

UNIVERSITE DE LILLE

Ecole Gradué Biologie Santé de Lille (n°446)

Doctorat

Aspects Moléculaires et Cellulaires de la Biologie

Dimitri VANAUBERG

Régulation de l'enzyme pro-oncogénique *Fatty Acid Synthase* (FASN) par la modification nutrition-dépendante O-GlcNAcylation dans des cellules cancéreuses hépatiques

Thèse dirigée par le **Pr. Tony LEFEBVRE** au sein de l'Unité de Glycobiologie Structurale et Fonctionnelle (UGSF) de Lille

En vue de l'obtention du grade de Docteur

Soutenue publiquement le 22 Octobre 2025 devant le jury composé de :

Dr. Ganna PANASYUK, Présidente

Univ. Paris, Institut Necker-Enfants Malades, INSERM U1151/CNRS UMR 8253, Paris

Dr. Fabienne FOUFELLE, Rapporteuse

Univ. Paris, Centre de Recherche des Cordeliers, INSERM, Paris

Pr. Benjamin LAUZIER, Rapporteur

Univ. Nantes, Unité de Recherche de l'Institut du Thorax, INSERM U1087/CNRS UMR 6291, Nantes

Dr. Nicolas JONCKHEERE, Examineur

Univ. Lille, CHU Lille, INSERM U1277/UMR 9020 (CANTHER), Lille

Dr. Alain MARTORIATI, Examineur

Univ. Lille, Faculté des Sciences et Technologies, CNRS UMR 8576 (UGSF), Lille

Dr. Capucine VAN RECHEM, Membre invitée

Stanford University, Department of Pathology, Stanford, California

UNIVERSITY OF LILLE

Doctoral School of Biology and Health – Lille (n°446)

PhD Program

Molecular and Cellular Aspects of Biology

Dimitri VANAUBERG

**Regulation of the pro-oncogenic enzyme Fatty Acid Synthase
(FASN) by the nutrient-dependent modification
O-GlcNAcylation in hepatic cancer cells**

Thesis supervised by **Pr. Tony LEFEBVRE** within the Unit of Structural
and Functional Glycobiology (UGSF) in Lille

**Submitted in partial fulfillment of the requirements for the degree of Doctor
of Philosophy**

Publicly defended on October 22, 2025, with the jury composed of:

Dr. Ganna PANASYUK, Chair of the examination committee

Univ. Paris, Institut Necker-Enfants Malades, INSERM U1151/CNRS UMR 8253, Paris

Dr. Fabienne FOUFELLE, Reviewer

Univ. Paris, Centre de Recherche des Cordeliers, INSERM, Paris

Pr. Benjamin LAUZIER, Reviewer

Univ. Nantes, Unité de Recherche de l'Institut du Thorax, INSERM U1087/CNRS UMR 6291, Nantes

Dr. Nicolas JONCKHEERE, Examiner

Univ. Lille, CHU Lille, INSERM U1277/UMR 9020 (CANTHER), Lille

Dr. Alain MARTORIATI, Examiner

Univ. Lille, Faculté des Sciences et Technologies, CNRS UMR 8576 (UGSF), Lille

Dr. Capucine VAN RECHEM, Guest member

Stanford University, Department of Pathology, Stanford, California

« La persévérance, c'est ce qui rend l'impossible possible, le possible probable, et le probable réalisé. »

Robert Half

**« We must accept finite disappointment,
but never lose infinite hope. »**

Martin Luther King Jr.

A mon amour,
To my beloved,

ACKNOWLEDGMENTS/REMERCIEMENTS

Après ces quatre années passées à l'UGSF, je souhaiterais tout d'abord remercier le Directeur **Dr. Yann Guérardel**, pour m'avoir accueilli au sein de l'Unité ainsi que tout le **personnel** y travaillant.

Je souhaite également remercier l'ensemble des membres de mon jury, la présidente **Dr. Ganna Panasyuk**, les rapporteurs **Dr. Fabienne Foufelle** et **Pr. Benjamin Lauzier**, ainsi que les examinateurs **Dr. Alain Martoriati** et **Dr. Nicolas Jonckheere** pour avoir accepté d'évaluer mon travail de thèse et pour l'intérêt qu'ils y ont porté. Je remercie tout particulièrement **Dr. Ganna Panasyuk**, **Pr. Vanessa Dehennaut** et **Dr. Nicolas Jonckheere** pour les précieux conseils donnés lors de mes deux comités de suivi individuel. Je remercie nos autres collaborateurs : **Dr. Stéphanie Olivier-Van Stichelen** et **Dr. Fabrice Bray** pour la spectrométrie de masse, **Dr. Yannick Rossez** pour la lipidomique et l'équipe du **Dr. Marc Lensink** pour les analyses bioinformatiques. Je te remercie particulièrement **Guillaume** pour ton implication dans le projet depuis mon Master 2. Nos échanges ont toujours été très enrichissants.

Merci également au **Dr. Capucine Van Rechem** de me permettre prochainement d'intégrer son équipe au sein de la prestigieuse Université de Stanford. Mon rêve d'avoir une expérience professionnelle aux Etats-Unis est sur le point de se concrétiser. Je me réjouis à l'idée de cette collaboration, que je sais déjà prometteuse. Merci d'avoir aussi accepté de participer à ma soutenance de thèse.

J'aimerais exprimer ma gratitude envers le **Pr. Tony Lefebvre**, le directeur de thèse que tout doctorant rêverait d'avoir... Sans vouloir exagérer, Tony, je te remercie grandement pour ton optimisme et ta sympathie quotidienne. Chacun de nos échanges m'a aidé à faire avancer mon projet de thèse, grâce au fait que tu te rendes facilement disponible pour les personnes que tu encadres. Merci notamment pour ta confiance qui m'a permis d'être un maximum autonome et de m'avoir laissé prendre du recul quand j'en avais besoin. Et un grand merci pour tes cours de biochimie : toujours clairs et motivants ! Ce fut un vrai plaisir de travailler ensemble et de me rendre chaque jour au labo, merci d'avoir toujours cru en moi et de m'avoir permis de participer à un congrès aux Etats-Unis. Malgré les nombreuses déceptions inhérentes à la recherche, je garderai un très beau souvenir de ces années de travail.

Je souhaite également remercier **Sadia** pour le temps qu'elle aura consacré à m'apporter les outils méthodologiques indispensables à la réalisation de mes expériences, notamment en culture cellulaire. Merci Sadia, ta patience et ton encadrement m'ont été d'une grande aide et m'ont motivé à poursuivre en thèse. Je te souhaite le meilleur pour la suite de ta carrière !

