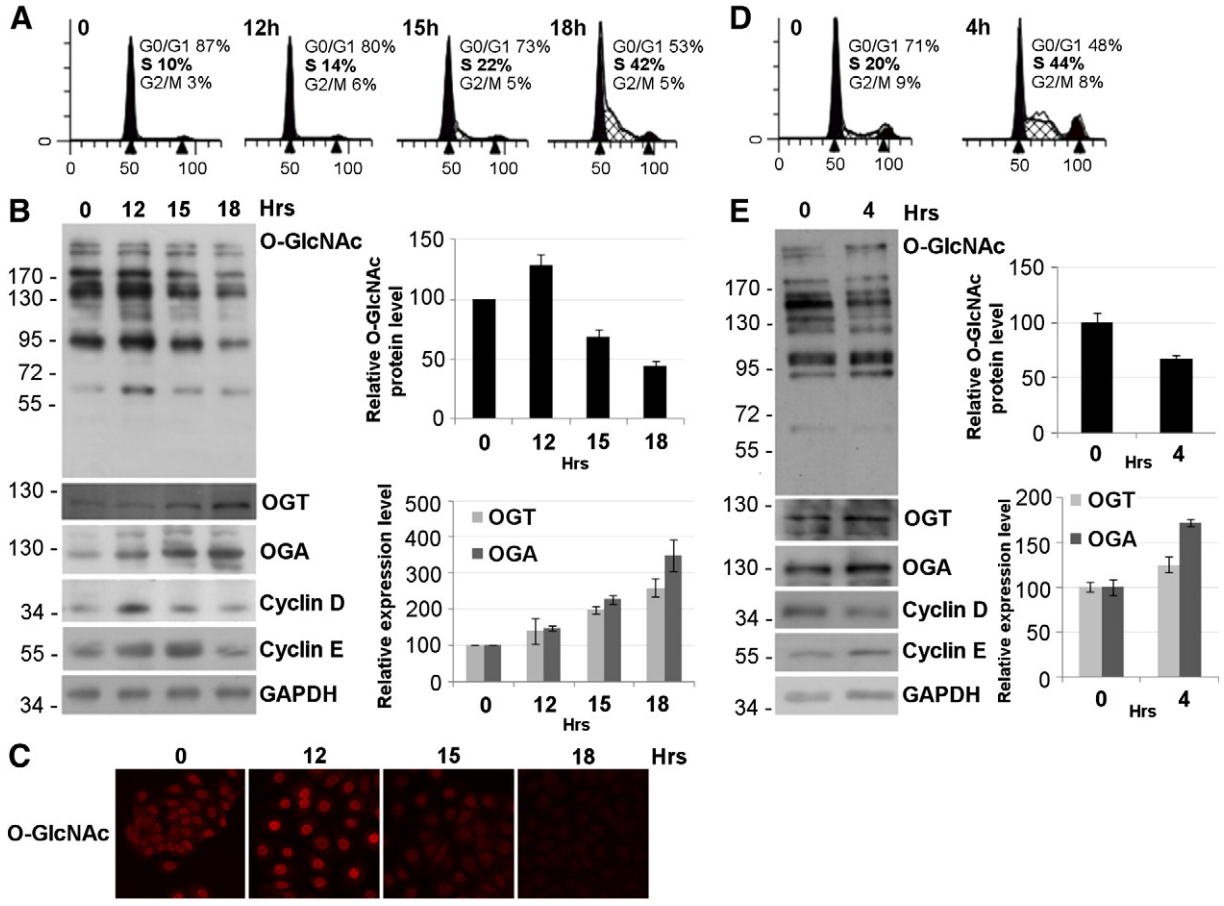

Fig. 1. *O*-GlcNAc cycling is modified during G1/S transition. A, MCF7 cells were synchronized in G0/G1 by serum starvation and released into the cell cycle by serum addition for the indicated times. Cell-cycle distribution was determined by flow cytometry analysis after staining of cells with iodide propidium. B, *O*-GlcNAc level, OGT and OGA protein levels were determined by immunoblotting using specific antibodies. Expression of G1- and S-phase specific cyclins, cyclin D and E were assessed by Western blotting. Equal loading was confirmed using GAPDH as a control. Histograms show the relative level of *O*-GlcNAc protein and relative expression of OGT and OGA determined by densitometric analysis and normalized to the loading control. Values are mean ± S.E. of 3 independent experiments. C, For each time, cells were fixed and analyzed by immunofluorescence microscopy using RL2 antibody. D, MCF7 cells were synchronized at the G1/S transition by a double-block treatment with 2.5 mM HU. After treatment, cells were either harvested (0 h) or release into cell cycle for 4 h. Accumulation of cells in S phase was assessed by flow cytometry analysis. E, Whole cell extracts were immunoblotted with antibodies directed against *O*-GlcNAcylated proteins, OGT and OGA. Cyclins D and E are used as controls of cell cycle progression through G1 and S phases and GAPDH as a loading control. The relative levels of *O*-GlcNAc proteins and the relative expression of OGT and OGA, represented in the histograms, were quantified by densitometry and normalized to GAPDH levels. Values are mean ± S.E. of 3 independent experiments.

that the observed reduction of *O*-GlcNAcylated protein level associated with S-phase entry may result from changes in the OGT/OGA expression level and more particularly in the increase of OGA level.

3.2. Cycloheximide blocks the serum-induced expression of OGT and OGA

To investigate whether the observed up-regulation of *O*-GlcNAc cycling enzymes levels during G1/S transition was dependent on translation process, the protein synthesis inhibitor CHX was used and the respective levels of OGA and OGT were assessed by Western blot analysis. CHX was added to G1-progressing cells, 12 h after serum addition. This caused a blockade of cells in G1-phase, consistent with the inhibition of serum-induced expression of cyclin D and cyclin E normally occurring during G1 phase (Fig. 2A). No variation of the protein level of OGT and OGA was observed in presence to CHX, supporting the idea that progression of cells towards S phase induces the expression of OGT and OGA proteins (Fig. 2A). Moreover, CHX induced a strong elevation in the steady-state level of *O*-GlcNAcylated proteins concomitantly to the blockade in G1 phase (Fig. 2A), suggesting that the observed variations in *O*-GlcNAc levels during the early steps of the cell cycle may result from a differential expression of *O*-GlcNAc cycling enzymes. However, CHX has been shown to slightly increase O-GlcNAc level in glucose-deprived cells, too [20]. Thus, we cannot exclude that part of the increased O-GlcNAcylation in presence of CHX occurred independently of the cell cycle.

3.3. OGA activity is increased at the G1/S transition

The observed variations in *O*-GlcNAc levels during the progression of cells towards S phase could be correlated with changes in the activity of *O*-GlcNAc cycling enzymes. To investigate this hypothesis, *in vitro* activity of endogenous OGA was measured in cell lysates (Fig. 2B). A significant 30% increase in OGA activity was observed when cells entered and progressed into S phase, consistent with the decrease of *O*-GlcNAcylated proteins level observed in S phase. The increased OGA activity is likely to be caused by the increased level of OGA protein through S phase (Fig. 1B). To determine whether inhibition of OGA activity could impair the progression of cells towards S phase, G1-cells were treated with the potent and selective OGA inhibitor Thiamet G to prevent deglycosylation of *O*-GlcNAcylated proteins [19]. Thiamet G markedly decreased by 60% the endogenous OGA activity (Fig. 2B) and induced a two-fold increase in the steady-state level of *O*-GlcNAcylated proteins, but had no effect on cell cycle progression (Fig. 2C). However, a 20% increase in OGA activity was observed at the G1/S transition in Thiamet G-treated cells (Fig. 2B), consistent with the slight decrease of *O*-GlcNAc level observed in these cells in S phase (Fig. 2C). The use of OGA inhibitors, including PUGNAc or GlcNAc-thiazoline, is known to raise OGA level and reduce OGT level in treated cells [15,16]. The 20% increase in OGA activity could therefore result from the effect of Thiamet G on O-GlcNAc cycling enzymes level. Indeed, Thiamet G induced a significant increase in OGA protein level at 18 h and impaired the increase in OGT protein observed in control cells (Fig. 2C).

3.4. Identification of nuclear and cytosolic proteins differentially *O*-GlcNAcylated during G1/S transition

In order to identify nuclear and cytosolic proteins that are differentially *O*-GlcNAcylated during the first steps of the cell cycle, quiescent serum-starved MCF7 cells were stimulated to reenter the cell cycle by addition of serum mitogenic factors and harvested 12, 15, and 18 h later. After subcellular fractionation, nuclear and cytosolic proteins from quiescent, G1 and S phase MCF7 cells were separated by 2-D electrophoresis and *O*-GlcNAc proteins were detected by Western blotting. The cytoplasmic and nuclear fractions show distinct patterns of *O*-GlcNAcylated protein spots (Fig. 3A and C respectively). In both fractions, the *O*-GlcNAc protein levels increased between G0 and G1 phase and slightly decreased as cells entered S phase, reinforcing data presented in Fig. 1. We hence focused on the identification of protein spots showing *O*-GlcNAc variation levels. The corresponding spots, numbered onto the enlarged regions of 2-D Western blot (Fig. 3B, D), were cut from the silver-stained 2-D gels of each fraction. Eventually, 58 cytoplasmic and nuclear proteins were identified by tandem mass spectrometry after trypsin digestion (Tables 1 and 2, respectively). Among them, 13 are novel potential *O*-GlcNAc proteins and 14 belong to a protein family for which *O*-GlcNAc modified members have previously been reported. Some proteins were found in several distinct spots, such as the heterogeneous nuclear ribonucleoprotein (hnRNP) K that was identified in 4 different spots of the cytosolic fraction (Table 1, Fig. 3B). This highlights the fact that a given protein can be reversely modified by various post-translational modifications at multiple sites, generating multiple distinct forms of the protein after 2-D electrophoresis

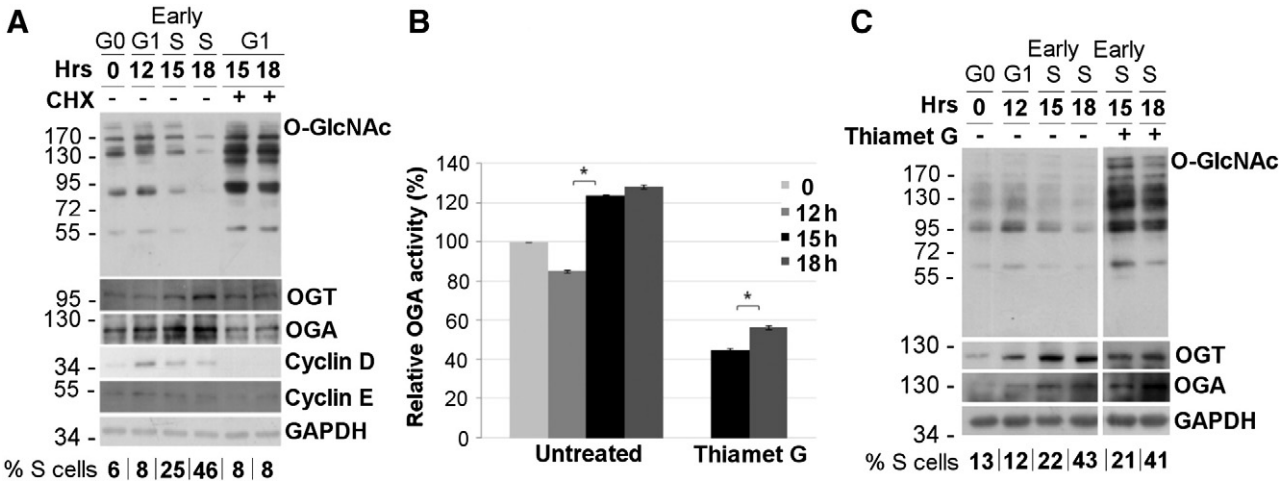

Fig. 2. S phase entry regulates *O*-GlcNAc dynamics. Quiescent (0) MCF7 cells were harvested 12, 15 or 18 h after serum stimulation. A, CHX was added 12 h after serum addition. Synchronized progression through the cell cycle was analyzed by flow cytometry. Western blot was performed as described above. B, Thiamet G was added 12 h after release into cell cycle. OGA activity assay was performed using pNP-GlcNAc as substrate as described under Materials and methods. Quiescent cells referred as 100% of the relative OGA activity. Standard deviation and *P*-value<0.05 (Student's *t*-test) were obtained from 3 independent experiments. C, Western blotting was performed using antibodies against *O*-GlcNAc, OGT and OGA. GAPDH was used as the loading control. Cell cycle progression was checked by flow cytometry analysis.

separation. Concerning the cytosolic fraction, cell cycle-dependent *O*-GlcNAc variations were mainly found on proteins involved in biosynthesis, folding, transport or turnover of proteins, such as heat shock proteins (HSP) HSP90A and HSP90B, epsilon and theta subunits of the T-complex protein 1(TCP) and importin-5 (Table 1, Fig. 3A, B). In the nuclear fraction, most of the identified *O*-GlcNAcylated proteins participate in the regulation of transcription and mRNA processing, such as RNA helicases DDX, hnRNP proteins and the splicing factor SF3A1 (Table 2, Fig. 3C, D). In addition, several structural *O*-GlcNAcylated proteins have been identified in both fractions, including lamin-A/C and lamin-B1, two components of the nuclear lamina that play important roles in nuclear assembly and chromatin organization.

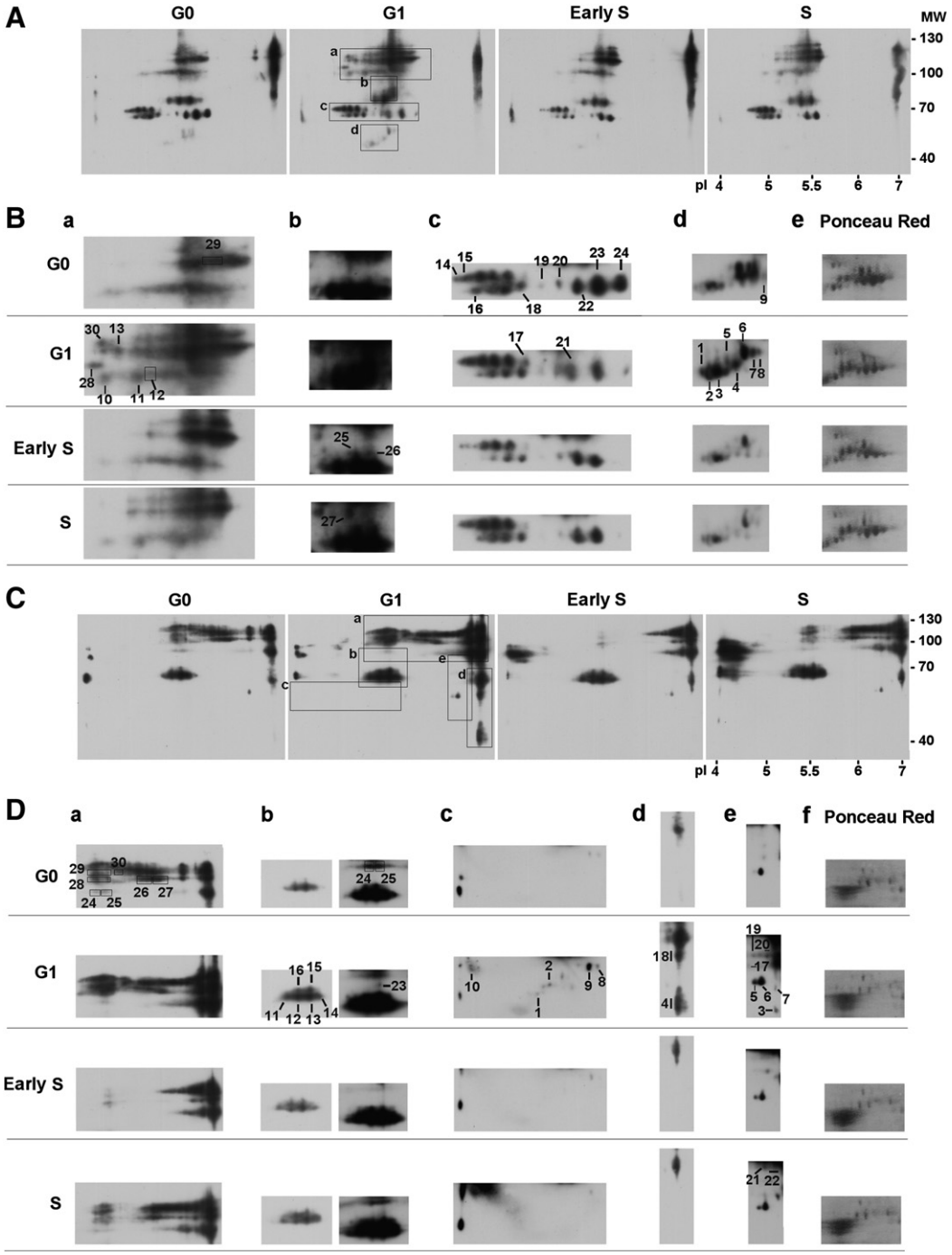

Fig. 3. Detection and identification of cell cycle-dependent *O*-GlcNAcylated proteins. Quiescent (G0) MCF7 cells were released into cell cycle after serum stimulation and harvested at the indicated phases of the cell cycle. The proportion of cells in G1, early S and S phases was checked by flow cytometry. After subcellular fractionation, proteins from the cytosolic (A) and nuclear (C) fractions were separated by 2-D electrophoresis and *O*-GlcNAc variations were detected by Western blot analysis using RL2 antibody. The approximate isoelectric point (pI) and molecular weight (MW, in kDa) scales are indicated. Regions of interest in which protein spots have been identified are drawn in the G1-phase image of the cytosolic (A) and nuclear (C) fractions. Enlarged images of *O*-GlcNAc Western-blot corresponding to these regions are shown respectively in B (*a*–*d*) and D (*a*–*e*). Images from longer exposures are shown for zooms *b* and *d* in B, and *b* (right panel), *c* and *e* in D. Spots which have been successfully identified by tandem mass spectrometry are numbered from 1 to 30 in each fraction. Protein candidates are reported in Table 1 for the cytosolic fraction and in Table 2 for the nuclear fraction. Enlarged region from the Ponceau red staining of the nitrocellulose membranes confirmed the equal loading (B, *e* and D, *f*). Images are representative of 4 experiments from 3 independent cellular lysate preparations.

More interestingly, we also reported that the minichromosome maintenance proteins MCM2, MCM3, MCM6 and MCM7 are differentially *O*-GlcNAcylated during G1/S transition (Table 2, Fig. 3D).

The *O*-GlcNAcylation status of 6 proteins during the G1/S transition was confirmed by immunoprecipitation and Western blot analysis (Fig. 4). We checked the *O*-GlcNAc variations on the hnRNP K protein, an abundant RNA-binding protein that shuttles between cytoplasm and nucleus and interacts with a wide range of partners to regulate multiple cellular processes including transcription, mRNA processing and translation [23]. Here we found that *O*-GlcNAcylation of hnRNP K decreased by 50% in G1-cells compared with quiescent cells and then increased in early and late S phase (Fig. 4A). Seven different cytokeratins (CKs) were identified in the cytosolic spots 1 to 8, for which an increase of *O*-GlcNAc level was observed in G1 phase (Fig. 3B, Table 1). However, it was difficult to get the individual variation of *O*-GlcNAcylation for each identified CK, since each of them localized in a minimum of two different spots (up to 8 spots for CK8). This observation underlies the complexity of post-translational modifications changes that may occur on multiple sites on a given CK during progression of cells towards S phase. Among them, CK8 is a major intermediate filament protein expressed in epithelial cells. As shown in Fig. 4B, the *O*-GlcNAcylation of CK8 was transitory increased by a two-fold factor as cells entered S-phase while the level of CK8 did not change. Of particular interest is the cell cycle-associated protein-1 (Caprin-1), a cytoplasmic phosphorylated protein which is required for normal proliferation [24]. We first confirmed by Western blotting that the level of Caprin-1 was increased in proliferating cells compared with resting cells [25]. More interestingly, we showed that *O*-GlcNAcylation of Caprin-1 was progressively reduced as quiescent MCF-7 cells moved towards S-phase (Fig. 4C).

The MCM2-7 complex is part of the multiprotein pre-replicative complex necessary to the initiation of DNA replication and functions as the replicative helicase [26]. Owing to the crucial role of MCM proteins in S phase, we further examined the *O*-GlcNAcylation status of the MCM proteins identified in the 2-D gels of the nuclear fractions. While the amount of MCM3, MCM6 and MCM7 proteins gradually increased as cells progressed into S phase (Fig. 4D–4F, upper panels), *O*-GlcNAcylation of MCM3 and MCM6 decreased up to 60% as cells progressed towards G1/S transition, then increased in S-phase cells (Fig. 4D, E). In contrast, *O*-GlcNAcylation of MCM7 increased nearly 3-fold when quiescent cells progressed towards G1/S transition, and then decreased in S-phase cells (Fig. 4F). It should be noted that, considering the method we used to extract cellular proteins for immunoprecipitation experiments, immunoprecipitated *O*-GlcNAc MCM proteins are likely to come from the chromatin-unbound fraction rather than the chromatin-bound fraction. These results indicate that several components of the nucleoplasmic MCM2-7 complex are differently modified by *O*-GlcNAc modification in a cell cycle-dependent manner.

4. Discussion

In the present study, we analyzed the dynamics of *O*-GlcNAc in the early phases of the cell cycle. We show that entry of quiescent human epithelial cells into the cell cycle by serum stimulation induces an overall increase in *O*-GlcNAc level during G1 phase progression, followed by a marked decrease in *O*-GlcNAcylated proteins when cells enter S phase. We also show for the first time that the reduction of *O*-GlcNAcylation of proteins in early S phase is consistent with an increase in OGA activity at the G1/S transition. In addition, *O*-GlcNAc level changes are accompanied by an elevation of OGT and OGA protein expression as cells progress towards S phase. These results are in agreement with the increase in OGT protein and *O*-GlcNAc level previously observed in nocodazole-sychronized HeLa cells and mouse embryonic fibroblasts progressing into G1 phase [10,11]. Our data thus enable to supplement the current knowledge of the pivotal role of *O*-GlcNAc cycling during cell cycle progression [7–16]. In addition, we observe that elevation of *O*-GlcNAcylation by inhibiting OGA activity in G1 phase does not impair

Table 1
Identified *O*-GlcNAcylated proteins in the cytosolic fraction.

Protein name	Entry name	Accession number	MW/pI	Mascot score[a] (matched peptides)	Spot[b]	References[c]
Structural component and cytoskeleton						
Actin, cytoplasmic 1	ACTB	P60709	41 470/5.32	118 (5)	2, 3	[21,37–41]
Keratin, type 1 cytoskeletal 9	K1C9	P35527	62 027/5.14	141 (7)	1, 2, 5, 7, 8	–(*)
Keratin, type 1 cytoskeletal 14	K1C14	P02533	51 529/5.09	311 (9)	4, 5	–(*)
Keratin, type 1 cytoskeletal 16	K1C16	P08779	51 236/4.99	251 (8)	4, 7	–(*)
Keratin, type 1 cytoskeletal 17	K1C17	Q04695	48 076/4.97	209 (8)	4, 7	–(*)
Keratin, type 1 cytoskeletal 18	K1C18	P05783	48 029/5.04	171 (5)	1, 2, 3	[7,21,29,40,42,43]
Keratin, type 2 cytoskeletal 5	K2C5	P13647	62 340/7.59	306 (12)	4, 7	–(*)
Keratin, type 2 cytoskeletal 8	K2C8	P05787	53 671/5.52	521 (7)	1-8	[7,29,43]
Protein biosynthesis, folding, transport and turnover						
Eukaryotic initiation factor 4A-I	eIF4A-I	P60842	46 125/5.32	363 (13)	7, 8	[37,44]
Heat shock protein HSP60, mitochondrial	HSP60	P10809	61 016/5.70	414 (20)	19-22	[38]
Heat shock cognate 71 kDa	HSP7C	P11142	70 854/5.37	479 (21)	25, 26	[37,41,45,46]
Heat shock protein HSP 90-alpha	HS90A	P07900	85 006/4.94	1149 (61)	10-12	[37,41]
Heat shock protein HSP 90-beta	HS90B	P08238	83 554/4.97	1430 (73)	11, 12	[37,41]
Importin subunit alpha-7	IMA7	O60684	59 991/4.89	23 (3)	16	–(*)
Importin-5	IPO5	O00410	123 550/4.83	34 (2)	28	–(*)
Stress-70 protein, mitochondrial	GRP75	P38646	73 635/5.87	35 (2)	26	[37,46]
T-complex protein 1 subunit epsilon	TCPE	P48643	59 633/5.45	94 (12)	23, 24	–(*)
T-complex protein 1 subunit theta	TCPQ	P50990	59 583/5.42	127 (10)	23, 24	[37,39,41]
Ubiquitin carboxyl-terminal hydrolase 5	UBP5	P45974	95 725/4.91	33 (2)	13	[37]
Ubiquitin-like modifier-activating enzyme 1	UBA1	P22314	117 774/5.49	118 (4)	29	[37]
UV excision repair protein RAD23 homolog B	RD23B	P54727	43 145/4.79	87 (4)	14, 15	[39,47]
Valosin-containing Protein	VCP	P55072	89 300/5.14	174 (24)	27	[38]
mRNA processing						
Heterogeneous nuclear ribonucleoprotein K	HNRPK	P61978	50 944/5.39	184 (10)	17-19, 21	[21,37,40]
Heterogeneous nuclear ribonucleoprotein U	HNRPU	Q00839	90 528/5.76	37 (4)	30	[37,40,41]
Caprin-1	CAPR1	Q14444	78 489/5.14	100 (6)	12	[41]
Others						
UPF0364 protein C6orf211	CF211	Q9H993	51140/5.48	64 (4)	9	–

(*) Proteins from the same family of the candidate have been reported to be *O*-GlcNAcylated.
[a] The higher score obtained for each of these candidates is reported here.
[b] Spots are numbered according to Fig. 3B.
[c] References within protein candidates have been reported to be *O*-GlcNAcylated.

Table 2
Identified *O*-GlcNAcylated proteins in the nuclear fraction.

Protein name	Entry name	Accession number	MW / pI	Mascot score[a] (matched peptides)	Spot[b]	References[c]
Structural component and cytoskeleton						
Actin, cytoplasmic 1	ACTB	P60709	41 470/5.32	3446 (173)	1, 2	[21,37–41]
Alpha-actinin-1	ACTN1	P12814	102 993/5.25	40 (2)	29	[37]
Alpha-actinin-4	ACTN4	O43707	104 788/5.27	728 (30)	24, 25	[37]
Coronin-1A	COR1A	P31146	50 994/6.25	150 (9)	5, 6	–
Ezrin	EZR1	P15311	69 370/5.94	490 (24)	19, 20	[37]
Lamin-A/C	LMNA	P02545	74 095/6.57	803 (29)	18	[44]
Lamin-B1	LMNB1	P20700	66 368/5.32	264 (8)	1	[44,48]
Protein biosynthesis and folding						
Stress-70 protein, mitochondrial	GRP75	P38646	73 635/5.87	552 (23)	15, 16	[37,46]
Heat shock 70 kDa protein 1A/1B	HSP71	P08107	70 009/5.48	1471 (57)	13, 14	[21,41,42,45,46]
UV excision repair protein RAD23 homolog B	RD23B	P54727	43 145/4.79	135 (5)	10	[39,47]
Heat shock cognate 71 kDa	HSP7C	P11142	70 854/5.37	2283 (99)	11, 12	[37,41,45,46]
mRNA processing and transcription regulation						
ATP-dependent RNA helicase DDX39	DDX39	O00148	49 098/5.46	77 (3)	9, 23	–(*)
Cleavage and polyadenylation specificity factor subunit 3	CPSF3	Q9UKF6	77 436/5.37	33 (1)	23	–
Cleavage stimulation factor subunit 2	CSTF2	P33240	60 920/6.36	114 (4)	17	–
Heterogeneous nuclear ribonucleoprotein G	HNRPG	P38159	42 306/10.06	406 (15)	4	[39,41]
Heterogeneous nuclear ribonucleoprotein L	HNRPL	P14866	64 092/8.46	63 (1)	18	[38,41,46]
Heterogeneous nuclear ribonucleoprotein M	HNRPM	P52272	77 464/8.84	58 (2)	18	–(*)
Heterogeneous nuclear ribonucleoprotein U	HNRPU	Q00839	90 528/5.76	547 (23)	26-28, 30	[37,39–41,46]
HnRNP D0 = AU-rich element RNA-binding protein 1	HNRPD	O14979	38 410/7.62	80 (2)	3	[37]
Matrin-3	MATR3	P43243	94 565/5.96	164 (8)	26	–
Polypyrimidine tract-binding protein 1 (hnRNP I)	PTPB1	P26599	57 186/9.22	50 (1)	7	–
Probable ATP-dependent RNA helicase DDX17	DDX17	Q92841	72 326/8.82	181 (7)	18, 20	[46]
Probable ATP-dependent RNA helicase DDX5	DDX5	P17844	69 105/9.06	310 (13)	18	–(*)
Probable ATP-dependent RNA helicase DDX6	DDX6	P26196	54 382/8.85	51 (1)	7	[46]
Splicing factor 3A subunit 1	SF3A1	Q15459	88 831/5.15	367 (17)	26, 27	–
Transcription intermediary factor 1-beta	TIF1B	Q13263	88 493 /5.52	69 (1)	28	–
U4/U6 small nuclear ribonucleoprotein Prp31	PRP31	Q8WWY3	55 421/5.63	230 (10)	8, 9	–
116 kDa U5 small nuclear ribonucleoprotein component	EFTUD2	Q15029	109 366/4.84	196 (7)	28	–
DNA replication						
DNA replication licensing factor MCM2	MCM2	P49736	101 832/5.34	53 (1)	28	–(*)
DNA replication licensing factor MCM3	MCM3	P25205	90 924/5.53	424 (19)	24, 25	–(*)
DNA replication licensing factor MCM6	MCM6	Q14566	92 831/5.29	1065 (49)	24	[37]
DNA replication licensing factor MCM7	MCM7	P33993	81 257/6.08	1001 (37)	21, 22	[37]
X-ray repair cross-complementing protein 6	XRCC6	P12956	69 799/6.23	118 (4)	17	–(*)
Others						
Kinectin	KTN1	Q13263	156 179/5.52	39 (2)	29	–
Protein phosphatase 1 regulatory subunit 12A	MYPT1	O14974	115 211/5.31	38 (1)	29	[46,49]
Putative oxidoreductase GLYR1	GLYR1	Q49A26	60 518/9.27	64 (2)	18	–
Trifunctional enzyme subunit beta, mitochondrial:3-ketoacyl-CoA thiolase	TP-beta	P55084	51 262/9.45	105 (4)	3	–

(*) Proteins from the same family of the candidate have been reported to be *O*-GlcNAcylated.
[a] The higher score obtained for each of these candidates is reported here.
[b] Spots are numbered according to Fig. 3D.
[c] References within proteins from the same family of the candidate have been reported to be *O*-GlcNAcylated.

S-phase progression, consistent with previous observations using the potent OGA inhibitor GlcNAc-thiazoline [16]. Inhibition of OGA activity by Thiamet-G does not impair either the decrease in *O*-GlcNAc level observed in S phase entry. These data suggest that the difference in *O*-GlcNAc level between G1 and S phase is more important than the level of *O*-GlcNAc by itself. This idea is further supported by the fact that in response to pharmacological inhibition of OGA, it seems that G1 phase-cells attempt to thwart Thiamet-G effect and hold a dynamic of *O*-GlcNAc in favor of deglycosylation by maintaining a low level of OGT and increasing the amount of OGA protein, as previously observed in OGA inhibitor-treated cells [15,16].

The decrease in *O*-GlcNAc level in S phase entry is not related to protein degradation since inhibitors of lysosomal-, proteasomal- or protease-dependent degradation pathways did not prevent this reduction (data not shown). One possible explanation for the S-phase associated *O*-GlcNAc decrease is a switch in glucose metabolism to regulate energy production and precursor biosynthesis required for DNA duplication. This hypothesis is consistent with a recent work demonstrating that glycolysis is elevated in G1 phase and decreases during S phase [27]. Instead of being directed to glycolysis pathway, glucose-6-phosphate may be diverted into the pentose phosphate pathway to support the nucleotide biosynthesis needed for DNA replication. This may reduce the rate of the nucleotide-sugar UDP-GlcNAc, donor of the GlcNAc group, which is synthesized *via* the hexosamine biosynthetic pathway.

To get further insight into protein networks that are differentially *O*-GlcNAcylated during G1/S transition, we performed a 2-D electrophoresis-based proteomic study on nuclear and cytosolic fractions from quiescent, G1-, and S-phase MCF7 cells. This proteomic approach allowed the identification of 58 proteins that present putative cell cycle-dependent *O*-GlcNAc variations. These results give an overview of cellular processes regulated by *O*-GlcNAcylation during cell cycle progression, including mRNA processing, transcription regulation, protein biosynthesis and folding, and DNA replication. Although most of the identified proteins are known to be *O*-GlcNAcylated (see references in Tables 1–2), our work highlights their *O*-GlcNAcylation status in the biological context of cell cycle progression. More particularly, we report for the first time that *O*-GlcNAcylation of hnRNP K is transitory reduced in G1-phase cells compared with quiescent and S-phase cells, and that the level of *O*-GlcNAcylated Caprin-1 decreases as cells progress towards S phase. *In silico* prediction of *O*-GlcNAc sites using the YinOYang prediction server (*www.cbs.dtu.dk/services/YinOYang*) reveals that Caprin-1 possesses several

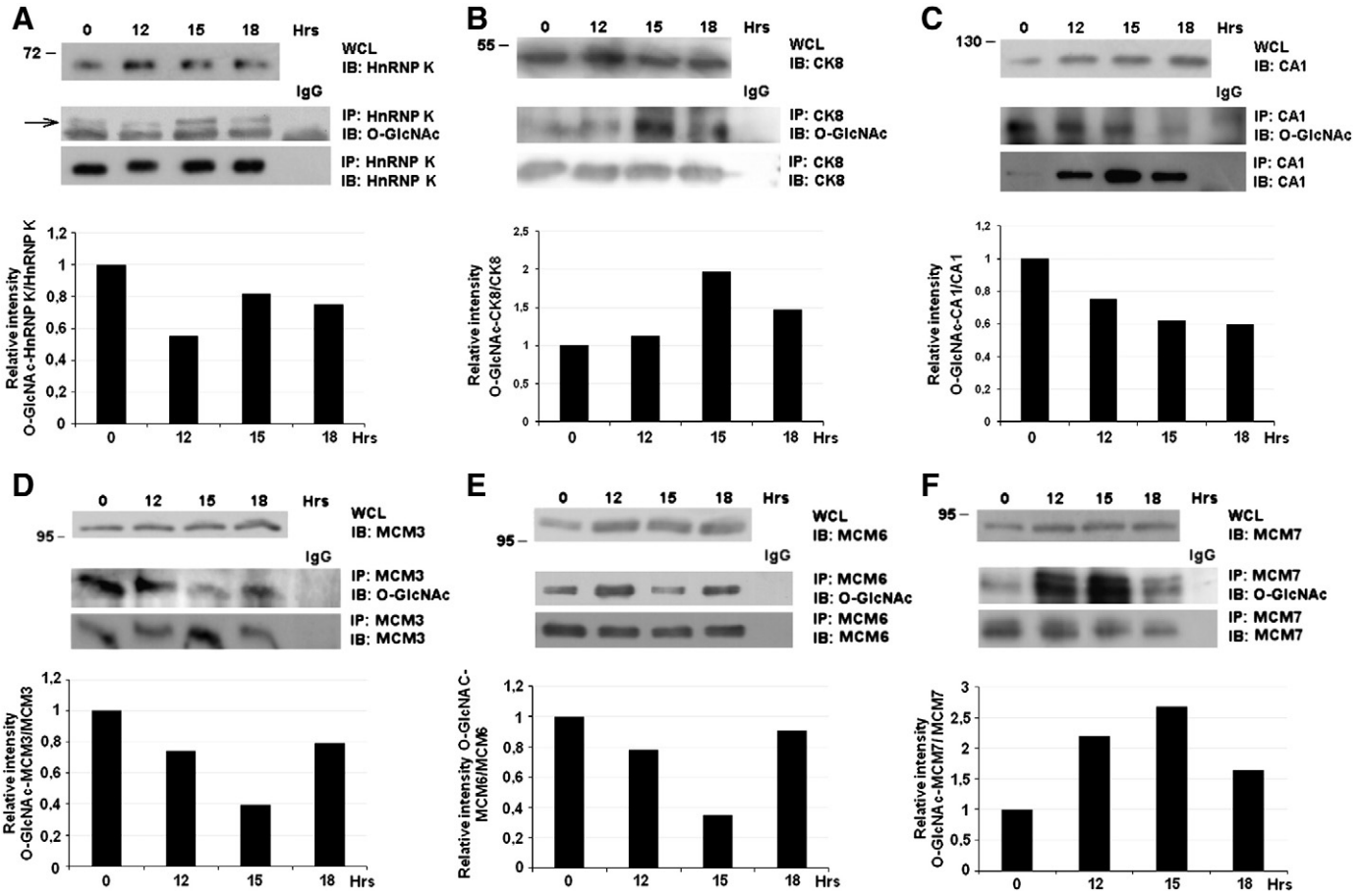

Fig. 4. Cell cycle-dependent *O*-GlcNAcylation of hnRNP K (A), CK8 (B), Caprin-1 (C), MCM3 (D), MCM6 (E) and MCM7 (F) was confirmed by immunoprecipitation (IP). MCF7 cells were synchronized in G0 by serum starvation and released into the cell cycle for the indicated times. Cell-cycle distribution was determined by flow cytometry analysis. Whole cell lysates (WCL) and immunoprecipitated candidate protein were revealed by immunoblotting (IB) using either RL2 antibody (*O*-GlcNAc) or protein-specific antibody. IB signals were quantified by densitometry. For each protein, the relative quantification of *O*-GlcNAc level, based on the ratio IB O-GlcNAc of IP Protein/IB Protein of WCL, is shown (*lower panel*). Results are representative of 3 independent experiments.

high-scoring potential *O*-GlcNAc sites, some of them being phosphorylated during mitosis [28]. Although results of such predictive tools should be taken with caution and have to be further confirmed by site mapping strategies, these data suggest that in addition to phosphorylation, the biological activity of Caprin-1 is regulated by *O*-GlcNAcylation.

In this study, *O*-GlcNAc cell cycle-dependent variations have also been found on several fundamental organizers of cellular architecture such as actin, cytokeratins, alpha-actinin 1, and lamin A/C. More particularly, we show that *O*-GlcNAcylation of CK8 peaks at S-phase entry. An elevation of *O*-GlcNAcylation of CK8/CK18 was previously observed in mitotic-arrested cells [29,30]. Moreover, an increase in phosphorylation of CK8/CK18 has been reported in S phase [31]. Taken together, these data support a role for *O*-GlcNAc and phosphorylation PTMs in the reorganization of intermediate filaments during cell cycle progression, probably by modulating solubility properties of keratin filaments, as previously demonstrated [31,32].

More importantly, we demonstrate for the first time that the nucleoplasmic DNA replication licensing factors MCM2, MCM3, MCM6 and MCM7 are *O*-GlcNAcylated in a cell cycle-dependent manner. Interestingly, although they interact together in the heterohexameric MCM2-7 complex, the *O*-GlcNAc variations observed in the first steps of the cell cycle differ between MCM7 protein, and MCM3 and MCM6 proteins. Whereas the functional impact of *O*-GlcNAcylation on MCM proteins is completely unknown, phosphorylation has been shown to regulate the activity of MCM2-7 complex, by modulating for instance the recruitment of MCM proteins and partners into the pre-replicative complex [33–35] or by regulating the helicase activity of the complex [36]. Further investigations are needed to localize the *O*-GlcNAc sites on MCM proteins in order to determine the functionality of *O*-GlcNAc residues and to examine whether *O*-GlcNAc modification could regulate the binding of MCM2-7 complex on chromatin.

To conclude, our findings show that there is a close link between the dynamics of *O*-GlcNAc and the progression of cells into the early steps of the cell cycle, through the regulation of OGA activity and protein levels of OGT and OGA. This study also provides a descriptive overview of differentially *O*-GlcNAcylated proteins at the G1/S boundary, highlighting the potential role of *O*-GlcNAcylation in key cellular processes that drive cells towards DNA synthesis. Further works on *O*-GlcNAc functions on modified proteins will provide a better understanding of the role of O-GlcNAcylation in the maintenance of genome integrity during DNA replication.

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Publication N°6

The FASEB Journal • Research Communication

Insulin signaling controls the expression of *O*-GlcNAc transferase and its interaction with lipid microdomains

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ABSTRACT **Lipid microdomains (rafts) are cholesterol-enriched dynamic ordered lipid domains belonging to cell membranes involved in diverse cellular functions, including signal transduction, membrane trafficking, and infection. Many studies have reported relationships between insulin signaling and lipid rafts. Likewise, links between insulin signaling and *O*-GlcNAcylation have also been described. However, the potential connection between *O*-GlcNAc and raft dynamics remains unexplored. Here we show that *O*-GlcNAc and the enzyme that creates this modification, *O*-GlcNAc transferase (OGT), are localized in rafts. On insulin stimulation, we observe time-dependent increases in OGT expression and localization within rafts. We show that these processes depend on activation of the phosphatidylinositol 3-kinase (PI3K) pathway. Inhibition of OGT does not significantly affect cholesterol synthesis and raft building but decreases insulin receptor expression and PI3K and mitogen-activated protein kinase pathway activation. Taken together, these findings indicate that *O*-GlcNAcylation, lipid rafts, and signaling pathways are spatiotemporally coordinated to enable fundamental cellular functions.—Perez-Cervera, Y., Dehennaut, V., Aquino Gil, M., Guedri, K., Solórzano Mata, C. J., Olivier-Van Stichelen, S., Michalski, J.-C., Foulquier, F., Lefebvre, T. Insulin signaling controls the expression of *O*-GlcNAc transferase and its interaction with lipid microdomains. *FASEB J.* 27, 3478–3486 (2013). www.fasebj.org**

Key Words: lipid rafts · OGT · PI3K pathway

Lipids were long considered as the fifth wheel among the biological molecule families. This concept was overturned in the early 1970s when Singer and Nicolson (1) proposed the fluid mosaic model, in which membranous components are spatiotemporally organized and wherein integral proteins diffuse laterally through the membrane. They showed that the lipid bilayer membrane is in a liquid state and that individual lipid molecules move laterally in the plane of the membrane. During the late 1980s, the discovery of lipid microdomains was another major breakthrough for the understanding of the biological roles of lipids in the field of cellular signaling. It was proposed that apical proteins are clustered together with lipids in the trans-Golgi network into microdomains enriched in glycosphingolipids (2). This proposal partly explained how apically transported proteins are segregated to the basolaterally resident compounds. The use of nonionic detergents enabled extraction of these lipid rafts and the analysis of their composition. Rafts are enriched in glycosphingolipids and cholesterol, which self-associate to form liquid-ordered domains and enable glycolipid-protein complexes to become detergent insoluble (3). Rafts have since been identified as crucial effectors of fundamental cell functions such as cell signaling (4). Numerous studies have reported the pivotal function of rafts in insulin signaling (5). It was observed 3 decades ago that insulin receptors in adipocytes are mostly

Abbreviations: Ac-5SGlcNAc, 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-5-thio-α-D-glucopyranose; CaMKIV, calcium/calmodulin-dependent kinase IV, Cdk1, cyclin-dependent kinase 1; CHAPSO, 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate; DRM, detergent-resistant membrane; FCS, fetal calf serum; MAPK, mitogen-activated protein kinase; MES, 4-morpholineethanesulfonic acid; OGA, *O*-GlcNAcase; OGT, *O*-GlcNAc transferase; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-triphosphate; PPO, PIP-binding activity of OGT; PUGNAc, *O*-(2-acetamido-2-deoxy-D-glucopyranosylidenamino) *N*-phenylcarbamate; siRNA, small interfering RNA; TBS, Tris-buffered saline

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clustered (6), whereas in hepatocytes they are evenly dispersed on the cell surface. In adipocytes, these receptors are sequestered in caveolar invaginations of the plasma membrane (7), whereas in hepatocytes, which lack caveolin, the main cholesterol-binding structural component of caveolae, the receptors, localized in lipid rafts only on stimulation by insulin (8). In any case, cholesterol is crucial for the activation of the insulin receptor and for downstream signaling. More precisely, in β-cells it has been shown that noncaveolar lipid rafts participate in insulin signaling and that, depending on the insulin receptor isoform (A- or B-type driving the expression of insulin or glucokinase, respectively), the activation occurred within distinct plasma membrane domains (9). Recently, it was suggested that insulin resistance might be the result of lipid microdomain disturbances. In 3T3-L1 adipocytes, an increase in ganglioside GM3 content correlates with a decrease in the insulin receptor content in lipid microdomains and leads to defective insulin signaling (10). Intriguingly, recent studies have proposed that *O*-GlcNAc transferase (OGT) is recruited to the plasma membrane through an interaction with phosphatidylinositol 3,4,5-triphosphate (PIP3) *via* a phosphoinositide-interaction domain of OGT [PIP-binding activity of OGT (PPO)] domain involved in the termination of the insulin transduction cascade (11). Similarly, Whelan *et al.* (12) observed that OGT relocalizes to the cytoplasm on insulin stimulation. At the molecular level, OGT interacts with the insulin receptor, which in turn tyrosine phosphorylates the enzyme, reinforcing the existence of a OGT pool close to the plasma membrane. In a second report, the same researchers (13) identified PDK1 as another interacting partner of OGT in response to insulin and showed that perturbation of *O*-GlcNAc cycling interferes with the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. These data strongly suggest that OGT actively participates in the regulation of cell signaling. The physical interaction between OGT and the plasma membrane led us to ask whether the glycosyltransferase may be recruited to lipid microdomains in response to insulin stimulation. Here we investigate the relationships between insulin signaling, raft formation, and the highly dynamic post-translational modification *O*-GlcNAcylation.

MATERIALS AND METHODS

Cell culture and treatments

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Cergy Pontoise, France) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 5 IU/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere enriched with 5% CO_2. Cells were cultured on dishes (100-mm diameter) preliminarily treated with 0.1% (w/v) porcine gelatin (Sigma-Aldrich, Lyon, France) as described previously (14). For starvation, cells were washed with 10 ml of phosphate buffered-saline (PBS), incubated in serum-free medium for 24 h, and then incubated with 100 nM human recombinant insulin (Life Technologies) for the indicated time periods.

For OGT silencing, HepG2 cells were reverse-transfected with Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's instructions using 10 nM small interfering RNA (siRNA) targeting OGT (15) or a control siRNA (Mission siRNA universal negative control no. 1; Sigma-Aldrich). After 24 h, cells were FCS-deprived for 48 h and then stimulated with insulin. For OGT inhibition experiments, cells were incubated with 100 μM 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-5-thio-α-D-glucopyranose (Ac-5SGlcNAc) 30 min before insulin stimulation.

Subcellular fractionation

HepG2 cells were fractionated using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions.

Preparation of detergent-resistant membrane (DRM) fractions

The procedure was adapted from a previously described protocol (16). In brief, cells were washed in cold PBS and lysed in 4-morpholineethanesulfonic acid (MES)-buffered saline (MBS; 25 mM MES and 150 mM NaCl, pH 6.5) containing either 1% (w/v) 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate (CHAPSO) or 1% (v/v) Triton X-100 (*t*-octylphenoxypolyethoxyethanol), 10 mM $MgCl_2$, 10 mM NaF, 2 mM Na_3VO_4, 1 mM EGTA, 5 mM dithiothreitol, and a cocktail of protease inhibitors. The lysate was passed 10 times through a 21-gauge needle fitted to a 2-ml syringe and incubated on ice for 30 min. An equal volume of the cell lysate was added to 90% (w/v) sucrose in MBS, and 2 ml of the mixture was placed in a 12-ml ultracentrifuge tube. A discontinuous sucrose gradient was formed on the top of the mixture by casting 6 ml of 35% (w/v) sucrose in MBS and then 4 ml of 5% (w/v) sucrose in MBS. The tubes were placed in a Beckman SW41 rotor (Beckman Coulter, Fullerton, CA, USA) and centrifuged for 23 h at 30,000 rpm (112,700 *g*) at 4°C. Twelve fractions of 1 ml each were collected from the top to the bottom and subsequently analyzed. Alternatively, samples were also concentrated by ethanol precipitation before analysis. The DRM fractions corresponding to lipid microdomains were identified by Western blot using an anti-flotillin-1 antibody.