Je tiens à remercier **tous les membres de l'équipe** de m'avoir intégré, aidé quotidiennement et de m'avoir permis de réaliser ma thèse dans d'excellentes conditions de travail. La bonne humeur était présente chaque jour, c'est pourquoi il était très agréable de venir travailler. Je te remercie tout particulièrement **Céline**. Ta rigueur, ta patience et ton aide m'ont beaucoup apporté. Merci de m'avoir aussi bien formé en biologie moléculaire et de t'être toujours rendue disponible quand j'en avais besoin. Merci pour tous les services que tu m'as rendus ! **Marlène**, même si tu es partie en retraite durant mon M2, merci pour ton aide et ta gentillesse. **Peggy**, merci pour ton aide également, notamment pour les midipreps ! **Dorothée**, je te remercie pour la gestion du laboratoire L2, mon endroit préféré du C9. 😊

Merci à **Ikram, Anne-Sophie, Caroline et Stéphan** pour votre suivi de près ou de loin de mon travail de thèse, notamment durant les réunions d'équipe. Grâce à votre expertise, nos échanges m'ont permis de faire avancer mon projet. Merci de faire de la *O-GlcNAc* team une équipe chaleureuse et accueillante ! 😊 Même si étions dans des bureaux différents, je remercie notre chef de bureau **Quentin**. 😊 Je te remercie beaucoup pour ton aide constante au sein du labo. Tu as toujours été à l'écoute quand j'en avais besoin, je te souhaite vivement de réussir dans tes projets à venir. Une pensée également pour **Ferdinand**, je me souviendrai toujours de tes blagues dans le labo et j'espère que tu t'épanouis entièrement dans tes nouvelles fonctions. **Gabriela**, estoy « hasta la madre » escribiendo esta tesis ahahah. Quel plaisir d'avoir pu réexercer un peu d'espagnol ensemble, même si j'ai encore du chemin à faire pour être trilingue ! Pour ta grande aide en cytométrie en flux, muchas gracias. Je te souhaite un bon retour au Mexique et j'espère pouvoir venir te rendre visite un jour. Hasta pronto ! 😊 Mon petit **Thomas**, ne lâche rien ! Comme moi, tu enchaînes les galères depuis le M2 mais je n'ai aucun doute en ta réussite. C'était un plaisir de travailler ensemble, merci pour l'intérêt que tu m'as toujours porté, pour nos nombreuses conversations. Je te souhaite de réussir brillamment ta thèse !

Je ne sais pas si j'ai besoin de citer de nom en parlant de café noisette... **Jodie** ?! Grâce à toi je commence petit à petit à me faire au « café » (toujours pas pur mais ça viendra peut-être...), que j'ai d'ailleurs pas mal consommé durant l'écriture de cette thèse ! Merci pour ton aide également au labo, j'espère que tu réussiras ce concours dans la police scientifique ! **Awatef**, encore désolé d'avoir autant rigolé le jour où tu t'es prise cette fameuse porte... 😊 Bon tu ne m'en veux pas, tu sais que je suis bon public. Merci pour ta bonne humeur et de nous avoir fait rire durant ces années, je suis très content de savoir que tu te plais dans ton nouveau travail.

Emilie, même si tu n'as plus été là à partir de ma seconde année de thèse, encore merci pour ton aide en bio cell durant ma première année ! C'était un plaisir de discuter ensemble tôt le matin au bureau, je te souhaite le meilleur pour la suite de ta carrière. **Anastasia** et **Clara**, merci pour les ondes positives apportées au bureau 115, ahah. C'était un plaisir de discuter et rigoler ensemble, je vous souhaite de réussir au mieux vos projets de thèse ! Thank you **Taiki**. 😊 Comme tu parles de mieux en mieux français je vais éviter l'anglais : grâce à toi j'ai pu perfectionner mon anglais, merci pour les nouveaux mots que tu m'as appris ahah. It was a pleasure to exchange together and I wish you all the best for the future! **Christer**, content de savoir que tu te plais à Paris, bonne continuation en post-doc ! **Marie**, bravo pour ton beau parcours et la belle réussite de ton M2. J'espère avoir de tes nouvelles et que tu t'épanouiras pleinement dans ton futur travail. Bravo **Jean-Sébastien** pour la réussite du concours de l'ED, que ta thèse soit remplie de beaux résultats. **Sarah** et **Harmony**, je vous souhaite aussi de poursuivre dans la voie que vous souhaitez. **Maxime**, bonne continuation en thèse, ne t'épuise pas trop avec les allers-retours entre le C9 et ONCOLille ! **Clara** (du deuxième étage), bon courage pour l'arrivée de ta troisième année ! C'était toujours un plaisir de discuter avec toi, merci pour ta bonne humeur. **Angélina**, content de savoir que tu aies trouvé un post-doc à ONCOLille, je te souhaite de t'y épanouir pleinement. Encore merci pour ton encadrement quand j'étais en L2 avec Malory, ce fut pour nous une belle découverte de la recherche. **Agnès**, **Sophie**, **Marie-Ange**, **Yoshiko**, **Kenji**, **Mathieu** et **Fabrice** : merci pour votre accueil et nos échanges.

Laurence, merci pour ton travail dans les démarches administratives. Merci **Adeline** et **Olivier** pour mes nombreuses demandes de séquençage. Merci aussi à **Ingrid** (ONCOLille) pour les

cellules Hep3B et les analyses à l'Incucyte. Je remercie la plateforme PAGés, notamment **Nao** et **Louise**, pour la GC-FID ainsi que la plateforme BICeL pour la microscopie confocale.

Merci à mes **parents** de m'avoir soutenu et d'avoir rendu possible l'obtention de ma superbe Dacia. Ma **sœur**, même si on se voit peu, je te souhaite le meilleur avenir personnel et professionnel possible. A mes **beaux-parents** : un grand merci pour votre aide durant toutes ces années... Merci de chouchouter nos ninis quand on part en vacances ! **Mathéo**, **Helen** et **Teyla** : hâte de venir vous revoir à la Réunion ! Merci pour votre soutien. Vous nous manquez, on pense tous les jours à notre pitchoune internationale...

Mon amour, **Malory**, comment te remercier... Malgré les journées à rallonge passées au labo, la fatigue, le stress, les déceptions et remises en question, tu as toujours été là pour me soutenir à 200 % dans mes projets et me permettre constamment d'aller de l'avant. L'avantage d'avoir étudié dans le même domaine m'a permis de pouvoir t'expliquer mon avancée au fil du temps, merci d'avoir été une oreille attentive durant toutes ces années même quand je pouvais trop en parler... Tu sais à quel point je peux être persévérant et passionné. Même si c'est à contre-cœur, merci de comprendre et de soutenir ma volonté de partir en post-doc aux Etats-Unis, tu sais à quel point ça compte pour moi. J'ai toujours mesuré la chance que j'avais de t'avoir pour être consolé et de pouvoir rentrer tous les jours sans avoir à me soucier des tâches ménagères. J'espère que ton souhait de devenir professeure des écoles s'exaucera au plus vite, tu es faite pour ça. Merci de t'occuper si bien de nos deux enfants poilus, **Tokyo** et **Tigree**, qui font notre bonheur depuis le début de ma thèse. Après bientôt dix années passées à tes côtés, je t'aime toujours aussi fort.

À toutes les personnes qui ont, de près ou de loin, soutenu, accompagné ou contribué à ce projet : merci du fond du cœur.

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PREFACE

This doctoral thesis was carried out at the Structural and Functional Glycobiology Unit (UGSF “Unité de Glycobiologie Structurale et Fonctionnelle” - UMR CNRS 8576) in Villeneuve d'Ascq, in the team “Regulation of the molecular and cellular mechanisms by O-GlcNAcylation” under the supervision of the team director, Pr. Tony Lefebvre.

This thesis was funded by the University of Lille and was the subject of publications, and oral and poster presentations presented below.