Protein and cholesterol assays

Samples were assayed for their protein content using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and for their cholesterol content using the Amplex Red Cholesterol Assay Kit (Invitrogen, Carlsbad, CA, USA) and according to the manufacturer's recommendations.

SDS-PAGE, Western blotting, and antibody staining

Equal amounts of protein were subjected to Western blotting. Samples were analyzed by SDS-PAGE under reducing conditions, and proteins were electroblotted on a nitrocellulose sheet (GE Healthcare, Templemars, France). Equal loading was verified using Ponceau red staining. Membranes were first saturated for 45 min with 5% (w/v) non-fatty acid milk in Tris-buffered saline (TBS)-Tween buffer [15 mM Tris/HCl, 140 mM NaCl, and 0.05% (v/v) Tween 20, pH 8.0].

Antibodies used were the mouse monoclonal anti-*O*-GlcNAc RL2 (1:1000; Ozyme, Saint-Quentin en Yvelines, France), mouse monoclonal anti-CD147 (1:2000; BioLegend, Saint-

Quentin en Yvelines, France), rabbit polyclonal anti-OGT AL28 (1:2000; generously provided by G. W. Hart, Johns Hopkins University, Baltimore, MD, USA) and TI14 (1:2000; Sigma-Aldrich), rabbit polyclonal anti-insulin receptor chain β (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-GAPDH (1:5000; Abcam, Paris, France), rabbit polyclonal anti-flotillin-1 (1:4000; Sigma), mouse monoclonal anti-flotillin-2 (1:2000; Santa Cruz Biotechnology), rabbit monoclonal anti-phospho-Ser473-Akt and mouse monoclonal anti-Akt (1:2000; Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-tubulin (1:1000; Santa Cruz Biotechnology); rabbit polyclonal anti-histone 2B (1:5000; Millipore, Billerica, MA, USA), rabbit polyclonal anti-phospho-Thr202/Tyr204-Erk1/2 (1:1000; Cell Signaling Technology), mouse monoclonal anti-Erk2 (1:1000; Santa Cruz Biotechnology) or anti-Erk1/2 (1:5000; Cell Signaling Technology), chicken anti-*O*-GlcNAcase (OGA) 345 (1:4000; generously provided by G. W. Hart), and rabbit polyclonal anti-actin (1:10,000; Santa Cruz Biotechnology).

Membranes were incubated with the different antibodies overnight at 4°C and then were washed 3 times with TBS-Tween for 10 min and incubated with either an anti-rabbit, an anti-mouse (GE Healthcare), or an anti-chicken (Abcam) horseradish peroxidase-labeled secondary antibody at a dilution of 1:10,000 for 1 h. Finally, 3 washes of 10 min each were performed with TBS-Tween, and detection was performed with enhanced chemiluminescence (GE Healthcare).

Immunofluorescence microscopy

Cells were grown on glass coverslips for 24 h, washed once with PBS, and fixed by incubation for 25 min with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at room temperature. The coverslips were rinsed twice with 0.1 M glycine in PBS for 15 min. The fixed cells were incubated for 1 h with primary antibodies diluted in the blocking solution [0.1% (v/v) Triton X-100, 1% (w/v) bovine serum albumin, and 2% (v/v) normal goat serum in PBS]. After washing with PBS, Alexa 488- or Alexa 568-conjugated secondary antibodies (Molecular Probes, Villebon sur Yvette, France) diluted in the blocking solution were applied for 1 h. Immunostaining was detected through an inverted Leica TCS SP5 (Leica, Wetzlar, Germany) confocal microscope. Data were collected by using a Leica TCS SP5 confocal microscope and processed in Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

RESULTS

Insulin increases membrane-associated OGT levels

To investigate the effects of insulin stimulation on OGT and *O*-GlcNAcylation levels, HepG2 cells were starved for 24 h before stimulation with insulin for 8 h. Cellular fractionation was then performed, and cytosolic, membrane, and nuclear proteins were isolated and analyzed by Western blot (**Fig. 1**). The efficiency of fractionation was first checked using established fraction-specific protein markers: β-tubulin for the cytoplasmic fraction, CD147 for the membrane fraction, and H2B for the nuclear fraction. The distribution of OGT was estimated within each fraction and, interestingly, although no changes were detected in the nuclear fraction, a significant increase in OGT levels was observed in the membrane fraction after insulin stimulation. This result demonstrates that OGT is recruited to membranes

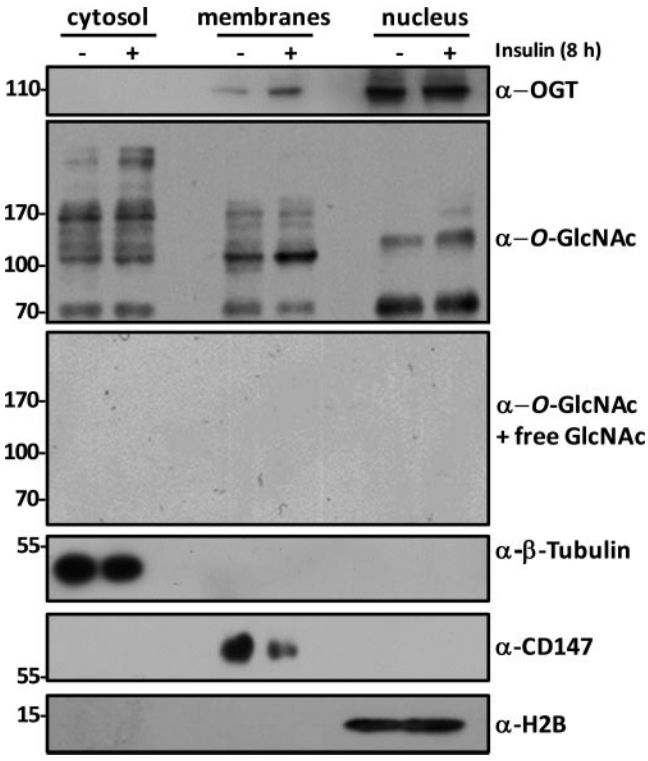

Figure 1. Insulin stimulates the localization of OGT to membranes. HepG2 cells were stimulated with insulin for 8 h and then were fractionated into cytosolic, membranous, and nuclear compartments. Efficiency of fractionation was evaluated by monitoring specific protein markers. Specificity of the anti-*O*-GlcNAc antibody was checked by coincubating it with 0.5 M free *N*-acetylglucosamine. Molecular mass markers are indicated on left (kDa).

after insulin stimulation, an observation in accordance with previous data (11). We next wondered whether the interaction between OGT and membranes was specifically localized to lipid microdomains.

O-GlcNAc and OGT are resident in rafts

The occurrence of *O*-GlcNAcylated proteins within lipid microdomains has never been investigated. To address this lack, we isolated DRMs from HepG2 cells using a buffer containing CHAPSO (see Materials and Methods for details) and fractionated them using discontinuous sucrose density gradient ultracentrifugation (16). Fractions were collected and probed using anti-flotillin-1 (an established protein-specific marker of rafts), anti-actin, anti-GAPDH, anti-OGT, and anti-*O*-GlcNAc antibodies (**Fig. 2*A***). In this way, we were able to detect the presence of OGT and *O*-GlcNAcylated proteins in fraction 5, corresponding to the lipid microdomain fraction. To check whether the interaction of OGT with lipid microdomains is detergent dependent, we compared the localization of the glycosyltransferase in rafts after extracting the cells with the anionic detergent CHAPSO and the nonionic detergent Triton X-100. After the ultracentrifugation step, the fractions collected were analyzed according to their cholesterol, protein, flotillin-

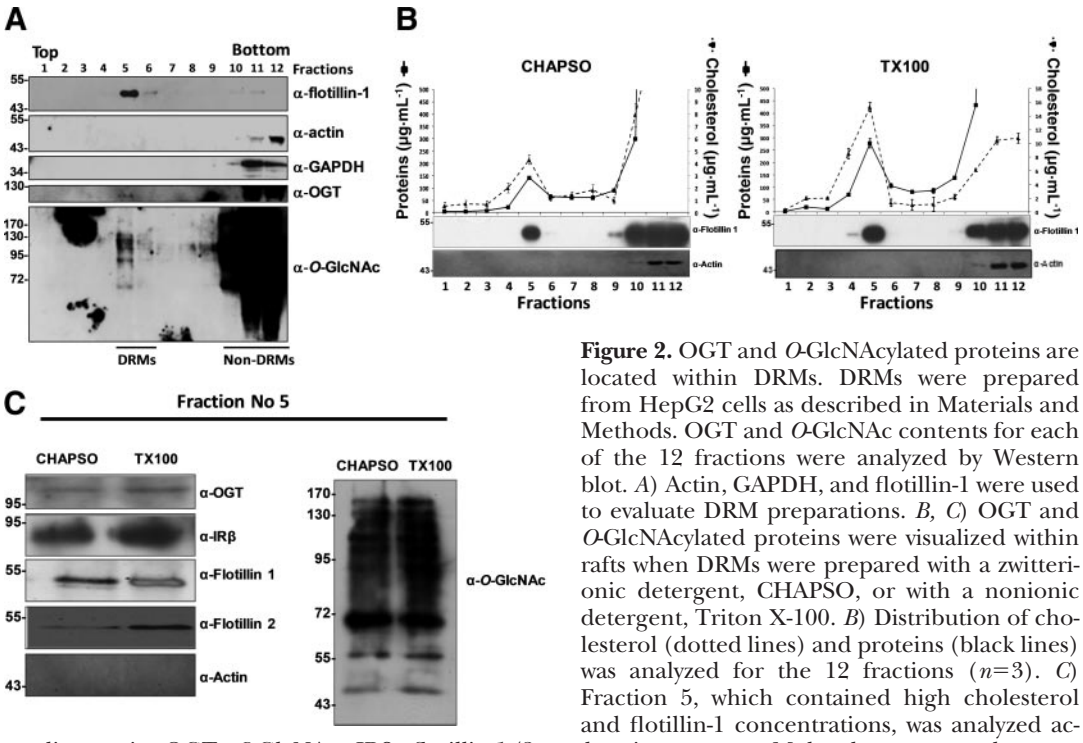

Figure 2. OGT and *O*-GlcNAcylated proteins are located within DRMs. DRMs were prepared from HepG2 cells as described in Materials and Methods. OGT and *O*-GlcNAc contents for each of the 12 fractions were analyzed by Western blot. *A*) Actin, GAPDH, and flotillin-1 were used to evaluate DRM preparations. *B, C*) OGT and *O*-GlcNAcylated proteins were visualized within rafts when DRMs were prepared with a zwitterionic detergent, CHAPSO, or with a nonionic detergent, Triton X-100. *B*) Distribution of cholesterol (dotted lines) and proteins (black lines) was analyzed for the 12 fractions (n=3). *C*) Fraction 5, which contained high cholesterol and flotillin-1 concentrations, was analyzed according to its OGT, *O*-GlcNAc, IRβ, flotillin-1/2, and actin contents. Molecular mass markers are indicated on left (kDa).

1, and actin contents (Fig. 2*B*). Fraction 5, corresponding to the highest cholesterol-enriched fraction, was analyzed using a panel of antibodies including anti-OGT and anti-*O*-GlcNAc (Fig. 2*C*). We found that DRMs contain both OGT and *O*-GlcNAcylated proteins independently of the detergent used.

Insulin stimulates the expression of OGT and promotes its targeting to rafts

Preliminary experiments performed on HepG2 cells demonstrated that in parallel to activation of insulin signaling-associated pathways, including the PI3K and mitogen-activated protein kinase (MAPK) pathways, the expression of OGT and its activity were enhanced (Supplemental Fig. S1), which is consistent with previous observations (11, 17). As depicted in Supplemental Fig. S1, although Akt and Erk are quickly activated in response to insulin (within a few minutes), OGT starts to increase later, between 15 min and 1 h, and *O*-GlcNAcylation only reaches a high level a few hours after hormonal stimulation. We next determined whether such a phenomenon occurs within DRMs (**Fig. 3**). First, cells were stimulated with insulin for 4 h, and levels of *O*-GlcNAcylation and OGT associated with the DRMs (Fig. 3*A*) were determined. We observed that in response to insulin, *O*-GlcNAc and OGT increase in both the DRMs and the non-DRM fractions. We then performed time-course experiments to evaluate the effect of insulin stimulation. Expression of OGT increases and is accompanied by a burst in *O*-GlcNAcylation (Fig. 3*B*, left panel); note that a slight increase in OGA is also observed. As expected and based on the above data (Fig. 3*B*, right panel), the *O*-GlcNAcylation level gradually increases in DRMs, and OGT significantly localizes to lipid microdomains in response to insulin. These experiments show that a pool of OGT is expressed and localized to DRMs in response to insulin stimulation. A control experiment showed that a total starvation time of 32 h did not increase the OGT level compared with that of cells starved for 24 h and stimulated with insulin for 8 h (data not shown).

Expression of OGT and its localization to DRMs is PI3K dependent

Because the expression of OGT and its recruitment to DRMs is mediated by the insulin-signaling cascade, we tested the effect of PI3K pathway inhibition on OGT expression and targeting to lipid microdomains (**Fig. 4**). We found that the PI3K inhibitor wortmannin abolishes the effect of insulin on OGT expression in whole-cell lysates (Fig. 4*A*), showing that the PI3K pathway contributes to the expression of OGT. Moreover, wortmannin also blocks insulin-mediated OGT targeting to the lipid microdomains (Fig. 4*B*). These results strongly suggest that expression and targeting of OGT to rafts are under the control of the PI3K pathway.

Effects of inhibiting PI3K on the localization of flotillin-2 and the insulin receptor

At 8 h after insulin stimulation, large clusters of insulin receptors colocalizing to the DRM-specific marker flotillin-2 were observed (**Fig. 5**). A previous study reporting a similar phenomenon showed that the human

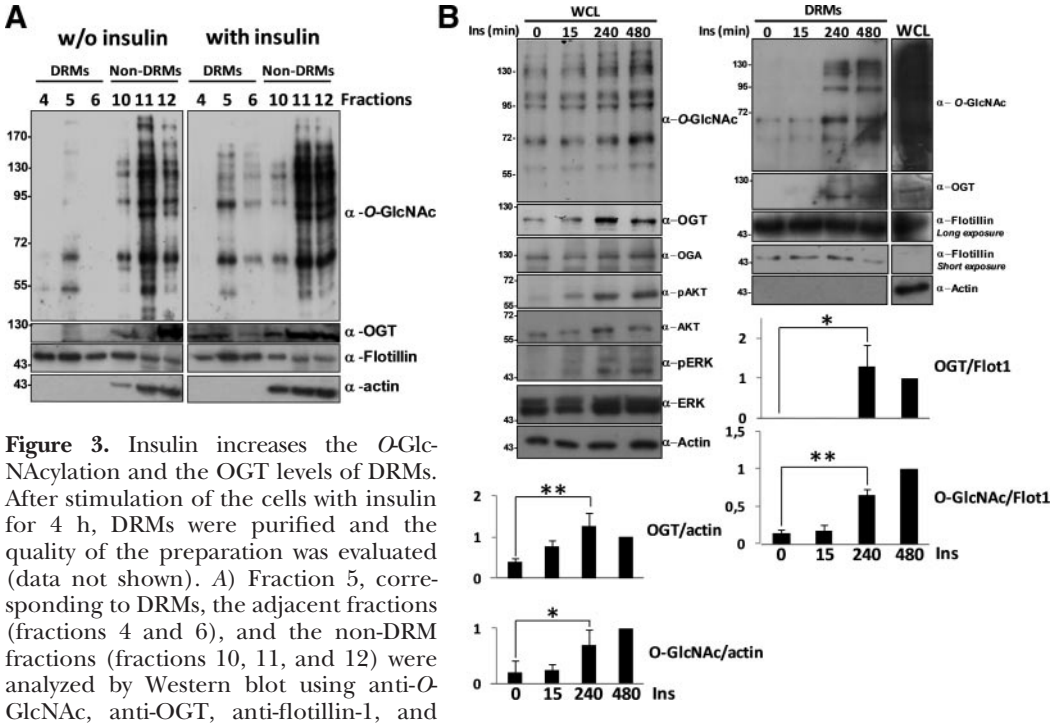

Figure 3. Insulin increases the *O*-GlcNAcylation and the OGT levels of DRMs. After stimulation of the cells with insulin for 4 h, DRMs were purified and the quality of the preparation was evaluated (data not shown). *A*) Fraction 5, corresponding to DRMs, the adjacent fractions (fractions 4 and 6), and the non-DRM fractions (fractions 10, 11, and 12) were analyzed by Western blot using anti-*O*-GlcNAc, anti-OGT, anti-flotillin-1, and anti-actin antibodies. *B*) Right panel: an insulin-stimulation time-course experiment was performed, and each DRM-containing fraction (fraction 5) was analyzed according to its *O*-GlcNAc and OGT content. Left panel: whole-cell lysates (WCL) were also analyzed by Western blot. Optical densities corresponding to OGT, *O*-GlcNAcylation, actin, and flotillin-1 (Flot1) bands (3 independent experiments) were measured and accordingly OGT/actin, *O*-GlcNAc/actin, OGT/flotillin-1, and *O*-GlcNAc/flotillin-1 ratios, respectively, were calculated for whole-cell lysates and DRMs. Values corresponding to time periods of 480 min were arbitrarily assigned to 1. Molecular mass markers are indicated on left (kDa). $*P < 0.05$; $**P < 0.01$.

hepatoma HuH7 cell insulin receptor is recruited to DRMs on insulin stimulation (8). To address whether this colocalization is dependent on the PI3K pathway, cells were first treated with wortmannin before insulin stimulation, and then colocalization of the 2 proteins was evaluated by confocal microscopy. We used lovastatin, a cholesterol biosynthesis inhibitor, as a control to check that our colocalization assay was correctly functional. As expected, we found that lovastatin treatment completely abolished the formation of flotillin-2/insulin receptor clusters, indicating that the assay reports on colocalization appropriately. Wortmannin treatment significantly relocalizes flotillin-2 and insulin receptor as a diffuse signal inside the cell, and only a few clusters persist. In contrast, on treating cells with U0126, an inhibitor of the MAPK pathway, we found only a slight diffuse intracellular relocalization of flotillin-2 and insulin receptor. This observation suggests that the formation of flotillin-2/insulin receptor clusters is probably dependent on the PI3K pathway.

OGT controls neither cholesterol synthesis nor raft assembly but its activity is necessary for IRβ expression and PI3K/MAPK pathway activation

To check whether OGT regulates raft building, we blocked OGT synthesis by using siRNA before insulin stimulation for 4 h. DRMs were purified, and cholesterol and proteins were assayed (**Fig. 6*A***). We showed that small interfering OGT (siOGT) does not prevent DRM-specific cholesterol and protein contents. Analysis of DRM fractions by Western blot indicates that OGT down-regulation does not affect raft building, as confirmed by the anti-flotillin-1 profile (Fig. 6*B*). Because we observed that on insulin stimulation OGT is expressed and targeted to rafts through activation of PI3K, we next wondered whether OGT in turn controls this pathway. We showed that OGT knockdown decreased phosphorylation of Akt and phosphorylation of Erk, the downstream effectors of the PI3K and MAPK pathways, respectively, in response to insulin (Fig. 6*C*). siOGT also dramatically reduces the expression of IRβ. This finding indicates that on insulin stimulation OGT activity is crucial for activation of the PI3K pathway and expression of IRβ. The same conclusions were reached by using Ac-5SGlcNAc (18), a potent OGT inhibitor (**Fig. 7** and Supplemental Fig. S2). Ac-5SGlcNAc prevents IRβ expression and reduces activation of PI3K and MAPK pathways in response to insulin (Fig. 7). On the other hand, OGT inhibition has no significant effect on the total cholesterol level or on DRM-specific cholesterol and protein contents (Supplemental Fig. S2*A*) and no affect on raft formation (Supplemental Fig. S2*B*).

Overall, these results indicate that OGT is expressed and targeted to lipid microdomains in response to

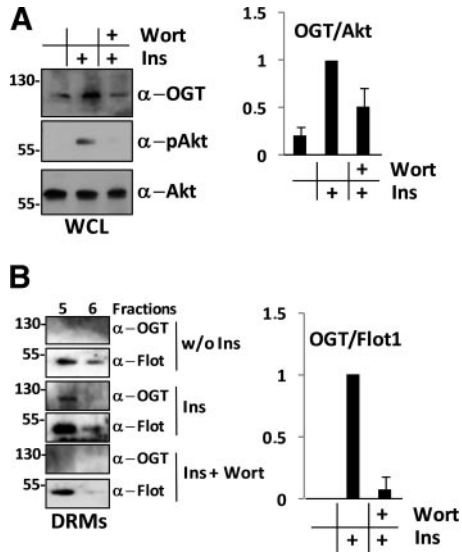

Figure 4. Insulin-stimulated expression and DRM localization of OGT are controlled by the PI3K pathway. HepG2 cells were treated with insulin (Ins) in conjunction with the PI3K inhibitor wortmannin (Wort) or vehicle. *A*) Whole-cell lysates (WCL) were analyzed by Western blot for their OGT content. Activation of the PI3K pathway was evaluated by measuring the phosphorylation of one of its downstream effectors, Akt. Optical densities corresponding to OGT and Akt bands (3 independent experiments) were measured, and, accordingly, OGT/Akt ratios were calculated ("insulin with vehicle" conditions were arbitrarily assigned the value of 1). *B*) Purification of DRMs was performed for each condition: starved cells, starved cells stimulated with insulin in conjunction with wortmannin or vehicle. Fractions 5 and 6 were evaluated using anti-OGT and anti-flotillin-1 (Flot) antibodies. Molecular mass markers are indicated on left (kDa).

insulin *via* the PI3K pathway. In turn, OGT potentiates PI3K activation and IRβ expression (**Fig. 8**).

DISCUSSION

In this work, we describe connections between the dynamics of lipid microdomains and *O*-GlcNAcylation in response to insulin stimulation. *O*-GlcNAc has received much attention in the past 10 yr (19) due in large part to commonalities between *O*-GlcNAcylation and phosphorylation. *O*-GlcNAc is much simpler than classic *N*- and *O*-glycosylation and is highly dynamic, making it able to control many fundamental cellular processes (20). Nevertheless, relatively few studies have described the regulation of signaling pathways by *O*-GlcNAcylation. Among these few examples, modification of the p65 subunit of NF-κB by *O*-GlcNAcylation on threonine 322 and 352 (21) is notable, although the functional role of *O*-GlcNAc on this transcription factor remains unclear. On the one hand, it was shown to decrease the interaction between NF-κB and the inhibitor IκBα, thus enhancing its activity; on the other hand, it was suggested that in cardiomyocytes *O*-GlcNAcylation leads to a decrease in the activation of the NF-κB pathway (22). With regard to the MAPK pathway, we previously demonstrated that inhibition of OGT in *Xenopus laevis* oocytes leads to its inactivation (23) and that an increase in the *O*-GlcNAcylation content results in its activation (24). We have also observed *O*-GlcNAcylation of Erk2, the downstream component of the MAPK pathway, but have not identified any functional role in this case (25). Other data strongly support the possibility that *O*-GlcNAcylation also participates in the regulation of calcium-dependent signaling (26, 27). In response to potassium, OGT is phosphorylated and activated by calcium/calmodulin-dependent kinase IV (CaMKIV) in a calcium-dependent manner (26). In turn, CaMKIV is *O*-GlcNAcylated in a region containing Thr200, phosphorylation of which activates the kinase activity (27). Activation of cells with ionomycin, which selectively induces calcium influx, decreases CaMKIV *O*-GlcNAcylation and increases Thr200 phosphorylation. These reports show that CaMKIV and OGT modify each other to control calcium-dependent signaling. Intriguingly, OGT seems to intervene in phosphorylation/dephosphorylation cascades that characterize many signaling pathways as

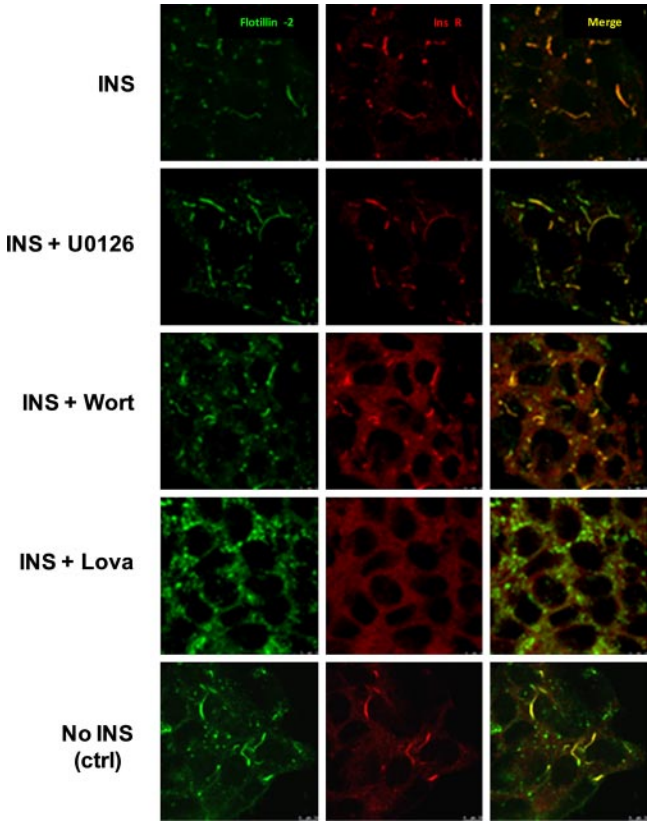

Figure 5. Localization of insulin receptor to flotillin-2 clusters depends on PI3K pathway. HepG2 cells were treated with insulin (INS) alone for 8 h or in conjunction with the MAPK inhibitor U0126, the PI3K inhibitor wortmannin (Wort), or the cholesterol synthesis inhibitor lovastatin (Lova). The negative control (ctrl), no insulin stimulation, is shown at the bottom. Cells were then incubated with specific antibodies against flotillin-2 and the insulin receptor, followed by incubation with fluorescent secondary antibodies. Green, flotillin-2; red, insulin receptor (Ins R); yellow, colocalization of flotillin-2 and the insulin receptor.

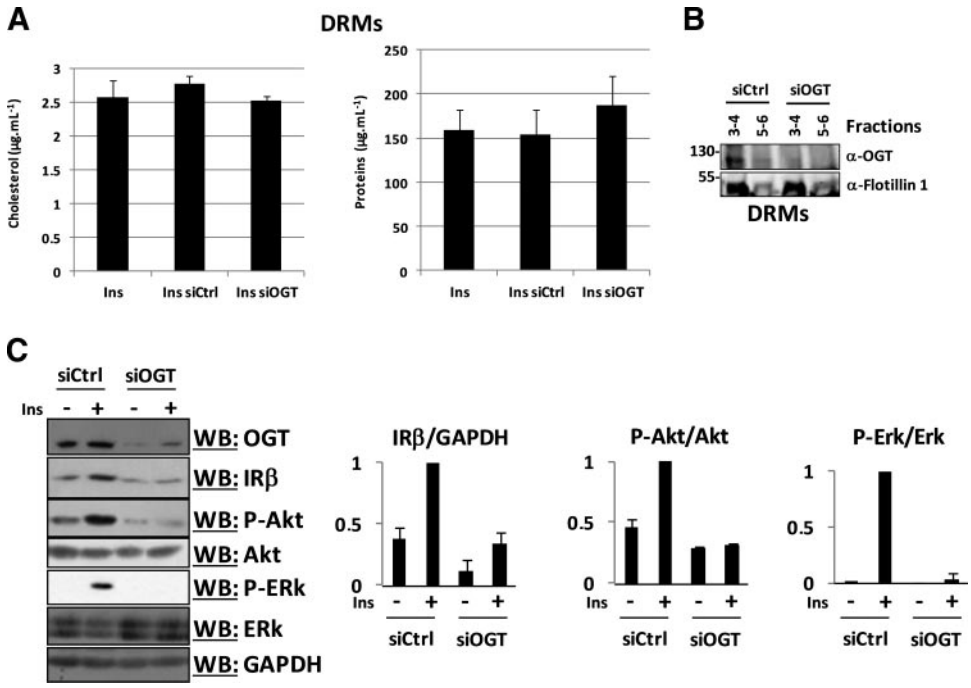

Figure 6. OGT is not necessary for cholesterol synthesis or for raft building but is required for IRβ expression. *A*) OGT was silenced by using siRNA. HepG2 cells were stimulated by insulin (Ins) for 4 h, and DRM cholesterol and protein contents were measured (n=3). *B*) DRM fractions were analyzed by Western blot with anti-OGT and an anti-flotillin-1 antibodies. *C*) Whole-cell lysates were analyzed by Western blot (WB) to control siOGT efficiency and to measure IRβ, P-Akt, Akt, P-Erk, Erk, and GAPDH expression. Optical densities of blots (n=3) were measured and accordingly IRβ/GAPDH, P-Akt/Akt, and P-Erk/Erk ratios were calculated and reported. Values corresponding to cells treated with small interfering control (siCtrl) and stimulated with insulin were arbitrarily assigned to 1.

proposed by Wang *et al.* (28). On OGT overexpression in HeLa cells, inhibiting phosphorylations of cyclin-dependent kinase 1 (Cdk1) on residues Thr14 and Tyr15 are increased. These modifications occur through activation of the kinase MYT1 and by decreasing the expression of both Cdk1 phosphatase cdc25c and Polo-like kinase 1, the latter being the upstream kinase that inhibits MYT1 and activates cdc25c. Such a detailed view of the regulation of the PI3K/Akt pathway has been also obtained. As an example, numerous components of the insulin-signaling pathway or proteins involved in the metabolism of glucose have been shown to be *O*-GlcNAcylated: Akt1 (29), Akt2 (30), GLUT1 (31), casein-kinase II, glycogen synthase kinase-3 (32), IRS1/2 (33), PDK1, the p110α unit of PI3K, and the β chain of the insulin receptor (11). Notably, because we show that decreasing OGT expression or inhibiting its catalytic activity reduces phosphorylation of Akt at Ser473, it was found that OGT knockdown also decreased Akt phosphorylation at Ser505 in *Drosophila*, a residue homologous to mammalian Akt Ser473 (34). In contrast, Kang *et al.* (35) showed that treatment of the insulinoma cell line INS1 and βTC6 cells with glucosamine reduces phosphorylation of Akt at Ser473. It is noteworthy that *O*-GlcNAcylation of keratins 8 and 18 is necessary for the activation of Akt1 by promoting the interaction between the intermediate filament and the kinase to protect the latter (36). It might be proposed that down-regulation of OGT reduces activation of Akt by reducing its interaction with

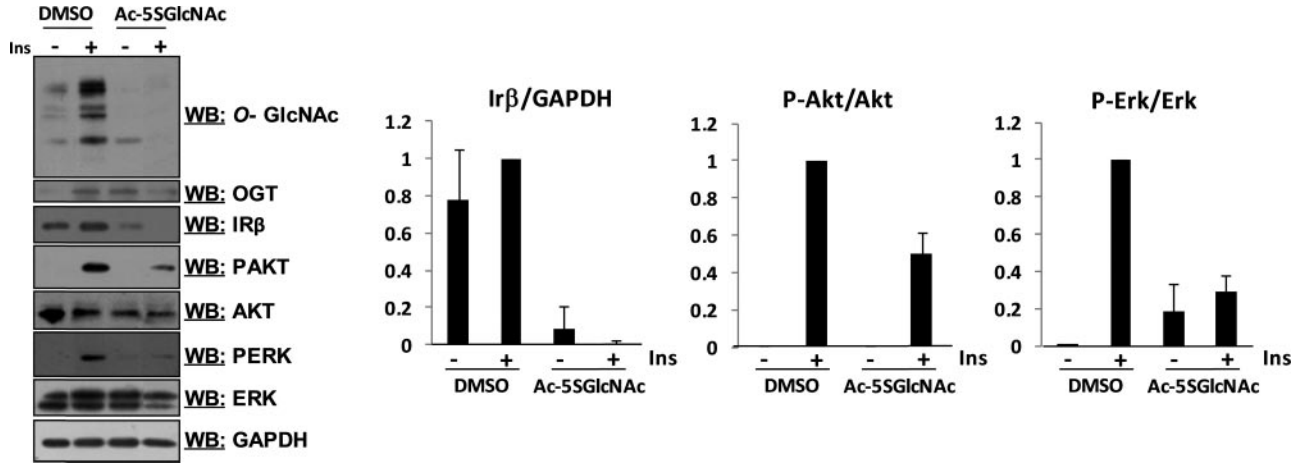

Figure 7. Blocking OGT inactivates PI3K and MAPK pathways. HepG2 cells were incubated with 100 μM Ac-5SGlcNAc 30 min before insulin stimulation for 4 h. Whole-cell lysates were analyzed by Western blot (WB) to control OGT inhibition by Ac-5SGlcNAc and to measure IRβ, P-Akt, Akt, P-Erk, Erk, and GAPDH expression. Optical densities of blots (n=3) were measured and accordingly IRβ/GAPDH, P-Akt/Akt and P-Erk/Erk ratios were calculated and reported. Values corresponding to cells treated with dimethyl sulfoxide (DMSO) and stimulated with insulin were arbitrarily assigned to 1.

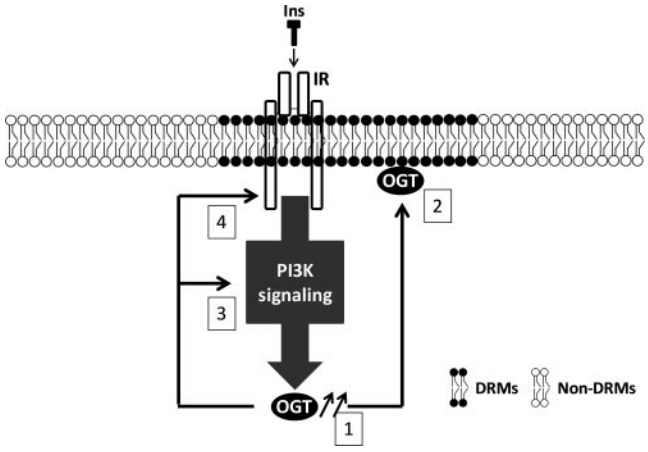

Figure 8. Insulin signaling potentiates OGT expression and localization to DRMs. On insulin stimulation, the OGT level is enhanced (*1*) and the glycosyltransferase localizes to lipid microdomains (*2*). OGT is essential for activation of the PI3K pathway (*3*) and IRβ expression on insulin stimulation (*4*).

keratins. An interaction of OGT with the insulin receptor and PDK1 has also been reported, and modification of the *O*-GlcNAcylation level perturbs insulin signaling (12, 13). Evans and colleagues (11) demonstrated that in response to insulin, OGT was recruited within 90 s to the inner face of the plasma membrane by PIP3 through a region adjacent to the catalytic domain II located at the carboxyl end of the transferase. Lysines 981 and 982 were identified as crucial for interacting with the acidic phosphate groups of the phosphoinositides through a newly described interaction domain of OGT named PPO, whereas another report indicated that mutations of 8 of the 10 lysines in the C-Cat domain did not affect OGT catalytic activity (37). In the same article, it was also reported that the exposure of 3T3-L1 adipocytes to high glucose concentrations or *O*-(2-acetamido-2-deoxy-D-glucopyranosylidenamino) *N*-phenylcarbamate (PUGNAc) decreased the phosphorylation of Akt at threonine 308, a known phosphorylated critical site. In contrast, phosphorylation of Erk1/2 in the MAPK pathway was unaffected. The phosphorylation of IRS1 was also modified under these conditions, because the treatment of adipocytes with PUGNAc or the adenoviral infection of adipocytes and Fao hepatoma cells with Ad-OGT increased the phosphorylation of IRS1 at Ser307 and Ser632/635, 3 sites that downregulate the insulin signaling pathway. Taken as a whole, this article demonstrates that OGT can perturb the insulin-signaling cascade by interacting with PIP3 at the plasma membrane. OGT modifies numerous components of the insulin-signaling pathway, lowering the response to insulin and, thus, in all likelihood, participating in the physiopathology of insulin resistance. However, intriguingly, it was found that individuals with Alzheimer's disease and type 2 diabetes have a decrease in activation of the PI3K pathway that correlates with a general decrease in *O*-GlcNAcylation (38). In this way and as previously shown for serum-stimulated MCF7 cells (39), we found in this study that OGT is necessary for activation of both PI3K and MAPK pathways in response to insulin and that it is also required for expression of the hormonal receptor. This finding raises the question of whether a low *O*-GlcNAcylation level is responsible for insulin resistance, Other data indicate that OGT silencing or overexpression (40) or inhibition (41) of OGA does not prevent or trigger insulin resistance in 3T3-L1 adipocytes. Quite obviously, apprehension of the regulation of signaling pathways by *O*-GlcNAcylation, especially in multifactorial pathological conditions such as type 2 diabetes and neurodegeneration, needs to be deepened and clarified.

Based on our findings, we propose that cells control their signaling pathways by increasing OGT levels and targeting the enzyme to rafts through activation of the PI3K pathway (Fig. 8). This ubiquitous mechanism may constitute a common regulatory system found in animal cells in response to external stimuli. One can expect that deregulation of this process could have dramatic effects on the etiology of diseases such as cancer, diabetes, and senile dementia. FJ

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Review

Dysregulation of the nutrient/stress sensor *O*-GlcNAcylation is involved in the etiology of cardiovascular disorders, type-2 diabetes and Alzheimer's disease

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ABSTRACT

O-GlcNAcylation is widespread within the cytosolic and nuclear compartments of cells. This post-translational modification is likely an indicator of good health since its intracellular level correlates with the availability of extracellular glucose. Apart from its status as a nutrient sensor, *O*-GlcNAcylation may also act as a stress sensor since it exerts its fundamental effects in response to stress. Several studies report that the cell quickly responds to an insult by elevating *O*-GlcNAcylation levels and by unmasking a newly described Hsp70–GlcNAc binding property. From a more practical point of view, it has been shown that *O*-GlcNAcylation impairments contribute to the etiology of cardiovascular diseases, type-2 diabetes and Alzheimer's disease (AD), three illnesses common in occidental societies. Many studies have demonstrated that *O*-GlcNAcylation operates as a powerful cardioprotector and that by raising *O*-GlcNAcylation levels, the organism more successfully resists trauma–hemorrhage and ischemia/reperfusion injury. Recent data have also shown that insulin resistance and, more broadly, type-2 diabetes can be controlled by *O*-GlcNAcylation of the insulin pathway and *O*-GlcNAcylation of the gluconeogenesis transcription factors FoxO1 and CRCT2. Lastly, the finding that AD may correspond to a type-3 diabetes offers new perspectives into the knowledge of the neuropathology and into the search for new therapeutic avenues.

1. Characteristics of *O*-GlcNAcylation

1.1. O-GlcNAcylation differs from other types of glycosylation

Glycosylation refers to a group of post-translational modifications (PTMs) that alter more than 50% of human proteins. The finding that 2%–4% of the genome encodes proteins involved in glycosylation processes underlines the fundamental role of these modifications. Among the types of glycosylation described so far, *O*-GlcNAcylation occurs on nucleocytoplasmic [1] and mitochondrial [2] proteins and consists of the addition of a single residue of *N*-acetylglucosamine to the hydroxyl group of serine and threonine. This *N*-acetylglucosaminyl moiety is neither epimerized nor elongated. Hundreds of proteins have been described so far as being *O*-GlcNAcylated, including cell signaling proteins such as the insulin receptor and its receptor substrates [3], the MAP kinase erk2 [4] and prohibitin [5], cell cycle regulators such as Myc [6] and beta-catenin [7], and several structural proteins such as vimentin [8] and actin [4]. *O*-GlcNAcylation was first demonstrated 25 years ago [9] and has been the subject of intensive investigation ever since. The discovery of *O*-GlcNAc groups constituted a major breakthrough in the field of glycobiology, in that it challenged two dogmas of our understanding of glycosylation: for the first time, a kind of glycosylation was found to occur at high levels in the cytosolic and nuclear compartments of eukaryotes, whereas glycosylation had until then been considered to be confined to the lumen of the endoplasmic *reticulum* and Golgi apparatus, to membranous proteins and to the secretory pathway; secondly, the versatility of *O*-GlcNAcylation demonstrated that glycosylation was not always static (as for *N*-glycans or the classic *O*-glycans). The discovery of *O*-GlcNAcylation led a new era in the world of PTM since it was then accepted that nucleocytoplasmic proteins possessed their own glycosylation.

1.2. O-GlcNAcylation is versatile, polyvalent and its level depends upon glucose availability

As mentioned above, one of the main features of *O*-GlcNAcylation is its versatility, and for this reason it is often compared to phosphorylation [10]. However, unlike phosphorylation–dephosphorylation processes, which are regulated by a set of perhaps a thousand kinases (the kinome) and fewer than two hundred phosphatases, the *O*-GlcNAcylation/de-*O*-GlcNAcylation process is only controlled by a few forms of two cytosolic and nuclear enzymes

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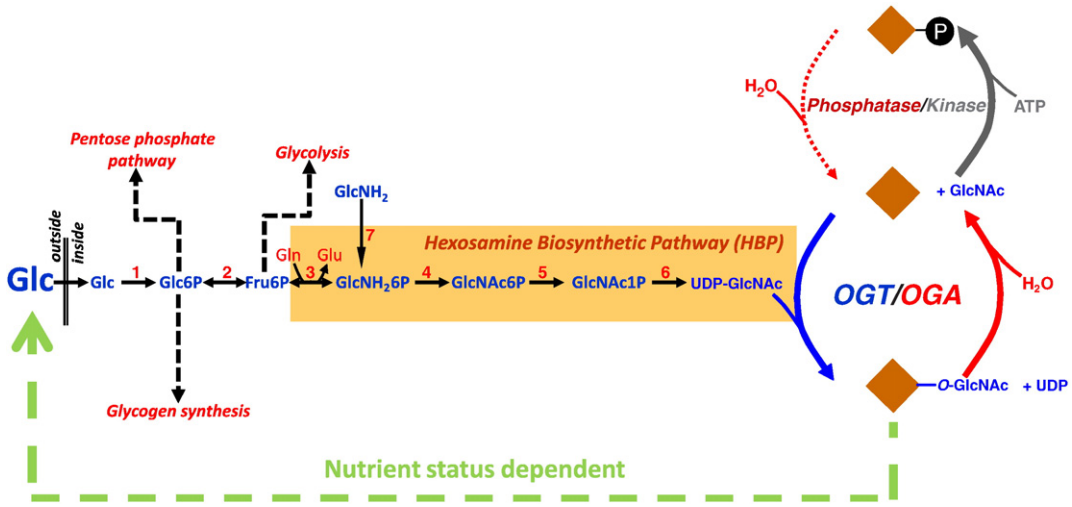

Fig. 1. The *O*-GlcNAcylation processes are dependent upon the nutrient status of the cell and are controlled by two enzymes: OGT and OGA. *O*-GlcNAcylation dynamics is managed by the unique glycosyltransferase OGT that transfers the GlcNAc moiety from UDP-GlcNAc to target proteins, and by the glycosidase OGA that hydrolyses the glycosidic bond. *O*-GlcNAcylation can compete with phosphorylation either at the same site or at an adjacent one. UDP-GlcNAc is the end-product of the hexosamine biosynthetic pathway, which flux is controlled by GFAT. The level of UDP-GlcNAc, and consequently of *O*-GlcNAcylation, is tightly dependent upon the nutrient status of the cell (green dashed arrow). **1**, hexokinase; **2**, phosphoglucose isomerase; **3**, glutamine:fructose-6-phosphate amido transferase (GFAT); **4**, glucosamine-6-phosphate acetyl transferase; **5**, phospho-*N*-acetylglucosamine mutase; **6**, uridine di-phospho-*N*-acetylglucosamine pyrophosphorylase; **7**, glucosamine kinase; **Fru6P**, fructose-6-phosphate; **Glc**, glucose; **Glc6P**, glucose-6-phosphate; **GlcNAc**, *N*-acetylglucosamine; **$GlcNH_2$6P**, glucosamine-6-phosphate; GlcNAc6P, *N*-acetylglucosamine-6-phosphate; **GlcNAc1P**, *N*-acetylglucosamine-1-phosphate; **Gln**, glutamine; **Glu**, glutamate; **OGA**, *O*-GlcNAcase (*O*-linked *N*-acetylglucosaminidase); **OGT**, *O*-GlcNAc transferase (*O*-linked *N*-acetylglucosaminyl transferase); **UDP**, uridine di-phosphate; **UDP-GlcNAc**, uridine di-phospho-*N*-acetylglucosamine.

[11]: OGT or *O*-linked *N*-acetylglucosamine transferase (E.C. 2.4.1.94), which transfers the monosaccharide from UDP-GlcNAc to the protein, and OGA or *O*-linked-*N*-acetylglucosaminidase or *O*-GlcNAcase (E.C. 3.2.1.52), which removes the GlcNAc moiety (Fig. 1). Three forms have been described for the former: the 110 kDa ncOGT (nucleocytoplasmic OGT), the 78 kDa sOGT (short OGT) and the 103 kDa mOGT (mitochondrial OGT) [12]. Two forms have been described for OGA [13]: a 130 kDa long form with two distinct activities, an N-terminal *O*-GlcNAcase activity and a C-terminal putative histone acetyl transferase activity, and a 75 kDa short form which lacks the second activity. In addition to being highly dynamic, *O*-GlcNAcylation displays the unique characteristic of competing with phosphorylation at the same site or in its vicinity, leading to mutual exclusion [14,15]. Consequently, owing to the physical differences created by the two types of PTM, this reciprocal relationship may result in behavioral differences in the modified protein: the neutrality of the GlcNAc moiety is in striking contrast to that of phosphate acidity, with the first remaining uncharged regardless of pH, whereas the second is negatively charged at physiological pH (conformational differences in the protein breed an assembly with distinct partners and then the activity is modified). Beyond the competition between *O*-GlcNAc and phosphate, the role played by *O*-GlcNAcylation is still unclear, in spite of abundant investigation. Nevertheless, it appears that *O*-GlcNAcylation plays a pivotal role in many fundamental cellular processes such as transcription [16], translation [17], cell signaling [18], cell trafficking [19,20], cell cycle control [8,21–23] and development

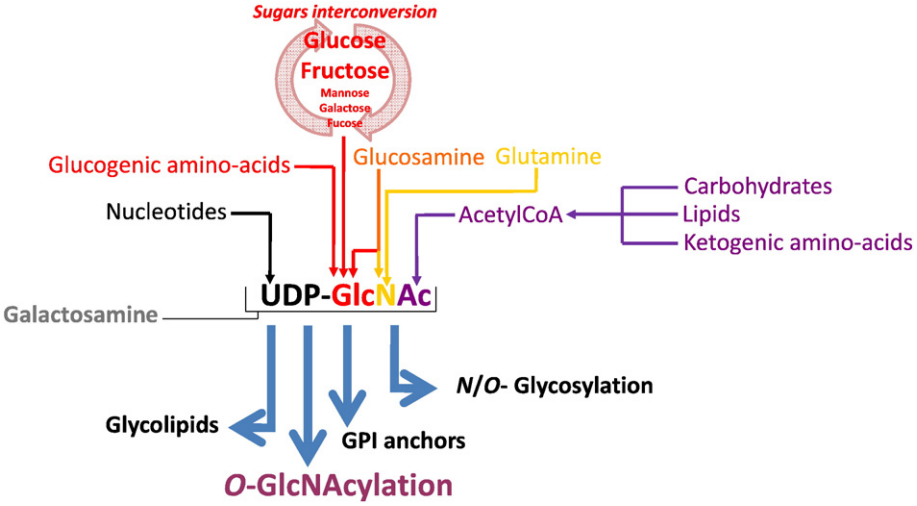

Fig. 2. UDP-GlcNAc is at the crossroad of many metabolisms. The level of UDP-GlcNAc is tightly correlated to the nutrient status of the cell, since many catabolic pathways converge on the nucleotide-sugar: nucleotides supply the UDP part of the molecule; the glucogenic amino acids and many carbohydrates, through interconversion reactions, contribute the carbohydrate backbone of UDP-GlcNAc; glucosamine directly participates in the elaboration of the glucosaminyl moiety (bypassing GFAT-mediated control); the amino group is donated by glutamine through the activity of GFAT; acetyl-coenzyme A (AcetylCoA) is provided by the beta-oxidation of fatty acids, by glycolysis and by metabolism of the ketogenic amino acids. Galactosamine can also quickly provide UDP-GlcNAc (interconversion of UDP-GalNAc and UDP-GlcNAc by 4-epimerase). UDP-GlcNAc is the donor of the GlcNAc group for many glycosylation processes.