PUBLICATIONS:

Dimitri Vanauberg, Céline Schulz, and Tony Lefebvre. Involvement of the pro-oncogenic enzyme fatty acid synthase in the hallmarks of cancer: a promising target in anti-cancer therapies (2023). *Oncogenesis* 12, 16. <https://doi.org/10.1038/s41389-023-00460-8>

Dimitri Vanauberg, Céline Schulz, Sadia Raab, Gabriela Fuentes-García, Emilie Cadart, Quentin Lemaire, Stéphanie Olivier-Van Stichelen, Fabrice Bray, Guillaume Brysbaert, Yannick Rossez, Stéphan Hardivillé, and Tony Lefebvre. O-GlcNAcylation of fatty acid synthase is required for its proper subcellular localization, expression level and activity (2025). *Journal of Biological Chemistry*. <https://doi.org/10.1016/j.jbc.2025.110497>

Dimitri Vanauberg, Céline Schulz, Guillaume Brysbaert, Nessim Raouraoua, Peggy Mistarz-Gruau, Marc Lensink, Anne-Sophie Vercoutter-Edouart, and Tony Lefebvre. Fatty Acid Synthase and O-GlcNAc Transferase preferentially interact *via* their respective N-terminal region (2025). *Biochemistry and Biophysics Reports*. *Submitted for publication*.

ORAL COMMUNICATIONS:

Dimitri Vanauberg and Tony Lefebvre. Regulation of FASN by the nutrient-dependent modification O-GlcNAcylation. 7th Latin American Glycobiology Congress (LAGC). Cuernavaca, Mexico (online). October 3rd - 6th, 2023.

Dimitri Vanauberg and Tony Lefebvre. Regulation of FASN by the nutrient-dependent modification *O*-GlcNAcylation in hepatic cancer. Satellite meeting of the 2024 SialoGlyco Congress. Lille, France. June, 4th, 2024.

Dimitri Vanauberg, Céline Schulz, Fabrice Bray, Guillaume Brysbaert, and Tony Lefebvre. Regulation of the pro-oncogenic enzyme FASN by the nutrient-dependent modification *O*-GlcNAcylation in cancer cells. 2024 Society for Glycobiology (SFG) meeting. Amelia Island, Florida, USA. November, 10th - 13th, 2024.

Dimitri Vanauberg, Céline Schulz, Sadia Raab, Emilie Cadart, Stéphanie Olivier-Van Stichelen, Fabrice Bray, Guillaume Brysbaert, Yannick Rossez, Stéphan Hardivillé, and Tony Lefebvre. Regulation of the pro-oncogenic enzyme FASN by *O*-GlcNAcylation in hepatic cancer cells. 4th PhD Students' Days. ONCOLille Institute, Lille, France. March, 13th - 14th, 2025.

POSTER PRESENTATIONS:

Dimitri Vanauberg, Sadia Raab, Steffi Baldini, Céline Schulz, Fabrice Bray, Guillaume Brysbaert, and Tony Lefebvre. Regulation of FASN by the nutrient-dependent modification *O*-GlcNAcylation in hepatic cancer. 16^{èmes} journées scientifiques du Cancéropôles Nord-Ouest (CNO). Deauville, France. May, 15th - 17th, 2024.

Dimitri Vanauberg, Céline Schulz, Fabrice Bray, Guillaume Brysbaert, and Tony Lefebvre. Regulation of the pro-oncogenic enzyme FASN by the nutrient-dependent modification *O*-GlcNAcylation in cancer cells. 2024 Society for Glycobiology (SFG) meeting. Amelia Island, Florida, USA. November, 10th - 13th, 2024.

AWARDS:

Student Travel Award from the SFG for the 2024 annual meeting headed in Amelia Island, Florida, USA. November, 10th - 13th, 2024.

RESUME

Du fait de leur prolifération anarchique, les cellules cancéreuses présentent un besoin accru en nutriments, incluant les lipides. Les lipides alimentaires n'étant pas suffisants pour satisfaire leurs besoins métaboliques, elles affichent généralement une lipogenèse *de novo* exacerbée. Ainsi, une des enzymes de la lipogenèse, l'acide gras synthase (FASN, *Fatty Acid SyNthase*), est souvent surexprimée dans les cancers. FASN est une « mégasynthase » possédant sept activités enzymatiques différentes et active sous la forme d'homodimères. En synthétisant des acides gras, cette enzyme ubiquitaire de 273 kDa contribue à la prolifération des cellules cancéreuses. En plus de favoriser la biogenèse des membranes cellulaires, FASN est aussi indirectement impliquée dans le stockage de l'énergie, la synthèse de messagers secondaires et l'acylation de protéines. FASN est ainsi capable de promouvoir différentes caractéristiques des cancers. Bien que n'étant pas capable à elle seule d'induire une transformation maligne, FASN est considérée comme étant une enzyme pro-oncogénique et constitue une cible thérapeutique intéressante.

D'autre part, les cellules cancéreuses sont aussi avides en glucose comme le démontre l'effet Warburg. Il est estimé que 2 à 3% du glucose entrant dans les cellules est redirigé vers la voie de biosynthèse des hexosamines (HBP). L'UDP-GlcNAc, produit final de la voie des hexosamines, est pris en charge par la O-GlcNAc transférase (OGT) qui catalyse le transfert du résidu GlcNAc sur l'hydroxyle d'une sérine ou thréonine d'une protéine cible. Cette modification, appelée O-GlcNAcylation, est réversible puisque la O-GlcNAcase (OGA) est capable d'hydrolyser le résidu de GlcNAc. La O-GlcNAcylation régule tout un ensemble de propriétés : interactions protéine-protéine, activité enzymatique, localisation subcellulaire, protéostase... De la même manière que FASN, l'OGT est active en homodimères et est fréquemment surexprimée dans les cancers. Favorisant de nombreuses caractéristiques des cancers, l'OGT constitue également une enzyme pro-oncogénique.

Les travaux de l'équipe se sont ainsi concentrés sur l'étude de la régulation croisée entre ces deux protéines pro-oncogéniques dans le contexte du cancer hépatique. Leur surexpression a notamment été démontrée par l'équipe dans des tissus cancéreux de patients. FASN est régulée par O-GlcNAcylation dans des foies de souris et dans la lignée HepG2. Cette modification favorise l'interaction de FASN avec la déubiquitineuse USP2a (*Ubiquitin-Specific Peptidase 2a*), réduisant sa poly-ubiquitination et par conséquent sa dégradation

protéasomale. L'OGT, de par son activité, favorise donc la stabilité de FASN. L'inhibition de l'OGT réduit significativement l'expression protéique de FASN. Inversement, l'inhibition de FASN diminue significativement l'expression protéique et l'activité de l'OGT. Ces perturbations résultent en une dérégulation du cycle cellulaire et une réduction de la viabilité des cellules HepG2.

Par diverses approches, nous avons dans un premier temps cherché à déterminer quels sites de *O*-GlcNAcylation de FASN étaient impliqués dans sa régulation. Dans un second temps, le but a été d'identifier les domaines d'interactions respectifs du complexe FASN-OGT. Nos résultats indiquent que les niveaux de *O*-GlcNAcylation régulent la synthèse d'acides gras des cellules HepG2. Le site T980 de FASN apparaît comme un site majeur de *O*-GlcNAcylation régulant nombreuses de ses propriétés : localisation, dimérisation, activité et protéostase. La modification de ce site soutient son caractère pro-oncogénique en promouvant la survie, la prolifération et la progression du cycle cellulaire de la lignée Hep3B. Enfin, FASN et OGT interagissent préférentiellement *via* leur partie *N*-terminal respective.

Ainsi, cibler le site T980 de FASN ou l'interaction FASN-OGT pourrait constituer une voie thérapeutique intéressante.

ABSTRACT

Due to their uncontrolled proliferation, cancer cells have an increased need for nutrients, including lipids. Dietary lipids being not sufficient to satisfy their metabolic needs, they generally display exacerbated *de novo* lipogenesis. Thus, one of the enzymes involved in lipogenesis, Fatty Acid Synthase (FASN), is frequently overexpressed in cancers. FASN is a « megasynthase » having seven different enzymatic activities, active in the form of homodimers. Synthesizing fatty acids from acetyl-CoA, malonyl-CoA and NADPH, H^+ , this ubiquitous 273 kDa enzyme contributes to cancer cell proliferation. As well as promoting the biogenesis of membranes, FASN is also indirectly involved in energy storage, the synthesis of second messengers and protein acylation. FASN is able to promote different hallmarks of cancers: resistance to apoptosis, migration/invasion, angiogenesis induction or even immune escape. Although not able on its own of inducing malignant transformation, FASN is considered as a pro-oncogenic enzyme and is an interesting therapeutic target.

On the other hand, cancer cells also have a high demand for glucose as demonstrated by the Warburg effect. It is estimated that 2 to 3% of the glucose entering cells is redirected to the hexosamine biosynthetic pathway (HBP). UDP-GlcNAc, the end product of HBP, is a nutritional sensor. Its synthesis depends on the metabolism of glucose, but also of amino acids, fatty acids and nucleotides. This nucleotide-sugar is handled by O-GlcNAc transferase (OGT) which catalyzes the transfer of GlcNAc on the hydroxyl group of a serine or threonine on a target protein. This modification, known as O-GlcNAcylation, is reversible as O-GlcNAcase (OGA) is able to hydrolyze the GlcNAc residue. O-GlcNAcylation regulates a whole range of protein properties: protein-protein interactions, enzymatic activity, subcellular location, stability... Likewise FASN, OGT is active in homodimers and is frequently overexpressed in cancers. Promoting many hallmarks of cancers, OGT is also a pro-oncogenic enzyme.

The team has thus focused on studying the cross-regulation between these two pro-oncogenic proteins in the context of hepatic cancer. Their overexpression has been demonstrated in patient cancer tissues. FASN is regulated by O-GlcNAcylation in mice livers and in the HepG2 cell line. This modification promotes FASN interaction with the deubiquitinase Ubiquitin-Specific Peptidase 2a (USP2a), reducing its poly-ubiquitination and consequently its proteasomal degradation. OGT's activity therefore promotes FASN stability. More recent work has highlighted the cross-regulation of these two enzymes. OGT inhibition significantly

reduces FASN protein expression. Conversely, FASN inhibition significantly reduces OGT protein expression and activity. These perturbations result in a deregulation of cell cycle and reduced viability of HepG2 cells.

By combining different approaches, we first aimed to determine which FASN *O*-GlcNAcylation sites were involved in its regulation. Secondly, the purpose was to identify FASN and OGT respective interaction domains. Our results indicate that *O*-GlcNAcylation levels regulate fatty acid synthesis in HepG2 cells. FASN T980 site appears to be a major *O*-GlcNAcylation site, positively regulating many of its properties: location, dimerization, activity and proteostasis. At the cellular level, modification of this site sustains its pro-oncogenic character by promoting survival, proliferation and cell cycle progression of the Hep3B cell line. At last, FASN and OGT interact preferentially *via* their respective *N*-terminal domain.

Thus, targeting FASN T980 site or FASN-OGT interaction could represent an interesting therapeutic avenue. Preventing FASN *O*-GlcNAcylation may reduce its pro-oncogenic properties, thereby slowing down tumor progression in patients with hepatic cancer.