[24,25], although each function controlled by *O*-GlcNAcylation remains to be more precisely defined and documented, and only general conclusions can be drawn concerning its impact.

Over the last few years, the concept that *O*-GlcNAcylation is a nutrient sensor has emerged [26,27]. The level of *O*-GlcNAcylation is tightly correlated to the concentration of UDP-GlcNAc, the end-product of the hexosamine biosynthetic pathway (HBP) (Fig. 1), which is itself dependent upon the availability of glucose (see also Section 4.2 of this review). The status of UDP-GlcNAc is virtually unique in the cell, since this nucleotide–sugar is at the crossroads of almost all metabolic pathways (Fig. 2): the "carbohydrate" skeleton of UDP-GlcNAc comes from glucose and other carbohydrates (through sugar interconversion reactions) and from glucogenic amino acids (all amino acids except leucine) through gluconeogenesis; the acetyl group is provided by lipids (β-oxidation of fatty acids), ketogenic amino acids (Leu, Lys, Phe, Tyr and Trp) and carbohydrates; UDP is provided by nucleotides and glutamine supplies the amine group. In other words, it can be assumed that an organism that is well fed, that possesses the right metabolism and therefore is healthy will undertake enough *O*-GlcNAcylation for all its cellular functions. Because of its nutrient sensor status, *O*-GlcNAcylation is likely to be the stress-related PTM, which may explain why the dysregulation of *O*-GlcNAcylation levels leads to cardiovascular disorders, type-2 diabetes and Alzheimer's disease.

2. *O*-GlcNAcylation and cell injury

2.1. O-GlcNAcylation levels are modified in response to stress

It has been observed that the cell rapidly changes its *O*-GlcNAcylation levels in response to stress [28–33] (Fig. 3). These fluctuations in *O*-GlcNAcylation are stress-, cell-, time- and dose-dependent. As an example, thermal stress provokes an increase in *O*-GlcNAcylation in the cell lines Cos7 [28], CHO, Hep3b [29] and HepG2 [33]. The burst of *O*-GlcNAcylation occurs very quickly, since it is observed a mere 5 minutes after heat shock in HepG2 [33] and around 15 minutes afterwards in other cells types [28,29]. Observations over longer periods have shown that when cells are heated for only 1 hour and then returned to 37 °C [28], *O*-GlcNAcylation continues to increase for a few hours and finally slowly decreases to reach its initial level, whereas *O*-GlcNAcylation is decreased to below its initial level when the heat shock is constantly applied to the cells [32]. The deletion of OGT using Cre–Lox recombination [28] and the down-regulation of OGT expression using RNA interference [28,33] have shown that the glycosyltransferase is needed for cells to resist thermal stress; OGT RNAi halves the thermotolerance of the cell. Like heat shock proteins, OGT responds to stress by increasing its activity [28], and it has been proposed that OGT can adopt a molecular chaperone function in response to heat stress [29]. In this context, it has been shown that in stably transfected HeLa cells, OGT possesses a particularly long half-life of 12–13 hours [34] that is higher than that of Hsp70 itself: 7 hours under heat shock conditions and as low as 2 hours under normal conditions [35]. This long OGT half-life may explain why in HeLa cells, RNAi exposure needs to take place for 96 hours (around 8 OGT half-lives) in order to lead to a decrease in endogenous OGT expression [33]. Note that other studies have shown that shorter exposure times of cells to OGT RNAi are sufficient for a decrease in OGT expression. As an example, the use of OGT RNAi for 36 hours in neonatal rat cardiac myocytes reduces OGT protein levels by half [36]. These observations suggest that OGT stability depends upon the cell line used. This characteristic of OGT is intriguing because it shows that the enzyme is particularly stable and suggests that it is less likely to be regulated by its turnover (synthesis–degradation) than by PTM and by interaction with partners such as OIP106, MYPT1, CARM1 [37], the stress–response protein p38MAPK [38], protein phosphatase 1 [39] and OGA [40]. Surprisingly, not all partners of OGT are *O*-GlcNAcylated proteins. For example, during glucose deprivation, OGT interacts with p38MAPK in the absence of either the *O*-GlcNAcylation of p38MAPK by OGT, or the phosphorylation of OGT by the kinase [38]. One can suppose that

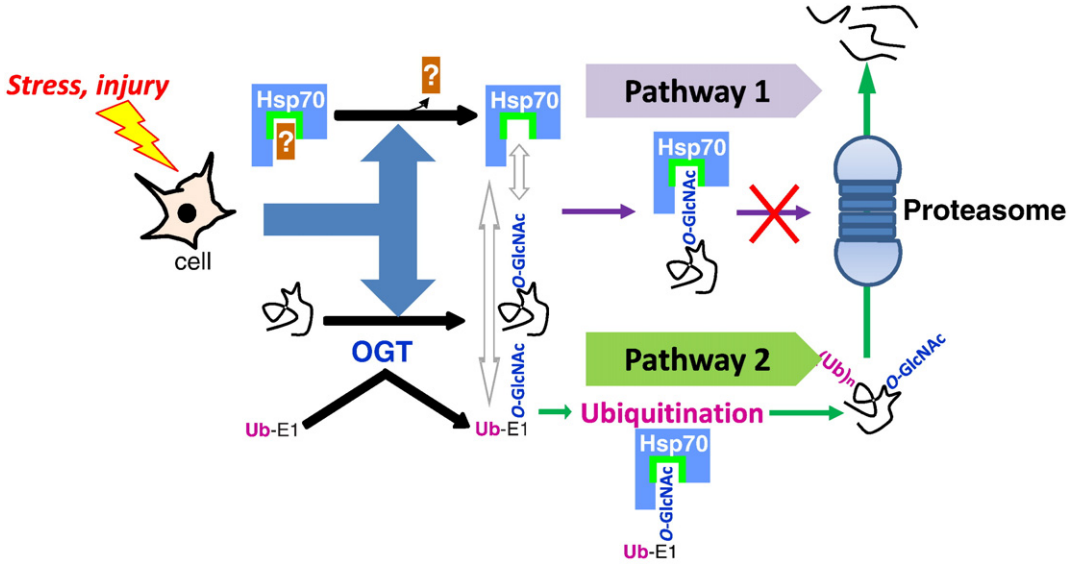

Fig. 3. Stress/injury modifies the cellular *O*-GlcNAcylation level and unmasks the *O*-GlcNAc binding property of Hsp70. It has been shown that the cell quickly elevates its *O*-GlcNAcylation levels and triggers its HGBA in response to stress. When stressed, the hydrophobic segments of proteins may become exposed on the surface. This conformational change leads to protein aggregation, and can provoke cell toxicity. It has been proposed that OGT functions as a chaperone by recognizing the exposed hydrophobic segments and by transferring a GlcNAc group to them; this could transiently increase the solubility of the protein, avoiding its aggregation. In parallel with the elevation of *O*-GlcNAcylation, the HGBA is triggered, possibly by the release of a putative factor (marked by a "?") that under physiological conditions masks the lectin region of Hsp70. Through their HGBA, Hsp70 and the *O*-GlcNAcylated proteins can interact by forming a complex that also decreases the insolubility of the denatured proteins and their propensity to aggregate, and similar to the glucosylation-deglucosylation cycle found within the endoplasmic *reticulum*, Hsp70 attempt to correctly refold the proteins, thus allowing them to temporarily escape proteasomal degradation (pathway 1). It has been also shown that the ubiquitination process is in part under the control of *O*-GlcNAcylation. This regulation may be controlled by the interaction between Hsp70 and the ubiquitin-activating enzyme E1 in a lectin/*O*-GlcNAc recognition dependent manner (pathway 2). If the refolding process carried out by Hsp70 fails (pathway 1), the *O*-GlcNAcylated protein is secondarily modified by poly-ubiquitination and destroyed by the proteasome (pathway 2).

OGT regulates many functions inside the cell not only by *O*-GlcNAcylating its substrates but also by merely interacting with its partners.

2.2. The paradox of *O*-GlcNAcylation

Like ATP, UDP-GlcNAc is an abundant nucleotide found within the cell. As previously explained, since UDP-GlcNAc is the end product of the HBP, its level is dependent upon the concentration of glucose. It has been observed that cells express high levels of *O*-GlcNAcylation when cultured with high concentrations of glucose [31,41,42], and conversely, cells decrease their *O*-GlcNAcylation levels when incubated in glucose-poor media [30,31,43]. Contrarily, observations of an increase in *O*-GlcNAcylation status in glucose-deprived conditions have been also reported [32,38,44,45]. For example, in a recent study undertaken by our group [32], glucose deprivation experiments led to a decrease in *O*-GlcNAcylation in HepG2 cells, as has been previously shown [30,31], but to an increase in HeLa cells. To explain this paradoxical finding, it has been proposed that following glucose deprivation, OGT expression is increased [38,44] while OGA expression is diminished [44]. Cheung and Hart [38] have more precisely shown that the increase in OGT transcripts and protein expression in Neuro-2a cells is induced by the decrease in glucose directly, but that the increase in OGT activity is due to its interaction with p38MAPK. These observations indicate that glucose deprivation elevates *O*-GlcNAcylation in some cell lines by modulating OGT at its transcriptional, translational and enzymatic activity levels. Recently, Taylor et al. [45] have proposed that the increase in OGT mRNA and the subsequent burst of *O*-GlcNAcylation induced by glucose deprivation is mediated by a decrease in the flux through the HBP and a down-regulation of the *O*-GlcNAcylation of proteins. What we cannot explain, however, are the discrepancies between our studies and those of Taylor et al. with respect to the *O*-GlcNAcylation status of glucose-deprived HepG2 cells. One could argue that the strain of HepG2 used was different or that the experimental protocol for glucose deprivation was not the same. For example, Taylor et al. supplemented their glucose-free DMEM with 1% fetal calf serum (FCS), whereas we did not, although serum is essential for entry into the cell cycle. Numerous studies have demonstrated that *O*-GlcNAcylation dynamics is crucial for cells to progress correctly through the cell cycle [4,8,21–23]. The discrepancies observed between the two sets of experiments may be explained by the fact that the cells were in the G_0 phase in our studies, whereas the cells progressed asynchronously through the cell cycle in that of Taylor et al. It could also be the case that growth factors potentiated the HBP-mediated OGT increase in the study of Taylor et al.

2.3. Hsp70 displays a GlcNAc binding property

Starting from the postulate that *O*-GlcNAc is a signal for the nuclear transport of cytosolic proteins [46–49], our group tried to identify factors capable of specifically binding *O*-GlcNAc residues in the cytosol through their lectin properties and translocating *O*-GlcNAcylated proteins to the nucleus. After investigation, we showed that Hsc70 (the 70 kDa heat shock cognate protein) and Hsp70 (70 kDa heat shock protein) were endowed with an *O*-GlcNAc binding property [30–33]. Such an observation was previously made by Minic et al. [50], who identified the Hsp70 analog p78 as binding to GlcNAc- or $(GlcNAc)_3$-agarose. Although it had previously been demonstrated that Hsc70 and Hsp70 were actively involved in nuclear transport [51,52], we thought that this unusual property was more likely to be implicated in the protection of damaged proteins. In this context, we observed that Hsp70–GlcNAc binding activity (HGBA) was especially triggered by cell injury, for example, by heat shock [30–32], glucose deprivation [30,32], ethanol, arsenite or heavy metals exposure [31], as well as by proteasome inhibition [32] and protein misfolding after the exposure of cells to a proline analog [32]. In accordance with these findings, we have formulated the following hypothesis. When a cell is injured, it concomitantly and transiently increases its *O*-GlcNAcylation level and its HGBA, leading to the binding of *O*-GlcNAc residues to Hsp70 (Fig. 3). After exposure to the aqueous medium, hydrophobic peptides are *O*-GlcNAcylated by the chaperone OGT. This modification leads to a transient decrease in the self-aggregation of the damaged intracellular proteins, both by slightly increasing their hydrophilicity and by increasing their interaction with Hsp70 in an *O*-GlcNAc/HGBA-dependent manner (Fig. 3). This may allow defective proteins to temporarily escape proteasome-mediated degradation. In support of this view, it has been demonstrated that *O*-GlcNAcylation exerts a negative effect on the proteasomal machinery [53]: *O*-GlcNAcylation may protect unfolded proteins both by modifying them and by acting on the proteasome itself, leading to a synergistic effect. This mechanism can be compared with the reticular glucosylation–deglucosylation cycle that ensures quality control in newly synthesized *N*-glycosyl proteins. OGT can "recognize" abnormally hydrophobic proteins and transfer a GlcNAc residue to them, as UGGT (UDP-glucose:glycoprotein glucosyltransferase) does for *N*-glycosyl proteins in the endoplasmic *reticulum* by grafting glucose residues to the non-reducing end of N-glycans [54]. The action of Hsp70 could be similar to the function of membrane-bound calnexin and soluble calreticulin, two endoplasmic *reticulum*-resident lectins that verify the correct folding of newly synthesized *N*-glycosyl proteins. Our recent observation that ubiquitination processes are under the control of *O*-GlcNAcylation [33], and that unlike ubiquitinated proteins, *O*-GlcNAcylated proteins levels are insensitive to the proteasome inhibitor MG132, lends weight to the notion that *O*-GlcNAc protects intracellular proteins from proteasomal destruction.

In addition to sharing the same molecular weight, several parallels can be drawn between Hsc70/Hsp70 and CBP70 (70 kDa carbohydrate binding protein) as described by Felin et al. [55]. Initially, CBP70 was shown to be localized in the cytosol and the nucleus [56], but it was demonstrated that this protein is also associated with endoplasmic *reticulum* and the Golgi apparatus [57]. Interestingly heat shock proteins are ubiquitous and are found in numerous cellular compartments, from the cytosolic to the endoplasmic *reticulum* compartments (Hsc70, one of the cytosolic Hsp70 forms, and BiP, the endoplasmic *reticulum* Hsp70 form, have an identity of more than 65%). The same authors also observed the persistence of CBP70 and of its GlcNAc binding sites after heat shock treatment [58]. These observations reinforce the notion that CBP70 and Hsc70/Hsp70 are the same protein.

The occurrence of intracellular proteins with GlcNAc binding properties has also been elegantly demonstrated by Niikura et al. [59] and Nishio et al. [60] using neoglycolipid-coated CdTe QDs (cadmium–telluride quantum dots). Since the expression level of chaperones is known to increase in response to stress, these authors designed a GlcNAc-based chaperone probe in order to visualize stress inside the cell [59]. They found that GlcNAc-displaying QDs accumulated in digitonin-permeabilized HeLa cells, especially around the nucleus, in an ATP-dependent manner. Thorough investigation has indicated that the partner interacting with the GlcNAc-QDs could be the ER specific chaperone GRP78/BiP. Interestingly, other sugar-QDs (glucose, mannose and galactose) have proven to be unsuccessful at demonstrating lectin properties. Recently the same group has found that permeabilized cells bind more GlcNAc-QDs when exposed to heavy metals [60]. The authors propose that GlcNAc-QDs may represent a valuable tool for measuring cellular stress.

3. *O*-GlcNAcylation and cardioprotection

Many studies show that *O*-GlcNAcylation protects the cardiovascular system. In particular, it helps to resist a trauma such as ischemia–reperfusion or trauma–hemorrhage and prevents the

destruction of the vascular endothelium [61, and see also Chatham and Marchase in this issue].

3.1. Ischemia/reperfusion (I/R) trauma can be lowered by elevated O-GlcNAcylation

Ischemia results in a decrease in blood circulation to organs, leading to a reduced oxygenation of tissues and a decrease in the supply of nutrients. Reperfusion corresponds to the return of blood to ischemic organs. When blood circulation restarts, before the restoration of normal functioning, an inflammatory reaction and oxidative stress occur, leading to apoptosis. It has been shown that the pretreatment of rat hearts with glucosamine or glutamine before the induction of I/R trauma improves the recovery of cardiac function in conjunction with an increase in the *O*-GlcNAcylation levels [62–64]. Conversely, the treatment of hearts with the OGT inhibitor alloxan or the glutamine:fructose-6-phosphate amidotransferase (GFAT) inhibitor azaserine reverses the protective effect and decreases *O*-GlcNAcylation. The protective effect mediated by elevated *O*-GlcNAcylation levels seen in the different models probably operates through p38MAPK phosphorylation, the induction of which is usually observed following an ischemic trauma [64]. The beneficial effects of treatment with glucosamine or PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-*N*-phenylcarbamate), an inhibitor of OGA, have also been tested during reperfusion trauma [65]. Under these conditions, an increase in *O*-GlcNAcylation is associated with an improvement in the recovery of cardiac function. The authors have also reported a decrease in the level of troponin as well as a decrease in the calpain-dependent proteolysis of alpha-fodrin and calmodulin-dependent kinase II, suggesting that *O*-GlcNAcylation exerts its effects through an inhibition of the calcium-dependent stress response. Similar results have been obtained with neonatal rat ventricular cardiomyocytes [66]. The pretreatment of cells with glucose, glucosamine or PUGNAc leads to an increase in cell viability and *O*-GlcNAcylation that correlates with a reduction of apoptosis, necrosis and calcineurin activation, whereas pretreatment with alloxan exacerbates the effects of the I/R trauma. To confirm that the effectiveness of glucosamine in protecting cardiomyocytes is mediated by the HBP and not by another pathway, the authors overexpressed OGT in a second study and definitively proved that the protective effect was a result of *O*-GlcNAcylation [67]. Both glucosamine exposure and OGT over-expression, and to a lesser extent, exposure to NButGT, another OGA inhibitor, increased basal and I/R trauma-induced *O*-GlcNAcylation levels and attenuated the loss of cytochrome *c* and the hydrogen peroxide-induced loss of mitochondrial membrane potential. An increase in the expression of the mitochondrial anti-apoptotic factor Bcl2 was also observed but no differences were seen in Bad and Bax levels. Conversely, the down-regulation of OGT decreased *O*-GlcNAcylation levels and prevented the I/R-induced increase in *O*-GlcNAcylation and Bcl2. These results strongly argue for the regulation of apoptosis by *O*-GlcNAcylation.

In the same context, the group of Jones at the University of Louisville has shown that *O*-GlcNAcylation attenuates the effects of myocardial infarction, oxidative stress and hypoxia in mouse and rat cardiomyocytes [36,68,69]. They have demonstrated that the mitochondrial voltage-dependent anion channel (VDAC), a potent component of the mitochondrial permeability transition pore (mPTP) that is formed during certain pathological conditions such as ischemia and stroke and that is often responsible for cell death, is *O*-GlcNAcylated. Thus, in contrast to phosphorylation, which is unaffected during cardiomyocyte injury [70], this PTM appears to play a role in mitochondrial membrane permeability in response to hypoxia. Indeed an enhancement of *O*-GlcNAcylation levels in cardiomyocytes attenuates calcium-induced mPTP formation, allowing one to hypothesize that VDAC *O*-GlcNAcylation has a constraining effect in its interactions with other components of mPTP, such as the protein transporters of the inner and outer mitochondrial membranes, Tim and Tom, the adenine membrane translocase and cyclophilin-D.

The finding that *O*-GlcNAcylation can protect the heart from injuries may explain at least partly why it has been known since 1986 that preconditioning this organ with brief episodes of I/R renders it more resistant to myocardial infarction [71,72]. Indeed, stress may anticipate the response to cell damage by enhancing *O*-GlcNAcylation levels. Under conditions of I/R preconditioning, the heart elevates its *O*-GlcNAc levels in order to resist potential injuries, particularly infarction.

3.2. Enhanced O-GlcNAcylation thwarts trauma–hemorrhage

Beneficial effects of *O*-GlcNAcylation have been observed after the induction of a trauma–hemorrhage (bleeding) with or without subsequent fluid replacement (fluid resuscitation) [73–76]. The administration of glucosamine [73] or PUGNAc [75] during fluid replacement improves cardiac function recovery as well as brain, liver and kidney reperfusion, with an increase in *O*-GlcNAcylation levels in these organs [73,75]. The enhancement of *O*-GlcNAcylation levels is correlated with a decrease in the circulating inflammatory cytokines IL-6 and TNF-α [75], and a blockade of NF-κB (nuclear factor-kappa B) signaling pathway activation [76]. Lipopolysaccharide-induced NF-κB signaling pathway activation is also attenuated by glucosamine treatment and OGT over-expression but is exacerbated in neonatal rat ventricular myocytes in which siRNA is used to target OGT [76].

3.3. Endoluminal arterial injury: O-GlcNAcylation is a vasoprotective PTM

Inflammation is a pivotal factor influencing vascular diseases. It has been shown in ovariectomized rats that a carotid artery trauma rapidly decreases *O*-GlcNAcylation levels [77]. The administration of glucosamine or PUGNAc by intravenous injection before the trauma diminishes the inflammatory response in correlation with an increase in *O*-GlcNAcylation. It has thus been proposed that the elevation of *O*-GlcNAcylation constitutes a new anti-inflammatory and vasoprotective mechanism.

4. Glucose, HBP and *O*-GlcNAcylation: the link to type-2 diabetes?

4.1. O-GlcNAcylation favors the development of insulin resistance and type-2 diabetes

Although extensively studied, the etiology of diabetes, of which one of the hallmarks is chronic hyperglycemia, is unclear. A constant nutrient excess and low energy expenditure (sedentary lifestyle) are two predisposing factors for type-2 diabetes. Resistance to insulin exerts dramatic and deleterious effects at the level of insulin target tissues, including pancreatic beta-cells, the liver, adipocytes and skeletal muscle. Coupled with glucose toxicity, insulin resistance is responsible for complications such as cardiovascular disorders, retinopathies, nephropathies and erectile dysfunction in diabetics. At the biochemical level, insulin resistance and glucose toxicity induce the production of reactive oxygen species (ROS), the glycation of proteins and an enhancement of intracellular protein *O*-GlcNAcylation. Moreover, like hyperglycemia, hyperlipidemia and hyperinsulinemia also lead to an enhancement of protein *O*-GlcNAcylation.

Insulin resistance inhibits glucose uptake and glycogen synthesis in skeletal muscle and adipocytes, and it has been proposed that this resistance to insulin is an adaptation by the organism in response to a nutrient excess. In the early 1990s, Marshall and Traxinger [78], who should be considered pioneers in the field of HBP, remarked that the development of insulin resistance requires three key components: glucose, insulin and glutamine. If one of these three components is absent, resistance to insulin does not occur. The role of glutamine in the development of type-2 diabetes has been found to be the

regulation of GFAT, the rate-limiting and key enzyme in the HBP, which catalyzes the conversion of fructose-6-phosphate to glucosamine-6-phosphate [79,80] (Fig. 1).

4.2. O-GlcNAcylation level depends upon the flux through the hexosamine biosynthetic pathway

A few years ago it was established that *O*-GlcNAcylation was tightly linked to glucose metabolism [27,79, and see also Hanover et al. in this issue]. As evaluated by Marshall et al. [78], 2%–3% of extracellular glucose is used for the *O*-GlcNAcylation process through the HBP (Fig. 1). It has been postulated that *O*-GlcNAcylation acts as a nutrient sensor implicated in insulin resistance and decreased glucose uptake by cells [81,82]. First, in insulin target cells, there appears to be a negative feedback regulation of glucose transport by the flux of glucose through the HBP [82]. Second, Robinson et al. [83] have shown that when rat hemidiaphragms are incubated in the presence of glucosamine or with high concentrations of glucose, glucose uptake is decreased. Subsequently, this glucosamine-induced insulin resistance was shown to be accompanied by an increase in UDP-GlcNAc [84]. McClain et al. [85] observed a type-2 diabetic phenotype in transgenic mice over-expressing OGT. This observation links insulin resistance to *O*-GlcNAcylation processes. In the same context, the incubation of rat epitrochlearis muscles with PUGNAc induces an increase in *O*-GlcNAcylation levels and a reduction in glucose transport, suggesting that *O*-GlcNAcylation can induce insulin resistance [86]. Taken together, these observations support the pivotal role of *O*-GlcNAcylation in reduced glucose transport and insulin resistance through the HBP. Nevertheless, a couple of recent studies have brought into question the link between the increase in *O*-GlcNAcylation and insulin resistance. Robinson et al. [87] have shown that adenoviral OGA overexpression fails to restore the impaired activation of Akt in 3T3-L1 cells pre-incubated with high concentrations of glucose and low doses of insulin. An OGT knockdown does not reverse insulin resistance either. In the same cell type, it has been shown that the highly selective OGA inhibitor NButGT, in contrast to PUGNAc, increases the level of *O*-GlcNAcylation without either impairing the phosphorylation–activation of Akt or inducing insulin resistance [88]. Both groups suggest that *O*-GlcNAcylation in itself is not sufficient for the development of insulin resistance.