ABBREVIATIONS AND NOMENCLATURE

5-FU: 5-FluoroUracil

A

ACC: Acetyl CoA Carboxylase

AcCoA: Acetyl-Coenzyme A

ACLY: Adenosine triphosphate (ATP) citrate lyase

ACP: Acyl carrier protein

ACSL: Acyl-CoA synthetase

AGPAT: Acylglycerol-3-phosphate acyltransferase

AML: Acute myeloid leukemia

AMPK: AMP-activated protein kinase

ANOVA: Analysis of variance

APC/C: Anaphase promoting complex/cyclosome

ATF4: Activating transcription factor 4

Atg: Autophagy-related protein

ATRA: All-*trans* retinoic acid

B

BCL2: B-cell-lymphoma-2

BMAL1: Brain and muscle ARNT-Like 1

C

CAC: Colitis-associated cancer

CaMKII: Calcium/calmodulin-dependent kinase II

CD: Catalytic domain

CDC20: Cell division cycle 20

CDK: Cyclin-dependent kinase

CDKI: CDK inhibitor

cDNA: complementary deoxyribonucleic acid

cGAS: Cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase

Chk1: Checkpoint kinase 1

ChREBP: Carbohydrate responsive element binding protein

CLOCK: Circadian locomotor output cycles kaput

CPT1: Carnitine palmitoyltransferase 1

CRC: Colorectal cancer

CREB: Cyclic AMP-response element binding protein

Cryo-EM: Cryo-electron microscopy

D

DGAT: Diacylglycerol acyltransferase

DH: Dehydratase

DNA: Deoxyribonucleic acid

DNL: *De novo* lipogenesis

E

EC: Endothelial cell

EGF: Epidermal growth factor

eIF2 α : eukaryotic translation initiation factor 2 α

ELOVL: Elongation of very long-chain fatty acids

EMT: Epithelial-mesenchymal transition

ER: Endoplasmic *reticulum*

ER α : Estrogen receptor α

F

F-2,6-BP: Fructose-2,6-Bisphosphate

FA: Fatty acid

FABP5: Fatty acid-binding protein 5

FASN: Fatty acid synthase

FFA: Free fatty acid

FoxM1: Forkhead Box M1

G

GC-FID: Gas phase chromatography with a downstream flame ionization detector

GFAT: Glutamine:fructose-6-phosphate amidotransferase

GH: Glycoside hydrolase

GK: Glucokinase

GlcNAc: N-acetyl-D-glucosamine

GPAT: Glycerol-3-phosphate acyltransferase

GSK3 β : Glycogen synthase kinase 3 β

H

HAT: Histone acetyltransferase

HBP: Hexosamine Biosynthetic Pathway

HCC: Hepatocellular carcinoma

HCF-1: Host Cell Factor-1

HDAC: Histone deacetylase

HGS: Hepatocyte growth factor-regulated tyrosine kinase substrate

HK: Hexokinase

HSP: Heat shock protein

HUVEC: Human umbilical vein endothelial cell

I

IFN-I: Type I interferons

IL-10/17: Interleukin-10/17

IRS-1: Insulin receptor substrate-1

J

JNK: c-Jun *N*-terminal kinase

K

KR: Keto-acyl reductase

KS: Keto-acyl synthase

KSHV: Kaposi's sarcoma-associated herpesvirus

L

LCFA: Long-chain fatty acids

LC-MS/MS: Liquid chromatography coupled to tandem mass spectrometry

LPS: Lipopolysaccharide

M

MalCoA: Malonyl-Coenzyme A

MAPK: Mitogen-activated protein kinase

MAT: Malonyl/acetyl transferase

MCM: Minichromosome maintenance proteins

MDH: Malate dehydrogenase

MDM2: Murine double minute 2

ME: Malic enzyme

MHC II: Major Histocompatibility Complex

MMP-9: Metalloproteinase 9

MPF: M-phase promoting factor

mRNA: messenger ribonucleic acid

mTOR: mechanistic/mammalian Target Of Rapamycin

N

NAGK: N-acetylglucosamine kinase

NF- κ B: Nuclear factor-kappa B

NK: Natural killer

NLS: Nuclear localization sequence

NO: Nitric oxide

NSCLC: Non-small cell lung cancer

Nup62: Nuclear pore protein p62

O

OA-519: Oncogenic Antigen - 519

OAA: Oxaloacetate

OGA: *O*-GlcNAcase

O-GlcNAcylation: *O*-linked β -N-acetylglucosaminylation

OGT: *O*-GlcNAc transferase

OXPHOS: Oxidative phosphorylation

P

p70S6K: p70 ribosomal protein S6 kinase

PARP1: Poly[ADP-ribose] polymerase 1

PC: Pyruvate carboxylase

PDAC: Pancreatic ductal adenocarcinoma

PDH: Pyruvate dehydrogenase

PD-L1: Programmed death ligand 1

PFK1: Phosphofructokinase 1

PGDH: Phosphogluconate dehydrogenase

PI3K: Phosphatidylinositol 3-kinase

PIP3: Phosphatidylinositol-3,4,5-triphosphate

PKA: Protein kinase A

PKM2: Pyruvate kinase M2

PPI: Protein-protein interaction

PPO: PIP3-binding domain of OGT

PPP: Pentose Phosphate Pathway

PTM: Post-translational modification

R

Raf: Rapidly accelerated fibrosarcoma

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RTA: Replication and transcription activator

S

SCD: Stearoyl-CoA desaturase

SD: Standard deviation

SEC: SECRET AGENT

SEM: Standard error of the mean

SH: Sulfhydryl

siRNA: Small interfering RNA

SKP2: S-phase kinase-associated protein 2

Sp1: Specificity protein 1

SPY: SPINDLY

SREBP-1c: Sterol responsive element binding protein-1c

STAT3: Signal transducer and activator of transcription 3

STING: Stimulator of interferon genes

SUMOylation: Small ubiquitin-like modifier (SUMO)-ylation

T

TBP: TATA-box binding protein

TCA: Tricarboxylic acid cycle

TE: Thioesterase

TET 1/2/3: Ten-eleven translocation 1/2/3

TG: Thiamet-G

TKI: Tyrosine kinase inhibitor

TPR: Tetratricopeptide repeat

TS: Thymidylate synthase

U

Ub: Ubiquitin

UFA: Unsaturated fatty acids

ULK1: Unc-51-like autophagy activating
kinase 1

USP2a: Ubiquitin-Specific Peptidase 2a

V

VEGFR: Vascular endothelial growth factor
receptor

VLCFA: Very long-chain fatty acids

VLDL: Very low-density lipoproteins

W

WB: Western blot

WGA: Wheat Germ Agglutinin

X

XLID: X-linked intellectual disability

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Table 1: Summary of FASN inhibitors.

GENERALITIES

Part 1: The lipogenic and pro-oncogenic enzyme FASN

1. 1. The *de novo* lipogenesis pathway

Lipids constitute a heterogeneous and essential family of biomolecules that share the common property of hydrophobicity (Burdge & Calder, 2014). Fatty acids (FA) are the building blocks of a lot of lipids and are mainly supplied by the diet. However, FA can be synthesized *de novo* thanks to the activity of the lipogenic enzymes Acetyl-CoA carboxylase (ACC) and Fatty acid synthase (FASN).

First, glycolysis produces pyruvate that is imported into mitochondria (**Figure 1**). Pyruvate either forms acetyl-CoA through the activity of Pyruvate dehydrogenase (PDH), or it is carboxylated into oxaloacetate (OA) by Pyruvate carboxylase (PC). OA is in turn condensed to acetyl-CoA to form citrate, triggering the TCA (tricarboxylic acid cycle). Citrate is exported out of the mitochondria in the cytosol where it is cleaved into acetyl-CoA and OA by ACLY (ATP citrate lyase). OA allows pyruvate regeneration *via* the intermediary formation of malate, what consists in the citrate-malate-pyruvate shuttle.

Thus, acetyl-CoA enters the *de novo* lipogenesis (DNL) pathway with a carboxylation reaction catalyzed by ACC, producing malonyl-CoA. Acetyl-CoA and malonyl-CoA are then used by FASN with the co-substrate NADPH, H⁺ to produce free fatty acids (FFA) (Raab & Lefebvre, 2022). There are two isoforms of ACC: ACC1/ACC α and ACC2/ACC β , respectively encoded by the *ACACA* and *ACACB* genes. While ACC1 is cytosolic, ACC2 is located at the outer mitochondrial membrane. Malonyl-CoA produced by ACC2 allosterically inhibits Carnitine palmitoyltransferase I (CPT1) to regulate β -oxidation of lipids (Abu-Elheiga et al., 2000). ACC1 is therefore the main isoform involved in the DNL pathway.