The conversion of glucose to *O*-GlcNAc residues has been elucidated (Fig. 1). Once glucose enters the cell, it is rapidly converted to glucose-6-phosphate by hexokinase. Glucose-6-phosphate can then be epimerized to glucose-1-phosphate by phosphoglucomutase, to be converted to UDP-glucose for the activation of glycogen synthesis or converted to fructose-6-phosphate by phosphoglucose isomerase. Only a small amount of fructose-6-phosphate is converted to glucosamine-6-phosphate by GFAT, since the major proportion of fructose-6-phosphate is directed towards glycolysis. Glucosamine-6-phosphate is acetylated to *N*-acetyl glucosamine-6-phosphate through the action of glucosamine-6-phosphate *N*-acetyltransferase, epimerized to *N*-acetyl glucosamine-1-phosphate by phospho-*N*-acetylglucosamine mutase and then converted to UDP-GlcNAc by UDP-GlcNAc pyrophosphorylase. UDP-GlcNAc then serves to activate glycosylation processes, such as *O*-glycosylation including *O*-GlcNAcylation, *N*-glycosylation and the synthesis of the glycosylphosphatidylinositol (GPI) anchor and glycolipids (Figs. 1 and 2). Note that glucosamine itself is able to circumvent GFAT to enhance *O*-GlcNAcylation and to provoke insulin resistance.

4.3. O-GlcNAcylation is a down-regulator of the insulin signaling pathway

The phenomenon of insulin resistance and the corresponding decrease in glucose uptake are correlated with a defect in the translocation of the glucose transporter GLUT4 to the plasma membrane [89]. Under non-pathological conditions, vesicles that contain GLUT4 transporters are translocated to the plasma membrane in response to glucose stimulation, allowing glucose to enter adipocytes and skeletal muscle cells. The mechanism underlying the failure of GLUT4 to be integrated into the plasma membrane during the course of insulin resistance is not known, but the Akt/protein kinase-B (PKB) pathway could provide the answer [90] (Fig. 4). The binding of insulin to its receptor triggers an activation of the intrinsic tyrosine kinase activity of this receptor. Insulin receptor substrates (IRS) are recruited by the insulin receptor and are themselves tyrosine-phosphorylated. PI-3 (phosphoinoside-3) kinase (p85/p110) migrates to the plasma membrane and phosphorylates phosphatidylinositol-4, 5 bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5 triphosphate (PIP3). PDK-1 (phosphatidylinositol-dependent protein kinase-1) is then activated and phosphorylates Akt/PKB that in turn phosphorylates many substrates including GSK-3β and FoxO1 (Fig. 4). The link between Akt/PKB and GLUT4 has not really been established and it is not known how insulin resistance occurs at this stage. Nevertheless, numerous components of the insulin signaling pathway or involved in glucose metabolism have been shown to be *O*-GlcNAcylated: Akt1 [91], Akt2 [92], GLUT1 [93], casein-kinase II, glycogen synthase kinase-3 [94], IRS1/2 [95], PDK1, the p110α unit of PI3-kinase and the β chain of the insulin receptor itself [3] (Fig. 4). Based on these findings, it has been proposed that *O*-GlcNAcylation plays a pivotal role in the mechanism of insulin resistance by down-regulating the insulin signaling pathway [3]. Following stimulation by insulin, OGT is rapidly recruited (within 90 s after stimulation) on the inner face of the plasma membrane by PIP3 through a region adjacent to catalytic domain II at the carboxyl end of the transferase [3]. Yang et al. have identified lysines 981 and 982 as necessary for interacting with the acidic phosphate groups of the phosphoinositides. It appears that OGT does not possess any domain known to interact with the phosphoinositides similar to the pleckstrin homology (PH) domain that allows proteins such as PDK1, Akt/PKB, the βγ-subunits of heterotrimeric G proteins and protein kinase-C to interact with PIP3. The phosphoinositide-interaction domain of OGT has been called PPO for “PIP-binding activity of OGT.” In the same paper it has been reported that the exposure of 3T3-L1 adipocytes to high glucose concentrations or PUGNAc decreases the phosphorylation of Akt at threonine 308, a critical site known to be phosphorylated. In contrast, it seems that the phosphorylation level of the MAPK pathway, particularly the Erk1/2 kinases, is unaffected. The phosphorylation of IRS1 is also modified under these conditions, since the treatment of adipocytes with PUGNAc or the adenofection of adipocytes and Fao hepatoma cells with Ad-OGT increases the phosphorylation of IRS1 at Ser307 and Ser632/635, three sites known to down-regulate the insulin signaling pathway. Taken as a whole, this paper demonstrates that OGT allows the termination of the insulin transduction cascade by interacting with PIP3 at the plasma membrane. OGT then *O*-GlcNAcylates numerous components of the insulin signaling pathway, lowering the response to insulin, and thus in all likelihood participates in the physiopathology of insulin resistance. Moreover, in concordance with the intimate link between glucose, the *O*-GlcNAcylation processes and the insulin signaling pathway, it can be hypothesized that a defect in *O*-GlcNAcylation metabolism will be reflected by a decrease in glucose uptake through insulin resistance, and consequently by glucose toxicity.

4.4. Four important transcription factors involved in glucose homeostasis are O-GlcNAcylated

4.4.1. Insulin synthesis is under the control of O-GlcNAcylation (PDX-1 and NeuroD1)

As cited above, numerous components of the insulin signaling pathway are *O*-GlcNAcylated and interestingly, PDX-1 and NeuroD1, two pancreatic transcription factors that control insulin synthesis, are

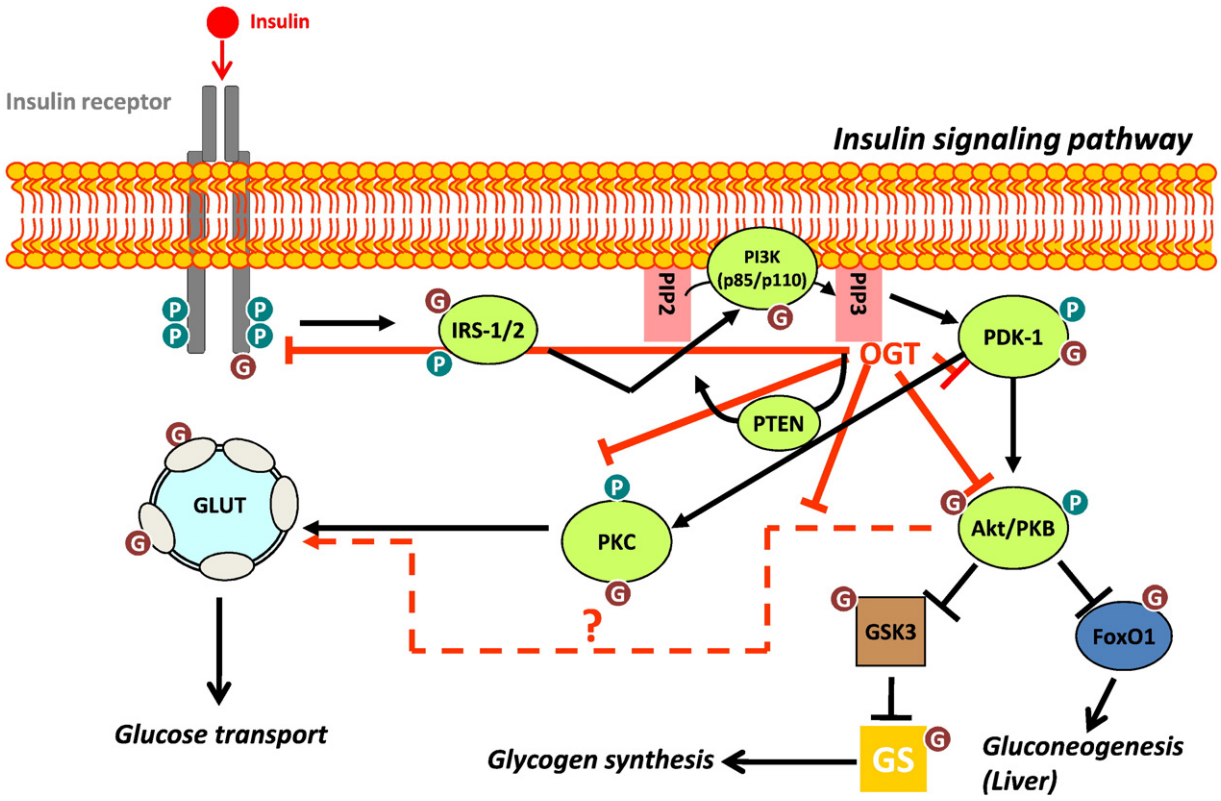

Fig. 4. The insulin signaling cascade is inactivated by *O*-GlcNAcylation. After it binds to its receptor, insulin engenders a phosphorylation cascade that activates (glycogen synthase, GLUT translocation to the plasma membrane) or inactivates (GSK-3, FoxO1) many targets, resulting in accelerated glucose transport, an activation of glycolysis and glycogen synthesis and an inactivation of gluconeogenesis (sequestration of FoxO1 in the cytosol). It has been demonstrated that upon insulin activation, the biosynthesis of PIP3 re-translocates OGT to the plasma membrane, leading to the extensive *O*-GlcNAcylation of signaling cascade components and to an inhibition of the insulin response (red inhibitory arrows). OGT is thought to be responsible for the insulin resistance phenomenon due to this activity. The problem is amplified by the non-inactivation of FoxO1, which regulates the hepatic production of glucose (gluconeogenesis), exacerbating glucose toxicity. In pancreatic beta-cells, the hyperactivation of FoxO1 participates in beta-cell mass destruction. **Akt**, RAC (related to the A and C kinases)-alpha serine/threonine protein kinase; **FoxO1**, Forkhead box O1; **GLUT**, Glucose transporter; **G**, *O*-GlcNAc residue; **GS**, glycogen synthase; **GSK-3**, glycogen synthase kinase-3; **IRS-1/2**, insulin receptor substrates 1 and 2; **OGT**, *O*-GlcNAc transferase; **PDK-1**, phosphatidylinositol-dependent protein kinase-1; **P**, phosphate residue; **PIP2**, phosphatidylinositol-4, 5 bisphosphate; **PIP3**, phosphatidylinositol-3, 4, 5 triphosphate; **PI3K**, PI-3 (phosphatidylinositol-3) kinase (p85/p110); **PKB**, protein kinase-B (Akt); **PKC**, protein kinase-C; **PTEN**, phosphatases and tensin homolog.

themselves modified by *O*-GlcNAcylation [96,97]. It has been shown that an enhancement of PDX-1 *O*-GlcNAcylation is correlated with an increase in its nuclear translocation and that the treatment of Min6 beta-cells with high glucose concentrations increases their DNA-binding activity and insulin secretion [96]. In the same Min6 cells, it has been observed that the subcellular localization of NeuroD1 depends upon its *O*-GlcNAcylated status [97]. When cells are treated with high glucose concentrations, NeuroD1 interacts with OGT, and this interaction is seemingly correlated with the nuclear localization of the transcription factor. Conversely, at low glucose concentrations, NeuroD1 interacts with OGA in its deglycosylated form. These results indicate that the subcellular localization of NeuroD1 depends upon its interaction with OGT or OGA, and more broadly, upon the concentration of glucose. Since the transcriptional activity of PDX-1 and NeuroD1 is controlled in part by their *O*-GlcNAcylation status, this suggests that glucose can stimulate insulin synthesis through an increase in the *O*-GlcNAcylation of these two key transcription factors.

4.4.2. Gluconeogenesis is controlled by O-GlcNAcylation (FoxO1 and CRTC2)

Under non-pathological conditions, insulin favors glycolysis and glycogen synthesis and inhibits gluconeogenesis; in diabetes, on the contrary, the excessive production of glucose by the liver is responsible for fasting hyperglycemia [98]. Recent studies focusing on the transcription factors FoxO1 [99–101] and CRTC2/TORC2 [102] have shown that *O*-GlcNAcylation also leads to insulin resistance and glucose toxicity through the control of gluconeogenesis. The groups of Issad (Cochin Institute, Paris) and Hart (Johns Hopkins Institute, Baltimore) have demonstrated that FoxO1 is *O*-GlcNAcylated, and the latter have observed that the *O*-GlcNAcylation of FoxO1 is elevated in diabetes and that insulin reduces its glycosylation [100]. The treatment of HepG2 or HEK293 cells with PUGNAc increases FoxO1 transcriptional activity targeting a glucose-6-phosphatase (the last enzyme of the gluconeogenesis pathway, which converts glucose-6-phosphate into glucose) reporter gene [99], and glucose activates FoxO1 transcriptional activity of several target genes including PEPCK (phospho-enol pyruvate carboxykinase), a second enzyme critical for the regulation of gluconeogenesis [100]. Many *O*-GlcNAcylated sites on FoxO1 have been mapped using ETD/MS/MS (electron transfer dissociation/mass spectrometry/mass spectrometry): Thr317, Thr318, Ser550, Thr648 and Ser654 [100]. The mutation of Thr317 to alanine reduces the activity of the transcription factor [100]. This result is consistent with those of Kuo et al. [99], showing that PUGNAc, on the other hand, does not affect the transcriptional activity of the constitutively nuclear T24A/S256A/S319A mutant. In yet another report, it has been shown that the transcriptional co-activator PGC-1α, which is itself *O*-GlcNAcylated, regulates FoxO1 *O*-GlcNAcylation and consequently its transcriptional activity, by recruiting and targeting OGT to FoxO1 [101]. Interestingly, both glucose-6-phosphatase and PEPCK are also transcriptionally regulated by CRTC2. *O*-GlcNAcylation also controls CRTC2 but, unlike the case of FoxO1, seems to do so through nuclear re-location since the two major *O*-GlcNAcylated sites that have been mapped sequester CRTC2 in the cytoplasm by a phosphorylation-dependent mechanism [102].

This series of papers elucidates the aberrant increase in gluconeogenesis that occurs during the pathophysiological mechanism of insulin resistance. FoxO1 is under the control of the insulin signaling pathway through Akt phosphorylation. The phosphorylation of FoxO1 by Akt triggers its association with 14-3-3, leading to its sequestration in the cytosol [103]. Even if *O*-GlcNAcylation promotes FoxO1-enhanced transcriptional activity by a mechanism independent of its subcellular localization, the glycosylation of FoxO1 contributes to excessive glucose production during insulin resistance, maintaining hyperglycemia. A vicious circle may thus be created: 1) The *O*-GlcNAcylation of FoxO1 increases its transcriptional activity; 2) glucose-6-phosphatase and PEPCK are expressed; 3) gluconeogenesis produces more glucose that reaches the blood circulation; 4) glucose exerts its deleterious effects on the pancreas, which in turn produces less insulin; 5) insulin fails to stimulate its target tissues; 6) FoxO1 is no longer phosphorylated but is increasingly *O*-GlcNAcylated (HBP is also over-activated) and translocated into the nucleus (return to 1) [98]. It must be noted that hyperglycemia also maintains the *O*-GlcNAcylation of FoxO1, accelerating its transcriptional activity and insulin resistance. It is also well known that glucose reduces beta-cell mass by increasing the apoptosis of beta-cells and decreasing their survival, and that FoxO1 participates in the mechanism of pancreatic cell death [104]. It can thus be envisioned that the *O*-GlcNAcylation of FoxO1 in the pancreas contributes to an increase in the death of pancreatic beta-cells [99].

4.5. Numerous deficits in diabetic patients may depend upon O-GlcNAcylation

It seems that many of the complications seen in diabetes are also linked to *O*-GlcNAcylation. Diabetic cardiomyopathy, in which cardiac contractility is impaired, is one of the leading causes of diabetes-related morbidity and mortality. It has been demonstrated that exposure to high glucose concentrations, treatment with glucosamine or over-expression of OGT in cardiomyocytes provokes an increase in *O*-GlcNAcylation levels and a dysregulation of the calcium flux through a decrease in the expression and activity of the calcium-dependent sarcoendoplasmic *reticulum* ATPase, SERCA2a [105]. This phenomenon is inverted when OGA is ectopically expressed, and its over-expression in streptozotocin (STZ)-induced diabetic rat hearts by the injection of OGA-expressing adenoviruses increases calcium signaling, improves heart contractility and enhances the expression of SERCA2a [106]. From these experiments, it can be concluded that *O*-GlcNAcylation in the diabetic heart contributes to cardiac dysfunction and that a reduction in *O*-GlcNAcylation may have a beneficial impact on diabetic cardiac function through the control of calcium metabolism. Recently, the involvement of the *O*-GlcNAcylation of putative mitochondrial proteins in the development of cardiomyopathy has been explored [2]. For the first time, *O*-GlcNAc-bearing proteins have been identified inside the mitochondria. Several of the proteins identified are members of complexes I, III and IV of the respiratory chain, and the authors have moreover identified one *O*-GlcNAcylation site on NDUFA9, one of the proteins of complex I. The exposure of cells to high glucose levels increases the *O*-GlcNAcylation of NDUFA9 and COX I (a complex IV protein) whereas glycosylation is decreased when OGA is over-expressed. Importantly, *O*-GlcNAcylation reduces the activity of complexes I, III and IV and lowers mitochondrial calcium concentration and ATP production. Taken together, these studies show that the enhanced *O*-GlcNAcylation seen in diabetes contributes to cardiomyopathy by lowering calcium flux through a decrease in SERCA2a expression and activity and by the diminution of the intra-mitochondrial calcium concentration. The failure of the respiratory chain due to an increase in the *O*-GlcNAcylation of many of its components leads to a depletion in ATP levels, which is reflected by poor myocardial contractility.

Erectile dysfunction is another disorder linked to diabetes [107]. Penile erection is mediated by nitric oxide (NO) [108] released from nerve endings close to the blood vessels of the penis [107]. NO is produced by eNOS (endothelial nitric oxide synthase) and targets guanylyl cyclase, the enzyme that in turn generates the second messenger cyclic GMP (cGMP). Under non-pathological conditions, eNOS is regulated by insulin through phosphorylation at Ser1177 by Akt/PKB. The exposure of bovine aortic endothelial cells to high glucose levels or to glucosamine blocks eNOS activity [109]. Intriguingly, it has been observed that hyperglycemia doubles eNOS *O*-GlcNAcylation and reciprocally decreases its phosphorylation at Ser1177. Identical results have been obtained with human coronary artery endothelial cells, and a dysregulation in the reciprocity of Akt *O*-GlcNAcylation and phosphorylation is thought to be involved in atherosclerosis, a pathology which progression is accelerated in diabetes [110]. In *in vivo* experiments conducted with male rats with alloxan-induced type-1 diabetes [111], the penis of diabetic rats shows an increase in Akt *O*-GlcNAcylation and a concomitant decrease in phosphorylation at Ser1177, and presents an erectile impairment. These results explain why in diabetes, hyperglycemia disrupts the erectile mechanism by elevating eNOS *O*-GlcNAcylation and by reciprocally decreasing its phosphorylation at the crucial Ser1177 Akt site.

The predisposition of diabetic patients to develop Alzheimer's disease will be discussed below (Section 5.3).

5. *O*-GlcNAcylation and Alzheimer's disease

Alzheimer's disease (AD) is the main cause of dementia in aging individuals. This pathology is characterized by memory and comprehension deficits, inconsistent behavior, hallucinations and mental confusion. In our western societies, life expectancy is constantly on the rise, and for this reason the number of individuals with AD is growing at a staggering rate: 24 million cases of AD have been documented in the world, with a prevalence of 1.5%–2% in people older than 75 years and 14% in people older than 90 years. Accordingly, AD has become a considerable social and economic problem. Like type-2 diabetes, Alzheimer's disease is associated with aging. A recent study has shown that *O*-GlcNAcylation levels are higher in the heart, brain and skeletal muscles of aging Brown Norway rats when compared to younger ones [112]. In this respect, it seems that *O*-GlcNAcylation may also be considered as a marker for aging or senescence.

5.1. O-GlcNAcylation occurs abundantly in the central nervous system

Among the 600 proteins that have been identified as bearing *O*-GlcNAc residues, many are neuronal proteins [113]. Numerous such *O*-GlcNAcylated neuronal proteins are implicated in the etiology of AD, including Tau (Tubulin-associated unit) and the beta-amyloid precursor protein (APP).

The importance of *O*-GlcNAcylation metabolism in neurons is underlined by four observations: 1) a study of *O*-GlcNAcylation in cerebellar neurons of healthy mice at different ages [114] has shown that this modification is ubiquitously present from embryonic day 10 until adulthood; 2) the synaptosome is enriched in *O*-GlcNAcylated proteins [115] and OGT [116] and the nerve terminal is enriched in enzymes regulating *O*-GlcNAcylation processes [117]; 3) cultured neuronal Kelly cells are more susceptible to an imbalance in *O*-GlcNAcylation/phosphorylation induced by the broad-spectrum phosphatase inhibitor okadaic acid than HeLa or Cos cells [118].

Griffith and Schmitz [119] were pioneers in the field of *O*-GlcNAcylation and AD. Using autopsied normal and AD human brains, they established that the expression of *O*-GlcNAcylated proteins was significantly up-regulated in AD brains in comparison to age-matched control brains. Curiously, some other studies contradict these initial

findings. For example, Yao and Coleman [120] did not find any difference in *O*-GlcNAcylated protein levels between AD and control brains (normal age-matched controls) except for the 160 kDa protein, AP-3, for which a marked decrease was observed in AD brains. More recently, Robertson et al. [121] have shown that *O*-GlcNAcylation is reduced in heat-stable Tau-enriched preparations from both AD and frontotemporal dementia patients. These authors impute the discrepancies between these results to the methods used to generate fractions and to label terminal sugars.

5.2. In patients with neuronal disorders, proteins form aggregates and the proteasomal machinery is impaired

Two characteristic features of neurological diseases are protein aggregation and a dysfunction of the ubiquitin-proteasome system (UPS) [122]. Protein misfolding followed by aggregation can occur either inside the cell, with the formation of inclusion bodies, or outside the cell. The aggregates usually consist of fibers that contain misfolded proteins with a beta-sheet conformation that is termed "amyloid." In Huntington's disease, huntingtin is the main deposited protein, and its aggregation is caused by a CAG repeat coding for a polyglutamine stretch, as for other polyglutamine diseases. In Parkinson's disease, the major aggregating component is alpha-synuclein, in prion diseases it is the prion protein itself that aggregates, and to date the factor that aggregates in amyotrophic lateral sclerosis is unknown. In AD, two kinds of aggregates are found: intracellular paired helical filaments (PHF, which in turn aggregate in neurofibrillary tangles or NFT) of which one of the major components is Tau [123], and extracellular senile plaques (neuritic plaques) in which the 42 amino-acid beta-amyloid peptide (Aβ) accumulates [124]. Aβ is generated by the sequential intracellular cleavage of the ubiquitously expressed type I plasma membrane protein APP. It has been shown that APP is *O*-GlcNAcylated, with the glycosylation probably occurring within the short cytoplasmic tail [125]. APP is the first plasma protein reported as bearing *O*-GlcNAc residues. To date the relationship between APP *O*-GlcNAcylation, its processing and AD remains unknown.

Protein aggregation may be the result of defective protein solubility or may result from an acceleration of protein synthesis or a deficit in its degradation. This idea is reinforced by the observation that ubiquitin is found in large amounts in aggregates and in neuronal cell death, leading to the conclusion that in AD, the proteasome is inhibited and the UPS is dysregulated.

It has been reported that the proteasomal machinery is modified by *O*-GlcNAcylation [53,126] and that after modification by OGT, the proteasome is inhibited [53]. Intriguingly, it has been proposed that a genetic impairment in the OGA gene results in proteasomal dysfunction through a lack of hydrolysis of the inhibitory *O*-GlcNAc residues of the 19S regulatory cap. Indeed, the OGA gene is located in the 10q locus [127,128], a chromosomal region frequently mutated in AD. The impairment of OGA in AD and the subsequent static *O*-GlcNAcylation of the proteasome may explain why the latter fails to degrade neuronal aggregates. In addition, it has been reported that *O*-GlcNAcylation reduces the sensitivity of intracellular proteins to proteasomal degradation by directly modifying them [43,129,130]. The two phenomena could thus act synergistically: a protein could escape degradation by means of its own *O*-GlcNAcylation and by the inhibitory effect of glycosylation on the proteasome, leading to a considerable decrease in the turnover of proteins that in turn may aggregate and cause neuronal death.

The role of *O*-GlcNAcylation in regulating the rate of protein degradation can be extended to the glycosylation of the de-ubiquitinating enzyme UCH-L1 (ubiquitin carboxyl hydrolase-L1), which has been observed in the synaptosome [115]. A failure of UCH-L1 *O*-GlcNAcylation may explain the resistance of aggregates to degradation, although the effect of this PTM on UCH-L1 has not yet been defined.

Our group has recently observed a link between *O*-GlcNAcylation and ubiquitination [33]. It appears that a dysregulation of *O*-GlcNAcylation dynamics has repercussions at the level of ubiquitination. We have also observed that the ubiquitin-activating enzyme E1 is itself modified by *O*-GlcNAcylation and that its glycosylation status changes according to cell culture conditions and stress. Moreover, E1 and Hsp70 seem to interact in an *O*-GlcNAcylation/lectin-dependent manner (Fig. 3). Crosstalk may exist between the *O*-GlcNAcylation and ubiquitination pathways through an increase in HGBA correlated with its interaction with *O*-GlcNAcylated E1. In this context, it should be interesting to look for the occurrence of *O*-GlcNAcylation on the E3 ligase parkin, found in Parkinson's disease, which catalyzes the addition of ubiquitin to specific UPS-targeted substrates.

5.3. Neuronal functions are tightly linked to glucose metabolism

The brain is critically dependent on glucose, which is almost its only energy source. The brain consumes close to 150 g of glucose per day under physiological conditions, and in neurons, glucose controls many cellular processes from neurotransmitter synthesis to ATP production. Glucose metabolism and consequently the supply of energy, is modified in the aging brain with respect to both insulin and acetylcholine signal transduction. There is cumulative evidence to suggest that during aging and AD, the brain actively adapts its glucose metabolism: glucose is conserved for anabolism, and the oxidative utilization of ketone bodies is enhanced [131]. Changes in brain glucose levels have been demonstrated in numerous neurodegenerative diseases, and these observations indicate that an adaptation in the use of this carbohydrate may be involved in the pathological process [132].

Interestingly, diabetic neuropathies have been linked to poor glycemic control. Diabetes, which is characterized by a deficiency in glucose uptake and utilization as previously mentioned, doubles the likelihood of developing AD [133]. Conversely, it is known that in the AD brain, glucose uptake and metabolism are impaired, and it seems that this failure could be a cause of neurodegeneration. Experiments performed on non-human primates have demonstrated that a decrease in cerebral glucose consumption is correlated with memory impairments [134]. What is the relation between the impairment of glucose utilization seen in AD and *O*-GlcNAcylation? A tentative response to this question has been provided by a comparison of the *O*-GlcNAcylation levels of cytosolic proteins from 19 AD brains and 15 controls, as a function of the post-mortem delay [135]. A non-linear regression analysis of the results shows that in AD brains, *O*-GlcNAcylation is 22% lower than in controls, indicating that *O*-GlcNAcylation is compromised in this disease. These results are in accord with our previous study showing that Tau hyperphosphorylation is associated with a decrease in its *O*-GlcNAcylation level [136]. Tau phosphorylation status increases during AD development (healthy Tau contains around 1.9 phosphate residues per molecule, while Tau found in PHFs contains 6–8 phosphate residues per molecule [137]) to the detriment of its *O*-GlcNAcylation level, which decreases [135]. However, we are faced with the problem of the hen and the egg: which PTM comes first? A recent observation argues for the hypothesis that *O*-GlcNAcylation occurs first and that its modification reflects on the phosphorylation status. De La Monte et al. at the Rhode Island Hospital and Brown Medical School have proposed the exciting hypothesis that AD represents a neuroendocrine disorder and that the term "type-3 diabetes" should be applied to describe this new mechanism of neurodegeneration [138–141]. After looking at the brains of AD patients (postmortem), they described reductions in insulin and insulin-like growth factors I/II (IGF I/II) levels in the frontal cortex, hippocampus and hypothalamus that are similar to those seen in diabetes [138]. Reductions in the

expression of the corresponding receptors were also observed. The same group also tested the effect of brain insulin depletion and of an impairment of the insulin signaling pathway using intracerebral streptozotocin injection in an *in vivo* model of rat [140]. Surprisingly, they observed the appearance of the hallmarks of AD among which are a reduction in the size of the brain, a loss of neurons in the temporal lobes, an increased expression of ubiquitin, an increased phosphorylation of Tau in the temporal cortex, an increase in the APP expression and an accumulation of Aβ in the brain, similar to what is found in AD [140]. Since the AD brain presents characteristics of both type-1 and type-2 diabetes, De La Monte et al. have combined the two previously described types of diabetes into a third, type-3. To push on a bit further, evidence has emerged that a reduction in glucose utilization and the subsequent energy depletion occur very early in the development of AD [142–144]: this convincingly argues for a role for an insulin signaling pathway failure in the progression of the disease. Gong and collaborators have recently proposed that diabetes may increase the risk for developing AD by impairing brain glucose uptake and metabolism [145]. From these intriguing observations, a link can be drawn through the HBP between the decreased glucose utilization found in the brains of AD patients and the ability of neurons to *O*-GlcNAcylate their intracellular proteins (Fig. 5). An impairment of the *O*-GlcNAcylation of crucial neuronal proteins such as Tau may favor their phosphorylation, thereby preventing the low molecular weight microtubule-associated protein from promoting microtubule assembly, stability and orientation. A blockade of neuronal transport ensues, leading inescapably to cell death (Fig. 5). In this view, it has been recently observed that hyperphosphorylated Tau in human brain contains four fold less *O*-GlcNAc residues when compared with non-hyperphosphorylated Tau [146]. In rat brain, inhibition of the HBP by using 5-oxo-6-diazo-norleucine (DON) results in a decrease of Tau *O*-GlcNAcylation and in an increase of its phosphorylation.

One way to tentatively block the putative type-3 diabetes-mediated hyperphosphorylation may be by counteracting the activity of Tau-directed kinases by lengthening the time of occupancy of phosphorylation sites or neighboring sites by *O*-GlcNAcylation. To this end, thiamet-G, a new inhibitor of OGA, has been successfully shown to decrease the *in vivo* phosphorylation of Tau [147]. This molecule is highly selective, can be orally administered and is capable of crossing the blood brain barrier. The administration of thiamet-G to rats diminishes phosphorylation at the pathological sites Thr231 and Ser396 in the cortex and in the hippocampus. Thiamet-G-induced hypophosphorylation is very quick, with the decrease in Ser396 phosphorylation attaining maximal levels only 4 hours after administration. According to the authors of this report, thiamet-G is thus a good candidate for altering disease progression in AD, and more broadly, in tauopathies.

6. Conclusion and future directions

O-GlcNAcylation has appeared in the last 10 years, of the same time of phosphorylation, as a crucial intracellular PTM regulating many cellular processes [1,15,148]. Increasing evidences also show that a loss of control of the dynamics of *O*-GlcNAcylation interferes with the normal progress of fundamental events and may be implicate in the appearance of pathological states. A better understanding of how this PTM takes part in such processes will indubitably go through the identification of the sites of glycosylation on the plethora of proteins bearing *O*-GlcNAc residues. Due to the lability of *O*-GlcNAcylation, it seems that ETD–MS is the good technology to succeed in doing such a mapping [100,149,150] since this tool preserves the GlcNAc group from detaching prior to the fragmentation of the peptide backbone. Many efforts will concentrate in the future years for elucidating the sites of *O*-GlcNAcylation of proteins

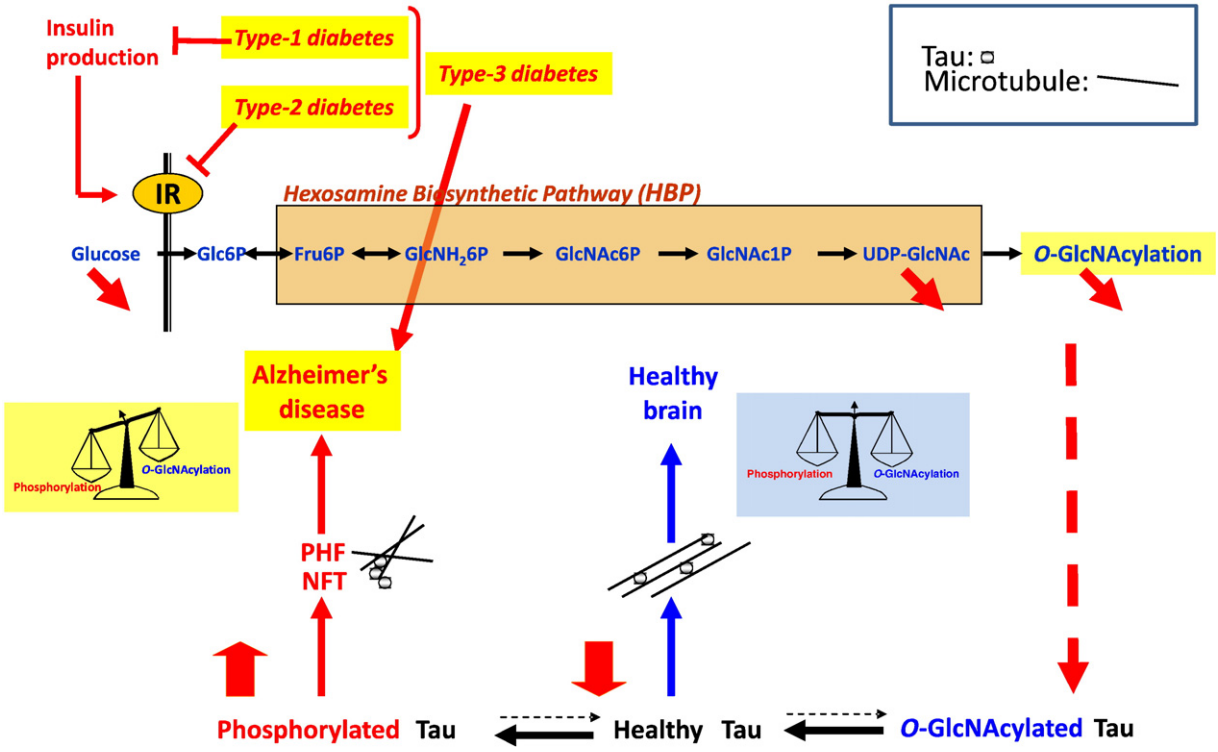

Fig. 5. Alzheimer's disease is seemingly a third type of diabetes. It has recently been proposed that AD patients present features in common with type-1 and type-2 diabetes. The failure of brain glucose metabolism should have repercussions on *O*-GlcNAcylation processes since under these conditions the HBP converts less glucose into UDP-GlcNAc than under non-pathological conditions. The hypo-*O*-GlcNAcylation of Tau proteins must have consequences for its phosphorylation. A dysregulation of the *O*-GlcNAcylation/phosphorylation "balance" of Tau leads to a depolymerization of the microtubule network, enhancing neuronal cell death. A deficiency in the *O*-GlcNAcylation of Tau could thus contribute to the etiology of AD. **Fru6P**, fructose-6-phosphate; **Glc6P**, glucose-6-phosphate; **GlcNH$_2$6P**, glucosamine-6-phosphate; **GlcNAc6P**, *N*-acetylglucosamine-6-phosphate; **GlcNAc1P**, *N*-acetylglucosamine-1-phosphate; **IR**, insulin receptor; **NFT**, neurofibrillary tangles; **PHF**, paired helical filaments; **UDP-GlcNAc**, uridine di-phospho-*N*-acetylglucosamine.

implicate in pathological processes such as type-2 diabetes and obesity (GLUTs, insulin receptor, IGF-R, PDK, Akt, ChREBP…) or neurological disorders like Alzheimer's disease (Tau, APP, neurofilaments…). As it may appear strange, this review was devoted to sicknesses worrying the western societies and no section describes the implication of *O*-GlcNAcylation in the cancerization processes. Very few things are known about this problem even if many studies have related the function of *O*-GlcNAcylation in the correct progress of cell cycle [8,21–23]. One can suppose that in the future much effort will be employed to decipher the impact of *O*-GlcNAcylation in many types of cancer and more particularly to colorectal, breast, bladder, pancreas and lung cancers, diseases largely widespread and which present a high morbidity and mortality rates. The knowledge of the relationship between the PTM and this group of pathologies, like for diabetes and neuropathies, will go through the identification of the key factors involved in the etiology of each kind of cancer and by the mapping of the exact sites of *O*-GlcNAcylation. It seems also that targeting the enzymes of the cycling glycosylation, OGT and OGA, by elaborating new specific inhibitors capable of operating in the organism will be an elevated challenge, especially for OGT since to date no worthwhile drug really blocks the glycosyltransferase activity. Today, what we can assert is that the next decade will be that of *O*-GlcNAcylation.

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Chapter 12

Antibodies and Activity Measurements for the Detection of *O*-GlcNAc Transferase and Assay of its Substrate, UDP-GlcNAc

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Abstract

Since the discovery of *O*-GlcNAc modification (*O*-GlcNAcylation) 20 years ago, much attention has been given to OGT (*O*-GlcNAc transferase), the unique enzyme responsible for the nuclear and cytosolic *O*-GlcNAcylation processes. This review focuses on protocols that are routinely used to analyze OGT expression and activity. First are detailed techniques using rabbit polyclonal anti-OGT antibodies, namely, Western blot, (co-)immunoprecipitation, and immunofluorescence. We also describe the measurement of OGT activity by using synthetic peptides as acceptors and radiolabeled UDP-GlcNAc. Finally, a sensitive HPAEC-based technique to measure the cellular content of UDP-GlcNAc, the donor substrate of OGT, is described in detail.

Key words *O*-GlcNAc transferase, Polyclonal anti-OGT antibodies, OGT activity assay, UDP-GlcNAc content assay

1 Introduction

OGT is a nucleocytoplasmic glycosyltransferase (uridine diphospho-N-acetylglucosamine:polypeptide β-N-acetylglucosaminyltransferase or *O*-GlcNAc transferase; EC. 2.4.1.255) assigned to the GT41 family in the CAZY (Carbohydrate-Active enZYme) database [1]. Three isoforms of OGT have been described: the 110 and 78 kDa forms of OGT are localized in the nuclear and cytoplasmic compartments whereas the 103 kDa form is localized in the mitochondria [2, 3]. Using UDP-GlcNAc as the donor substrate, this enzyme modifies thousands of proteins by adding a unique *N*-acetylglucosamine residue onto acceptor substrates mainly confined within cytosol and nucleus. The detection of OGT is quite easy to do since reliable antibodies have been developed, first by

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G. W. Hart's group which has discovered the *O*-GlcNAc modification [4], characterized and cloned OGT [5, 6], and by companies such as Sigma, which commercialize a panel of anti-OGT antibodies. The advisable utilization of these polyclonal antibodies allows OGT detection by Western blot (WB)—in different cell types, tissue, or organisms [7, 8]—and by immunofluorescence (IF) [9]. OGT antibodies can also be used in immunoprecipitation (IP), co-IP experiments to identify protein partners and in microinjection experiments to neutralize the enzyme [7].

Within the cell, OGT is not the only *N*-acetylglucosaminyltransferase using the nucleotide sugar UDP-GlcNAc; as an example, the UDP-*N*-acetyl-D-glucosamine:*N*-acetyl-D-glucosaminyldiphosphodolichol *N*-acetyl-D-glucosaminyltrans-ferase, EC. 2.4.1.141 is anchored within the endoplasmic *reticulum* membrane, participates in the biosynthesis of *N*-glycans and consequently competes with OGT for the use of UDP-GlcNAc since its catalytic domain is localized in the cytosol. Measurements of the enzymatic activity of OGT require specific substrates such as Nup62/p62 or casein kinase II (CKII) for macromolecular substrates, or c-Myc, Nup62/p62 or CKII-derived synthetic peptides [10]. The use of full-length proteins as substrate can be followed by the separation of [^{3}H]-GlcNAc-labeled proteins by SDS-PAGE and autoradiography of the dried gel. We show in this chapter that incorporation of radioactivity into peptide substrates is quantified by scintillation counting after fractionation by ion-exchange, gel-filtration, or HPLC.

The intracellular concentration of UDP-GlcNAc is closely dependent upon nutrient availability. Consequently OGT activity tightly correlates with the cellular nutritional status. Therefore, knowing the UDP-GlcNAc level can help in the understanding of *O*-GlcNAcylation processes. We have developed a two-step procedure, based on cation- and anion-exchange chromatography to measure cellular and tissue-derived UDP-GlcNAc contents [11, 12]. This highly sensitive method is presented in the last part of this chapter.

2 Materials

All buffers should be prepared using ultrapure water (18 MΩ water) except for electrophoresis (running) and electroblot buffers.

2.1 SDS-PAGE, Electro-transfer and Western Blot

For electrophoresis and Western blotting, refer to suppliers' recommendations.

2.2 Immunoblot

1. Rabbit polyclonal anti-OGT antibodies, DM17, SQ17 and TI14 were developed using synthetic peptides corresponding respectively to residues 740–756, 833–849 and 1024–1037 of human OGT (*see* **Note 1**).

2. HRP (Horseradish peroxidase)-linked whole Ab (from sheep) and ECL (Enhanced chemiluminescence) Plus (GE Healthcare, Velizy, France).
3. Cell lysis buffer, see IP lysis buffer (*see* Subheading 2.3).
4. TBS-Tween (TBS-T): 150 mM Tris–HCl, pH 8.0, 140 mM NaCl, 0.05 % (v/v) Tween-20 (Sigma, Saint Quentin Fallavier, France).
5. Blocking solution: 5 % (w/v) nonfat milk in TBS-T.
6. Hyperfilm (GE Healthcare).
7. Developer and fixer (Sigma).
8. CCD camera (ChemiGenius^2 bio imaging system, Syngene, Ozyme, Montigny le Bretonneux, France) (*see* **Note 2**).

2.3 Immunoprecipitation (IP) and Co-immunoprecipitation (co-IP) (See Note 3)

1. Protein A Sepharose™ 4 Fast Flow (GE Healthcare).
2. IP lysis buffer: 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 % (v/v) Triton X-100, 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) sodium dodecyl sulfate (SDS), Inhibitor cocktail tablets (Roche Diagnostics, Meylan, France).
3. TNE: 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA.
4. IP lysis buffer/NaCl: 0.5 M NaCl in IP lysis buffer.
5. IP lysis buffer/TNE: 50 % IP lysis buffer/50 % TNE (v/v).
6. Co-IP lysis buffer: 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 % (v/v) NP-40, 0.25 % (w/v) sodium deoxycholate, 0.1 % (w/v) sodium dodecyl sulfate (Sigma).
7. Laemmli buffer: 50 mM Tris–HCl, pH 6.5, 2.5 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 10 % (v/v) glycerol, 0.05 % (m/v) bromophenol blue (Sigma).

2.3.1 Silver-Staining of Proteins

1. Fixing Solution: 30 % (v/v) Ethanol, 5 % (v/v) acetic acid in H_2O.
2. Sensitizer Solution: 0.02 % (w/v) Sodium Thiosulphate (S6672, Sigma). Prepare 10 % (w/v) Sodium Thiosulphate and dilute in H_2O to obtain the final solution.
3. Silver Solution: 0.1 % (w/v) $AgNO_3$ (Ultrapure, Sigma) and 0.028 % (v/v) of 37 % formaldehyde solution (Sigma).
4. Developer Solution: 2.4 % (w/v) anhydrous Sodium Carbonate, 0.028 % (v/v) of 37 % formaldehyde solution, 0.0125 % (v/v) of 10 % (w/v) = Sodium Thiosulphate.
5. Stop solution: 4 % (w/v) Tris-base (Sigma), 2 % (v/v) acetic acid.
6. Destaining solution: 1.6 % (w/v) Sodium Thiosulphate and 1 % (w/v) potassium ferricyanide (Sigma). It has to be prepared just before use.

2.3.2 In-Gel Trypsin Digestion

1. Acetonitrile (ACN) (HPLC-grade).
2. Ammonium bicarbonate Solution: 50 mM NH_4HCO_3 (Sigma).
3. 25 mM NH_4HCO_3.
4. 50 % (v/v) ACN in 50 mM NH_4HCO_3.
5. Reduction Solution: 20 mM DTT in 50 mM NH_4HCO_3.
6. Alkylation Solution: 100 mM iodoacetamide in 50 mM NH_4HCO_3.
7. Trypsin solution: 10 ng/μL trypsin in 25 mM NH_4HCO_3 (20 μg trypsin/2 mL 25 mM NH_4HCO_3) (Trypsin, mass spectrometry sequence grade, G-BioSciences, Agro-Bio, La Ferté Saint Aubin, France).
8. Extraction solution: 45 % (v/v) ACN, 10 % (v/v) formic acid.

2.4 Indirect Immunofluorescence

1. PBS (Phosphate-buffered saline): 0.02 M Phosphate, pH 7.5, 150 mM NaCl.
2. Fixation solution: 3 % (w/v) PFA (paraformaldehyde) in PBS.
3. Neutralization solution: 50 mM NH_4Cl (ammonium chloride) in PBS.
4. Permeabilization solution: 0.1 % (v/v) Triton X-100 in PBS.
5. IF blocking solution: 10 % (v/v) goat serum in PBS (Lonza, Verviers, Belgium).
6. Texas Red-, FITC- or Alexa-conjugated secondary antibodies (Molecular Probes, Life Technologies, Invitrogen, Saint Aubin, France).
7. DAPI solution: Prepare a 10 mg/L stock solution of DAPI (4,6-diamino-2-phenylindole) in ultrapure water and store at 4 °C in the dark. Before use, dilute the stock solution to reach a final concentration of 50 ng/mL (1/200) in 10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, 10 mM mercaptoethylamine.
8. Mounting solution. Mowiol solution (Calbiochem, Merck chemicals, Nottingham, UK).

2.5 OGT Activity Measurement

1. Cell lysis buffer: 50 mM Tris–HCl pH 7.8, 150 mM NaCl, 1 % (v/v) NP-40, EDTA-free inhibitor cocktail (Roche Diagnostics).
2. OGT activity buffer [2×]: 100 mM Sodium Cacodylate, pH 6.5, 10 mM $MnCl_2$, 5 mM 5′-AMP.
3. Peptide-substrate YSDSPSTST (100 nmol/μL).
4. UDP-[^{3}H]-GlcNAc (50 μCi/500 μL).
5. Stop Solution: 100 mM formic acid.
6. Methanol (HPLC grade).

7. C18 phase Equilibration buffer: 50 mM formic acid.
8. C18 phase wash buffer: 1 M NaCl in 50 mM formic acid.
9. Elution buffer: 50 % ACN.
10. Sep-Pak C18 cartridges (200 mg Sorbent per Cartridge, 37–55 μm Particle Size, Waters, Milford, MA, USA).

2.6 UDP-GlcNAc Assay

This procedure is based on HPAEC (High pH Anion Exchange Chromatography).

1. Hypotonic lysis buffer: 10 mM Tris–HCl, pH 7.2, 10 mM NaCl, 15 mM 2-mercaptoethanol, 1 mM $MgCl_2$, protease inhibitors.
2. 1 M HCl.
3. Dowex 50WX2-400 in its H^+ form.
4. ProPAC-PA1 column (4×250 mm) (Dionex, Jouy en Josas, France).
5. Dionex HPLC system (Dionex).
6. UV spectrophotometer (Shimadzu, Marne la Vallée, France).
7. Neutralization buffer: 1 M Tris–HCl, pH 8.0.
8. Solution A: 20 mM Tris–HCl, pH 9.2.
9. Solution B: 2 M NaCl.

3 Methods

3.1 Western Blotting

1. Lyse cells in IP lysis buffer on ice (alternatively, cells may be directly homogenized in Laemmli buffer).
2. Centrifuge the cell extract at 20,000×*g* for 10 min at 4 °C.
3. Discard the membrane pellet.
4. Perform a Bradford, Lowry or BCA assay to determine protein concentration. Bovine serum albumin (BSA) is a frequently used protein standard. You can freeze samples at –20 °C (short time) or –80 °C for later use (protein concentration cannot be determined if cell lysis is performed in Laemmli buffer).
5. Add a volume of Laemmli buffer.
6. Boil the samples for 10 min.
7. Run proteins by SDS-PAGE (load the equivalent of 10–40 μg proteins per lane for a mini-gel).
8. Electrotransfer proteins onto nitrocellulose membrane.
9. Control the equal loading and transfer efficiency using Ponceau red staining (0.1 % (m/v) Ponceau S, 5 % (v/v) acetic acid).
10. Saturate the membrane and block unspecific sites with blocking buffer for 30 min at room temperature. It is not necessary to wash.