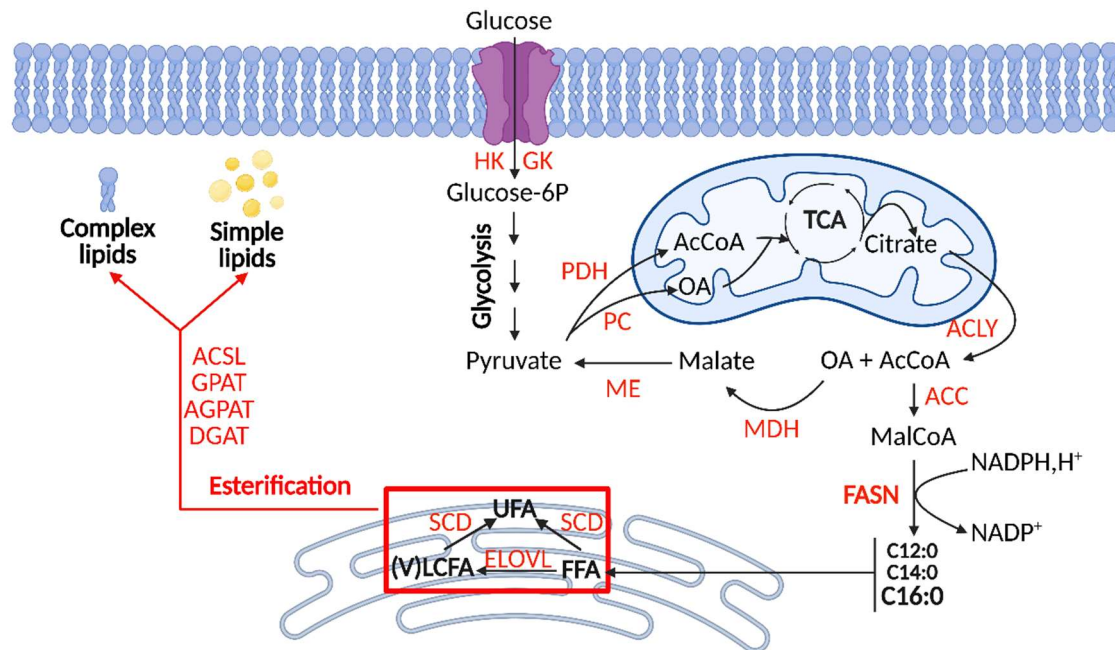


Figure 1: The synthesis of simple and complex lipids. Glycolysis generates pyruvate, which is converted to acetyl-CoA (AcCoA) in mitochondria by Pyruvate dehydrogenase (PDH) or oxaloacetate (OA) by Pyruvate carboxylase (PC). AcCoA and OA enter the TCA cycle, forming citrate, which is exported to the cytosol. ATP-citrate lyase (ACLY) cleaves citrate into AcCoA and OA. OA is reduced to malate by Malate dehydrogenase (MDH), then decarboxylated to regenerate pyruvate by Malic enzyme (ME), completing the citrate-malate-pyruvate shuttle. AcCoA is carboxylated by Acetyl-CoA carboxylase (ACC) to malonyl-CoA (MalCoA), the first step of *de novo* lipogenesis (DNL). Fatty acid synthase (FASN) uses AcCoA, MalCoA, and NADPH, H⁺ to produce fatty acids, primarily C16:0, but also C12:0 and C14:0. These fatty acids can be elongated and/or desaturated in the ER by Elongases (ELOVL) and Stearoyl-CoA desaturases (SCD) to form long-chain fatty acids (LCFA), very long-chain fatty acids (VLCFA) and unsaturated fatty acids (UFA), respectively. Final esterification by Acyl-CoA synthetases (ACSL), Glycerol-3-phosphate acyltransferase (GPAT), Acylglycerol-3-phosphate acyltransferase (AGPAT), and Diacylglycerol acyltransferase (DGAT) leads to the formation of simple (e.g., triglycerides) and complex lipids (e.g., phospholipids). HK: Hexokinase; GK: Glucokinase.

1. 2. Structure, activity and regulation of FASN

FASN is a ubiquitous and high molecular weight enzyme of 273 kDa and made of 2,511 amino acids. It is the second enzyme of the DNL pathway. FASN produces FFA by means of its 7 enzymatic activities (**Figure 2**): keto-acyl synthase (KS), malonyl/acetyl transferase (MAT), dehydratase (DH), enoyl-reductase (ER), keto-acyl reductase (KR) and thioesterase (TE). The acyl carrier protein domain (ACP domain) enables substrate shuttling between these catalytic domains *via* its phosphopantetheine prosthetic group, which is covalently attached to serine 2156 (S2156) (Raab & Lefebvre, 2022).

First, the malonyl/acetyl transferase activity catalyzes acetyl-CoA and malonyl-CoA binding on FASN sulfhydryl (SH) groups respectively on KS and ACP domains (**Figure 3**). Acyl being synthesized is then elongated by KS activity, reduced by KR activity using NADPH, H^+ , dehydrated by DH activity and reduced a second time by ER activity. Acetylation occurs only once at the initiation step, whereas malonylation occurs repeatedly (n times with $n = 7$ maximum) during elongation until a C16-acyl chain is produced on ACP. FFA, mainly palmitic acid, are finally released by TE hydrolytic activity (Raab & Lefebvre, 2022). First steps involving KS and MAT activities are part of the “condensing cycle”, the other ones of the “modifying cycle” (W. Choi et al., 2025).

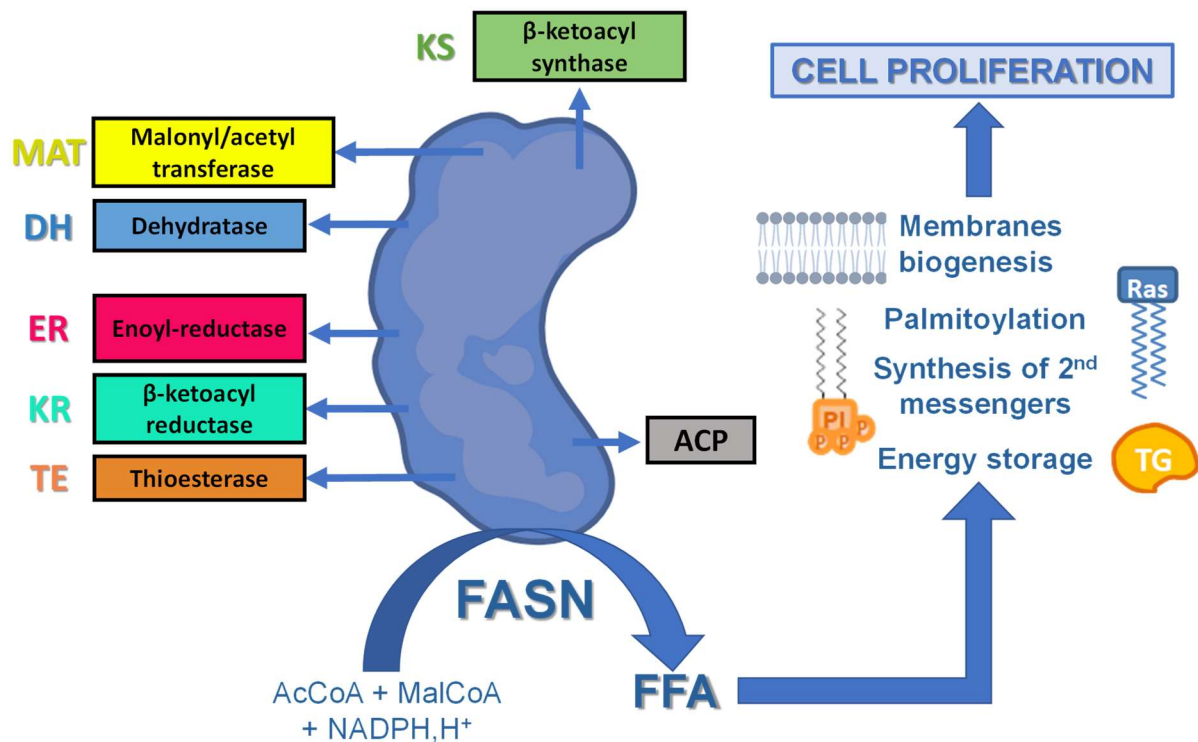


Figure 2: Structure, functions and enzymatic activities of FASN. FASN is represented with its different catalytic activities: KS, MAT, DH, ER, KR and TE. FASN synthesizes FFA from AcCoA, MalCoA and NADPH, H^+ . FFA are stored into lipid droplets and participate to the synthesis of second messengers, protein palmitoylation (e.g., Ras and Flotillin-1) and membranes building: thus, FASN is actively involved in cell proliferation.

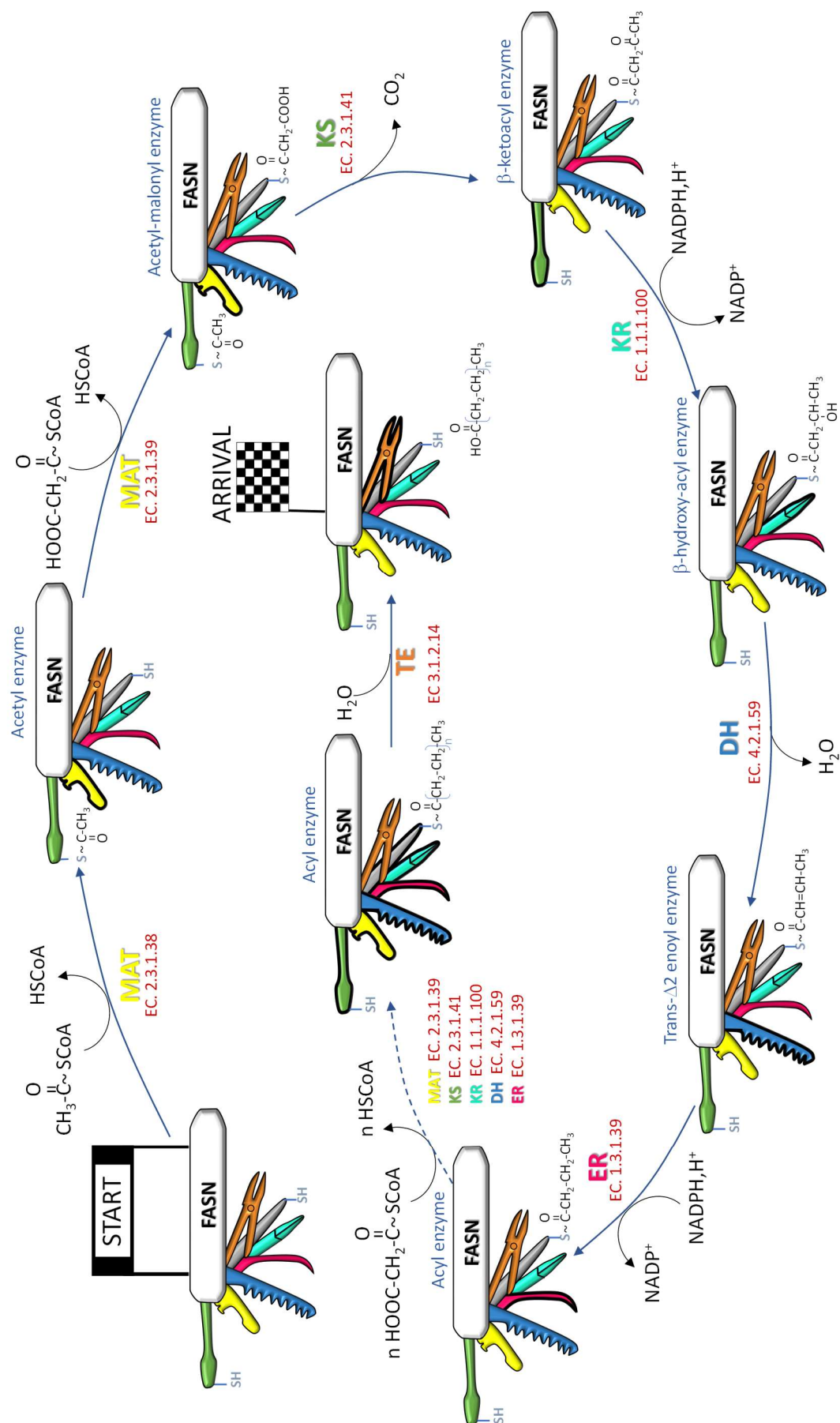


Figure 3: FASN catalytic cycle. To produce FFA, FASN first binds AcCoA and MalCoA *via* its MAT activity, loading them onto the KS and ACP domains, respectively. Next, the KS activity condenses AcCoA and MalCoA *via* a decarboxylation reaction. Then, the acyl being synthesized is reduced by KR, dehydrated by DH and reduced a second time by ER. This cycle of malonylation, decarboxylation, reduction, dehydration and second reduction can be repeated up to 7 times until a C16-acyl chain is produced on ACP. The fully synthesized FA is finally released from ACP thanks to the TE activity. Each FASN catalytic activity is accompanied by its EC (enzyme commission) numeration. Adapted from Raab & Lefebvre, 2022.

To produce FFA, FASN must be homodimerized in a head-to-tail (Smith et al., 2003) or head-to-head conformation (Witkowski et al., 2004). In the head-to-tail model, two sites for the synthesis of FFA are generated by combining KS, MAT and DH activities (“Domain I”) of one monomer with ER, KR, ACP (“Domain II”) and TE (“Domain III”) domains of the other monomer (**Figure 4**). The catalytic reactions in the “condensing cycle” carried out by the two monomers are unsynchronized (W. Choi et al., 2025). The central domain or interdomain, which has no enzymatic activity, has been shown as required in FASN homodimerization (Chirala et al., 2001). Interestingly, yeasts and mammals’ enzyme are called “type I FASN” compared to bacteria, plants and mitochondria which produce a “type II FASN”. Type II FASN consists of individual enzymatic activities each encoded by a distinct gene (Raab & Lefebvre, 2022).