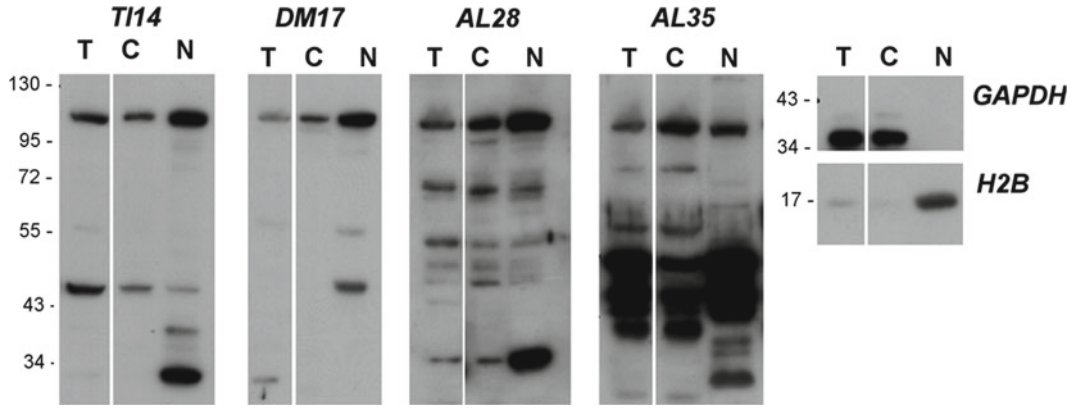

Fig. 1 Immunoblots obtained with anti-OGT. Cytosol and nuclei were prepared from MCF7 cells (not described). Cytosolic (C), nuclear (N), and total proteins (T) were run on a 10 % SDS-PAGE, electroblotted onto nitrocellulose sheet and analyzed by Western blot using anti-OGT antibodies (TI14, DM17, AL28, and AL35). GAPDH was used as a marker of cytosol and H2B (Histone H2B) as a nuclear control. Molecular mass markers are indicated at the *left* (kDa)

11. Dilute the anti-OGT into the blocking buffer at a final dilution of 1:1,000–1:2,000.
12. Incubate the solution with the membranes either 1 h at room temperature or overnight at 4 °C.
13. Discard the solution containing the primary antibody and store it at −20 °C (anti-OGT may be reused at least two or three times).
14. Wash membranes three times for 10 min in TBS-T.
15. Incubate membranes with anti-rabbit IgG HRP-labeled secondary antibody at a dilution of 1:10,000.
16. Wash membranes three times for 10 min in TBS-T.
17. Prepare ECL reagent just before use according to the manufacturer's recommendations.
18. Perform the development of the reaction using Hyperfilms, BioMax films of a CCD camera (Fig. 1).

3.2 Immunoprecipitation

All procedures should be carried out on ice (*see* **Note 4**).

3.2.1 Cell Lysis

1. Wash cells twice with ice-cold PBS (10 mL each wash for a 100 mm diameter Petri dish).
2. Place the cell culture dishes on ice and proceed to cell lysis by adding IP lysis buffer (0.5–1 mL for a 100 mm diameter Petri dish) for 10 min. Avoid exaggerated shaking.
3. Centrifuge cell lysates at 20,000 × *g* for 10 min at 4 °C.

4. Collect supernatants.
5. Perform a Bradford, Lowry or BCA assay to determine protein concentration. You can freeze samples at –20 °C (short time) or –80 °C for later use.

3.2.2 Preclearing the Lysates (See Note 5)

1. Prepare the Sepharose beads: Rinse the beads twice in PBS (to remove ethanol) and finally dilute them 1:1 (v/v) in IP lysis buffer.
2. Add 50 µL of protein A-coupled Sepharose to each sample (0.5–1 mg) for 1 h at 4 °C, under rotation.
3. Spin in micro-centrifuge at 1,500 rpm at 4 °C for 5 min.
4. Discard bead pellet and keep supernatant for immunoprecipitation.

3.2.3 OGT Immunoprecipitation

1. On ice, add 10 µL of rabbit polyclonal anti-OGT antibody to the precleared sample (final dilution of 1:100–1:500).
2. Place the tubes at 4 °C overnight under gentle agitation or rotation.
3. Mix the slurry well and add 30 µL of the beads to each sample. Incubate the lysate beads mixture at 4 °C under rotary agitation for 1 h.
4. Gently centrifuge beads for 1 min at 4 °C.
5. Carefully discard the supernatant using a vacuum water pump.
6. Wash the beads by adding successively 1 mL IP lysis buffer, IP lysis buffer/NaCl, IP lysis buffer/TNE and TNE alone and by vortexing for 1 min. Repeat **steps 4** and **5** between each wash.
7. Remove the last supernatant and add 25–50 µL of 2× Laemmli buffer. Boil for 5 min. You can then freeze the samples or run them on a SDS-PAGE gel.

3.3 Co-immunoprecipitation

All procedures should be carried out on ice (*see* **Note 4**). Wear powder-free gloves at all stages to avoid keratin contamination of the samples.

3.3.1 Cell Lysis

1. Wash cells twice with ice-cold PBS (10 mL each wash for a 100 mm diameter Petri dish).
2. Place the cell culture dishes on ice and proceed to cell lysis by adding co-IP lysis buffer (0.5–1 mL for a 100 mm diameter Petri dish) for 10 min. Avoid shaking.
3. Centrifuge cell lysates at 20,000 × *g* for 10 min at 4 °C.
4. Collect supernatants.
5. Perform a Bradford, Lowry, or BCA assay to determine protein concentration. Bovine serum albumin (BSA) is a frequently used protein standard. You can freeze samples at –20 °C (short time) or –80 °C for later use.

3.3.2 Preclearing the Lysates, Co-immunoprecipitation of OGT and Partners

This is the same procedure than described in the Subheading 3.2.

1. On ice, add 10 μL of rabbit polyclonal anti-OGT antibody to the precleared sample.
2. Place the tubes at 4 °C overnight under gentle agitation or rotation.
3. Mix the slurry well and add 30 μL of the beads to each sample. Incubate the lysate beads mixture at 4 °C under rotary agitation for 1 h.
4. Gently centrifuge beads for 1 min at 4 °C.
5. Carefully discard the supernatant using a vacuum water pump.
6. Wash beads four times by adding 1 mL co-IP lysis buffer and by vortex for 1 min very gently. Repeat **steps 4** and **5** between each wash.
7. Remove the last supernatant and add 25–50 μL of 2× Laemmli buffer. Boil at 95–100 °C for 5 min. You can then freeze the samples or run them on a SDS-PAGE gel.
8. Resolve proteins by SDS-PAGE (*see* **Note 6**).

3.3.3 Silver-Staining

Silver staining is used for sensitive detection of proteins separated by SDS-PAGE with detection limits from 0.5 to 5 ng. However its linearity is limited only over a short detection range. Gently agitate. Wear powder-free gloves at all stages and use clean staining trays to avoid keratin contamination of the gel. Use ONLY ultrapure water for all the solutions as well as for all the wash steps. Make Sensitizer, Stain and Developer Solutions *fresh* before each use.

1. Remove gel from glass and place in Fixing Solution for 10 min.
2. Replace Fixing solution and shake for at least 1 h.
3. Wash the gel in ultrapure water for 15 min. Repeat twice.
4. Sensitize the gel in Sensitizer Solution for only 1 min.
5. Wash the gel in ultrapure water for 1 min. Repeat once.
6. Incubate the gel for 45 min to 1 h in silver nitrate solution (*see* **Note 7**).
7. Wash the gel with ultrapure water for 30 s.
8. Discard water and add Developer solution. Shake for 30 s to remove excess of silver nitrate.
9. Change Developer solution and agitate until the staining is sufficient (5–10 min). Do not develop for more than 10 min.
10. Discard developer solution and add Stop solution. Shake for at least 10 min.
11. Wash the gel with ultrapure water.

3.3.4 In-Gel Trypsin Digestion

Wear powder-free gloves at all stages and work on a clean surface to avoid keratin contamination. Use only ultra pure water for all the solutions that have to be prepared freshly. It is advisable to prepare small volumes of solutions in a small flask rather than in plastic tubes to avoid contaminations with plastic polymers that interfere with mass spectrometry analysis. For low-level proteins (<1 pmol), reduction and alkylation is recommended to increase the sequence recovery by mass spectrometry.

1. Add 100 µL (enough to cover gel pieces) of 50 mM NH_4HCO_3 and vortex for 10 min.
2. Discard the supernatant and add 100 µL of 50 mM NH_4HCO_3/50 % ACN. Vortex for 10 min.
3. Discard and add ACN. Vortex for 10 min.
4. Discard ACN and dry the gel pieces by SpeedVac centrifugation to complete dryness (5–10 min).
5. Add 100 µL (or enough to cover) of Reduction Solution to the dried gel pieces and incubate the tubes at 56 °C for 1 h.
6. Discard supernatant and add 100 µL of Alkylation Solution. Allow the reaction to proceed in the dark for 45 min at room temperature. Vortex every 15 min.
7. Discard supernatant. Wash gel pieces with 100 µL of 50 mM NH_4HCO_3. Vortex for 10 min.
8. Discard supernatant and repeat successively with 50 mM NH_4HCO_3/50 % ACN and ACN to dehydrate gels.
9. Dry (SpeedVac) the gel pieces to complete dryness.
10. Add trypsin solution to just barely cover the gel pieces (~10–30 µL). Rehydrate the gel pieces on ice or at 4 °C for 20–30 min.
11. Remove excess of trypsin solution and add 25 mM NH_4HCO_3 as needed to cover the gel pieces.
12. Incubate at 37 °C overnight.

3.3.5 Extraction of Peptides

1. Spin briefly and Transfer the digest solution (aqueous extraction) into a clean 0.5 mL tube.
2. Add 50–70 µL (enough to cover) of extraction solution (45 % ACN/10 % formic acid) and vortex for 20 min.
3. Transfer the extracted solution into the 0.5 mL tube. Repeat once.
4. Vortex the extracted digests and dry (SpeedVac) the extracted peptides to complete dryness (~2 h).
5. Analyze with LC-MS/MS.

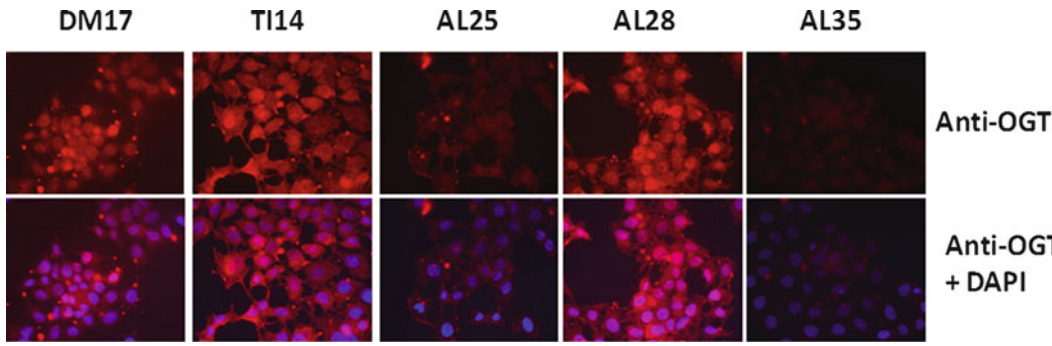

Fig. 2 Indirect IF pictures obtained with anti-OGT. MCF7 cells were prepared as described in Subheading 3.3 and analyzed with a panel of anti-OGT antibodies (TI14, DM17, AL25, AL28, and AL35). A DAPI staining was performed to specifically visualize the nuclei. Note that TI14, DM17, and AL28 are particularly efficient for staining OGT by IF

3.4 Immunofluorescence

Glass coverslips must be handled with care.

1. Cells are grown on glass coverslip in 60-mm plate dishes or on a six-well plate.
2. Gently rinse cells three times with ice-cold PBS.
3. Fix cells in fixative solution for 15 min at room temperature.
4. Wash with PBS.
5. Eliminate excess of PFA by incubating cells with neutralization solution for 10 min.
6. Wash with PBS.
7. Incubate cells with the permeabilization buffer for 5 min.
8. Block nonspecific sites with IF blocking solution for 30 min at room temperature.
9. Incubate coverslips with polyclonal anti-OGT antibody AL28 at a dilution of 1:100 in the IF blocking solution for 1 h. Alternatively, incubation can be performed at 4 °C overnight.
10. Wash three times with PBS.
11. Incubate coverslips with Texas Red-, FITC- or Alexa-conjugated secondary antibodies at a dilution of 1:100 in the IF blocking solution for 1 h at room temperature, in the dark.
12. Wash with PBS.
13. Stain nuclei with the DAPI solution for 30 min. Do not wash.
14. Perform mounting of coverslips in Mowiol solution (5 μL) onto the microscope slides (Fig. 2).

3.5 OGT Activity Assay

3.5.1 Preparation of Lysate from Cell Culture

1. Wash the cells with ice-cold PBS. Repeat once.
2. Place the cell culture dish in ice and add ice-cold lysis buffer. Allow the lysis to proceed for 15 min.
3. Scrape cells and transfer the lysate into a precooled microcentrifuge tube.
4. Spin at 20,000 × *g* for 20 min in a 4 °C precooled microcentrifuge.
5. Transfer the supernatant to a fresh tube kept on ice and discard the pellet.
6. Perform a Bradford, Lowry, or BCA assay to determine protein concentration of samples.

3.5.2 Labeling of OGT Peptide Substrate

1. Transfer 150 μL of protein extract (250–500 μg) into a precooled microcentrifuge tube in ice.
2. Add 160 μL of OGT activity buffer to 150 μL of protein extract (200–500 μg). Check the pH of the mix and adjust to pH 6.5 if necessary.
3. Add 1–10 μg of peptide substrate and 0.5 μCi UDP-[^{3}H]-GlcNAc. As a negative control of the assay, perform the reaction without any peptide substrate.
4. Allow the reaction to proceed for 1 h at room temperature.
5. Stop the reaction by adding 288 μL of 100 mM formic acid.
6. Spin at 10,000 × *g* for 5 min to clarify the mixture.
7. Transfer the supernatant into a clean microcentrifuge tube.

3.5.3 Removing Excess of UDP-[^{3}H]-GlcNAc and Desalting Labeled Peptide Substrate

1. Prepare the C18 phase by loading 10 volumes of methanol onto the top of the column. Methanol can be pushed through the C18 material by using a plastic syringe (e.g., 10 mL plastic disposable syringe).
2. Equilibrate the C18 phase by loading 10 volumes of 50 mM formic acid.
3. Load the sample onto the C18 phase. Slowly push the sample over the C18 phase.
4. Collect the flow-through fraction in a clean Eppendorf tube. Capture efficiency can be improved by passing the flowthrough over the C18 phase again.
5. Desalt the labeled peptide by loading successively 5 volumes of wash buffer, 5 volumes of equilibration buffer, and 5 volumes of H_2O onto the C18 tip. Buffers can be pushed through the C18 material by using a plastic syringe.
6. Elute the desalted peptide by loading 5 volumes of elution buffer onto the C18 column and slowly passing it over the C18 phase. Collect eluate in clean microcentrifuge tubes.

7. Dry the sample in a SpeedVac centrifuge. Alternatively, eluates can be frozen in a 15-mL conical tube and lyophilized.
8. Resuspend in H_2O and combine eluates in a microcentrifuge tube (200 μL).
9. Measure the incorporation of radioactive GlcNAc into the peptide substrate by scintillation counting.

3.6 UDP-GlcNAc Assay

1. Wash cells in ice-cold PBS.
2. Lyse cells in 1 mL of hypotonic buffer.
3. Add 50 μL of 1 M HCl to acidify the protein lysate. Perform a protein assay using the Bradford procedure (not BCA due to oxydoreduction interference with 2-mercaptoethanol).
4. Prepare a column using a Pasteur pipette with 1.5 mL of Dowex 50WX2-400 (H^+-activated).
5. Wash with 5 mL of ultrapure water.
6. Load the cell lysis onto the column.
7. Keep the unbound fraction into a 15 mL Falcon tube (polypropylene) in which 0.5 mL of neutralization buffer was previously added. Perform the collect on ice.
8. Wash the column with ultrapure water until the final volume reaches 10 mL in the Falcon tube.
9. Inject 250–1,000 μL of the unbound fraction using the ProPAC-PA1 column.
10. Use the following elution program: solution A for 1 min; elution gradient for 29 min with 85 % solution A and 15 % solution B; plateau of 5 min in these conditions; an elution gradient of 10 min until 100 % solution B is reached; maintain a plateau at 100 % solution B for 5 min.
11. Re-equilibrate the column in 100 % solution A.
12. Use a flow rate of 1 mL/min.
13. Detection is performed at 256 nm.

4 Notes

1. Alternatively rabbit polyclonal AL25, AL28, and AL35 antibodies produced in Dr. Gerald Hart's laboratory work also very well [6].
2. Films are usually more sensitive than CCD cameras. However regarding grey levels, the dynamic range is near 3.5–4.5 logs for CCD cameras against 2 logs for films.
3. Add protease inhibitors to all buffers (Inhibitor cocktail tablets, Roche Diagnostics, or Sigma).

4. Controls for the immunoprecipitation specificities of the polyclonal antibodies were performed with normal rabbit IgG (Santa Cruz Biotechnology, Heidelberg, Germany).
5. Preclearing the lysate is recommended to reduce non specific binding of proteins to agarose or Sepharose beads. It is advisable to use pipette tips with the end cut off to prevent damage to the beads.
6. A gradient gel will provide a greater separation of proteins. Usually 7.5–15 % SDS-PAGE allows separation of proteins ranging from 250 to 10 kDa.
7. Staining is enhanced with cold $AgNO_3$.

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Résumé

Une mauvaise hygiène alimentaire et certains désordres métaboliques sont décrits depuis plusieurs années comme des facteurs de risque majeurs du cancer colorectal. Ainsi, les individus diabétiques montrent une probabilité deux fois plus élevée de développer un cancer colorectal que des individus sains. Néanmoins, les mécanismes moléculaires reliant ces facteurs à la cancérisation colique et rectale restent mal compris. Comment un désordre nutritionnel ou métabolique influence-t-il l'initiation et la progression tumorale ? Pour tenter de répondre à cette question, nous nous sommes focalisés sur une voie de signalisation essentielle à l'initiation tumorale colique, la voie Wnt/β-caténine. La β-caténine, élément central de cette voie, contrôle la transcription de nombreux gènes parmi lesquels c-myc et la cycline D1. La régulation de cette activité transcriptionnelle est régie par un système de dégradation efficace. Il consiste en une séquestration de la β-caténine dans un complexe de destruction entraînant sa phosphorylation au niveau d'une séquence appelée « Destruction-box ». Cette phosphorylation est suivie de l'ubiquitinylation puis de la dégradation de la β-caténine par le protéasome. Dans un contexte de cancérisation colique et rectale, la β-caténine est stabilisée, soit par sa propre mutation, soit par des mutations du complexe de destruction, empêchant son recrutement et sa dégradation. Nous nous sommes interrogés sur le fait que des désordres métaboliques ou nutritionnels pouvaient participer à la stabilisation de la β-caténine et, de ce fait, à l'initiation tumorale.

Pour cela, nous nous sommes intéressés à une autre modification post-traductionnelle de la β-caténine susceptible d'affecter ses fonctions : la *O*-GlcNAcylation. Jusqu'alors, la modification de la β-caténine par un résidu de *N*-acétylglucosamine (GlcNAc) avait été décrite sans pour autant en comprendre la fonction. La *O*-GlcNAcylation est une modification réversible et dynamique, affectant les protéines cytosoliques, nucléaires et mitochondriales. Parce que phosphorylation et *O*-GlcNAcylation affectent les mêmes acides aminés, sérine et thréonine, il est fréquent que les deux modifications soient compétitrices l'une de l'autre. Un couple d'enzymes unique régule la *O*-GlcNAcylation des protéines : la *O*-GlcNAc transférase (OGT) et la *O*-GlcNAcase (OGA) qui catalysent respectivement l'ajout et l'hydrolyse de la GlcNAc. Ce caractère hautement dynamique en fait un élément essentiel dans la régulation de nombreux processus cellulaires physiologiques et pathologiques, au même titre que la phosphorylation. Par ailleurs, la *O*-GlcNAcylation est considérée comme un senseur nutritionnel puisque qu'elle retranscrit au sein de la cellule, le statut nutritionnel (et notamment la concentration en glucose extracellulaire) par l'intermédiaire de la voie de biosynthèse des hexosamines. De ce fait, la *O*-GlcNAcylation reflèterait au niveau protéique le comportement alimentaire et par extension un déséquilibre métabolique se répercuterait sur le dynamisme de cette glycosylation.

Au cours de la thèse, nous avons cherché à comprendre comment les caractéristiques de la β-caténine sont affectées par des variations de *O*-GlcNAcylation. Nous avons démontré que la *O*-GlcNAcylation de cette dernière la stabilisait, notamment en réponse à une augmentation du glucose extracellulaire. Afin de mieux comprendre ce mécanisme, nous avons entrepris la cartographie des sites de *O*-GlcNAcylation. La modification de la sérine 23 et des thréonines 40 et 41 concentre la *O*-GlcNAcylation de la β-caténine dans le domaine N-terminal impliqué dans les processus de dégradation. Nous avons mis en évidence une compétition directe entre *O*-GlcNAcylation et phosphorylation sur la thréonine 41. Une élévation de *O*-GlcNAcylation diminue l'ubiquitinylation et l'interaction de la β-caténine avec le suppresseur de tumeur APC, pièce essentielle du complexe de destruction. De ce fait, nous proposons qu'en réponse à une augmentation de la concentration en glucose, la *O*-GlcNAcylation entrave le déroulement de la séquence de dégradation de la β-caténine. Nous avons également démontré que la *O*-GlcNAcylation augmente l'activité transcriptionnelle de la β-caténine, et accélère la prolifération cellulaire.

D'autre part, l'expression de β-caténine, observée au cours de la reprise du cycle cellulaire, s'accompagne d'une interaction avec l'OGT. Par ailleurs, l'expression de l'OGT est elle-même augmentée pendant la phase G1, suggérant un rôle de l'enzyme dans les étapes précoces du cycle cellulaire. Il a été décrit qu'au cours de cette phase critique, la β-caténine contrôle l'expression de la cycline D1 nécessaire à la poursuite du cycle cellulaire. A ce niveau, l'OGT jouerait un rôle essentiel puisque son inhibition bloque l'activité transcriptionnelle de la β-caténine (et la synthèse de cycline D1).

Ainsi, l'interaction de la β-caténine avec l'OGT ainsi que sa *O*-GlcNAcylation subséquente joueraient un rôle essentiel sur son activité transcriptionnelle et potentiellement sur son caractère oncogénique, notamment au cours de la cancérogénèse colique. En effet, des analyses de lignées cancéreuses et d'extraits de tumeurs humaines coliques ont montré une augmentation significative des niveaux de *O*-GlcNAcylation et de β-caténine en comparaison respectivement à une lignée colique fœtale et aux tissus adjacents sains. De plus, la *O*-GlcNAcylation de la sérine 23 de la β-caténine a été retrouvée dans les tissus cancéreux.

L'influence de certains désordres métaboliques sur la cancérisation pourrait s'expliquer en partie par une stabilisation aberrante de la β-caténine liée à sa *O*-GlcNAcylation. Dans ce sens, nous avons montré qu'un régime alimentaire temporairement riche en glucose influence directement les niveaux de *O*-GlcNAcylation et de β-caténine dans les colons de souris C57Bl6. De même, une alimentation à long terme riche en sucres entraine le développement d'une intolérance au glucose et l'augmentation permanente de ces mêmes niveaux.

Nos travaux de thèse suggèrent qu'une stabilisation de β-caténine par *O*-GlcNAcylation en lien avec le régime alimentaire et plus largement l'hygiène de vie, impacterait sur ses propriétés et plus particulièrement son activité transcriptionnelle. Une mauvaise hygiène de vie et certains troubles métaboliques pourraient impacter la cancérisation colique et rectale par le biais d'une modification post-traductionnelle très largement exprimée, la *O*-GlcNAcylation.