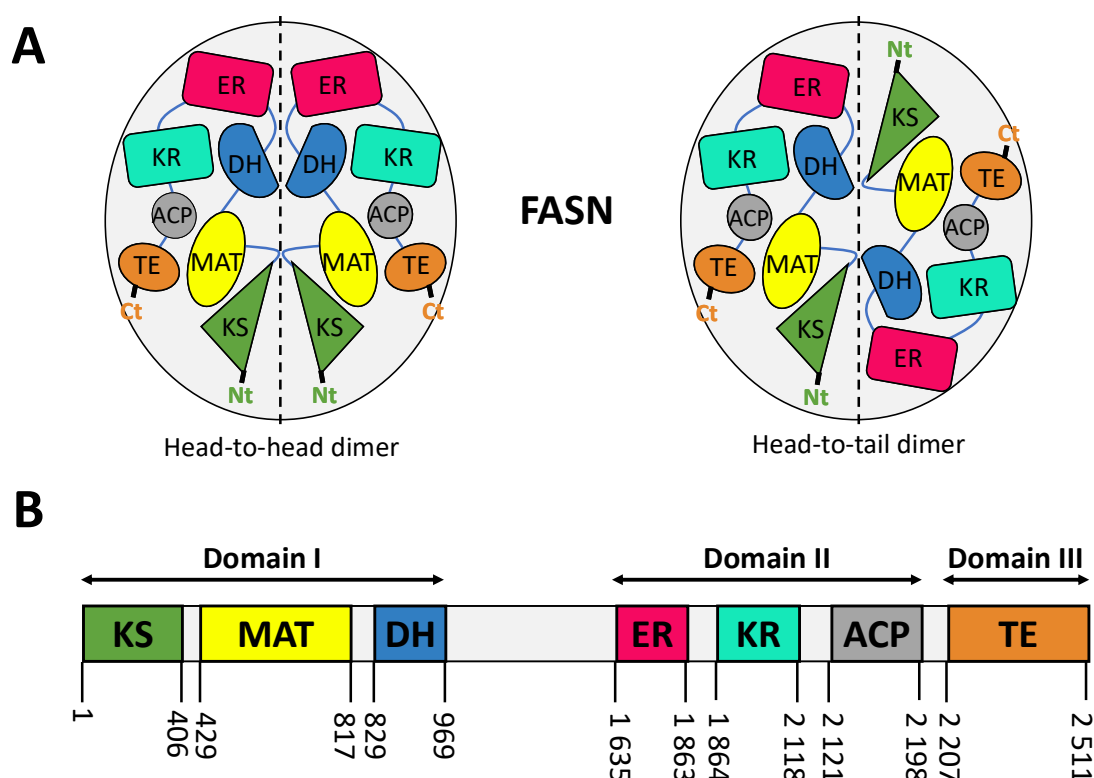


Figure 4: Dimerization models of FASN and delimitation of the catalytic activities. FASN is active in a “head-to-head” or “head-to-tail” conformation (**A**), adapted from Raab & Lefebvre, 2022. FASN is subdivided into three functional domains (**B**) with the KS, MAT and DH activities constituting the domain I, ER, KR and ACP for the domain II, and TE forming alone the domain III.

FFA produced by FASN are lauric acid (C12:0), myristic acid (C14:0) and mainly palmitic acid (C16:0). In the rat lactating mammary gland, although covalently linked to the polypeptide, an alternative TE (TE II) can interrupt prematurely the elongation of FA to release shorter FA (Libertini & Smith, 1978).

Thereafter, FFA can be elongated and/or desaturated by Elongation of very long-chain fatty acids (ELOVL) and Stearoyl-CoA desaturase (SCD) enzymes, respectively, to form (very) long chain and/or unsaturated FA (VLCFA/UFA) in the endoplasmic *reticulum* (ER). After esterification by various enzymes, FA form simple and complex lipids (**Figure 1**). For instance, esterification with glycerol mediated by the Glycerol-3-phosphate acyltransferase (GPAT) produces triglycerides (simple lipids) for energy storage. Complex lipids include glycerophospholipids (phospholipids), glyceroglycolipids and sphingolipids which have many functions (Raab & Lefebvre, 2022).

In response to food intake, FASN is regulated by the transcription factors ChREBP (Carbohydrate responsive element binding protein) and SREBP-1c (Sterol responsive element binding protein-1c), which relay the effects of glucose and insulin on glycolysis and lipogenesis, respectively (Baldini & Lefebvre, 2016). The mammalian/mechanistic Target Of Rapamycin (mTOR), involved in translation promotion, is known to positively regulate FASN expression (Calvisi et al., 2011; Hu et al., 2016; Raab et al., 2021). At the post-translational level, FASN stability is regulated by the Ubiquitin - proteasome pathway (Baldini et al., 2016; H.-S. Jang et al., 2024; Ye et al., 2023). In a lesser extent, some data show a regulation of FASN by autophagy. Upon nitrogen starvation, FASN is preferentially degraded by autophagy in yeast (Shpilka et al., 2015). This process depends on FASN interaction with Autophagy-related protein 8 (Atg8) and is essential for cell survival.

1. 3. Physiological roles of FASN

1. 3. 1. Systemic roles of FASN

Although ubiquitous, FASN is more heavily expressed in certain tissues including lungs, lactating mammary gland, liver and adipose tissues (Semenkovich et al., 1995). In lungs, particularly in alveolar epithelial cells, FASN is required for surfactant production and its activity is promoted by glucocorticoids (Rooney, 1989). Glucocorticoids can be administrated to pregnant women at risk to give birth prematurely in order to accelerate fetal lung development (Daskalakis et al., 2023). Thus, FASN is essential for embryonic development:

Fasn^{-/-} mice embryos die before uterine implantation (Chirala et al., 2003). FASN is particularly important for neurodevelopment, its genetic deletion in mice cortex leading to microcephaly (Gonzalez-Bohorquez et al., 2022). A proper lipid metabolism is required for cognitive functions as individuals homozygous for a gain-of-function variant of *FASN* (FASN-R1819W) have intellectual disability (Bowers et al., 2020). FASN also protects cardiomyocytes from a pathological calcium flux as shown in a mouse model of FASN knockout in the myocardium (Razani et al., 2011). On the contrary, FASN expression and activity were recently associated to cardiac remodeling and mitochondrial dysfunctions in a cardiac hypertrophy model of rats (Bharati et al., 2025).

1. 3. 2. Cellular functions of FASN

Recently, Köberlin and collaborators shed the light to a new cell cycle checkpoint involving FASN (Köberlin et al., 2024). By inducing cell cycle entry by Epidermal growth factor (EGF) treatment of serum-starved MCF-10A cells, the authors show that FASN activity is required for the G1/S transition (**Figure 5**). In G1, the lipidome is reshaped with an increase in UFA and neutral lipids. UFA increase membrane fluidity that may be important for a successful completion of mitosis and to promote the increased size of the ER during cell-cycle progression. In case of UFA insufficiency, an ER-stress is triggered, responsible for a G1 arrest mediated by increased p21 and, conversely, reduced Cyclin D1 and Rb phosphorylation. This lipid checkpoint is likely used by non-cancerous cells as cancer cells usually have mutated Rb, p21, or upregulated Cyclin-dependent kinase 4/6 (CDK4/6) activity (Köberlin et al., 2024).

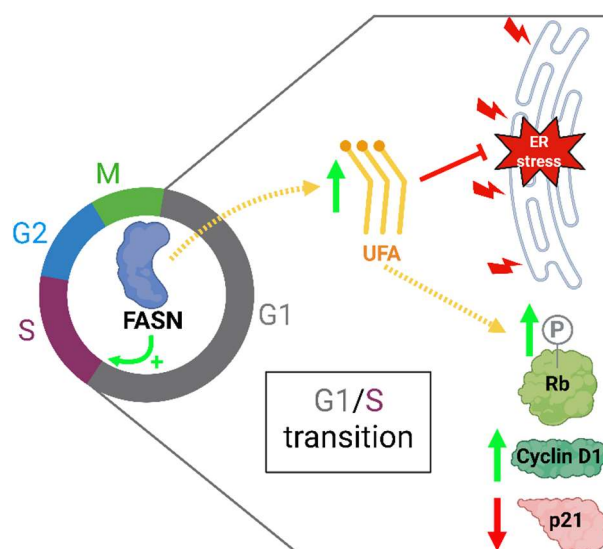


Figure 5: FASN-mediated cell cycle lipid checkpoint. In G1 phase, FASN indirectly increases UFA production. This prevents ER stress and indirectly promotes Rb phosphorylation as well as Cyclin D1 increase and p21 decrease. These mechanisms mediated by UFA increase allow G1/S transition.

By promoting cell cycle progression, FASN is involved in cell proliferation and stem cell maintenance. A study from Gonzalez-Bohorquez and collaborators demonstrated FASN involvement in neural progenitor cell proliferation, a pivotal process for embryonic brain development (Gonzalez-Bohorquez et al., 2022). FASN activity is also crucial for adult neurogenesis: deletion of *Fasn* in mice neural stem and progenitor cells significantly reduces cell proliferation (Knobloch et al., 2013). FASN is also involved in immune cell function. For instance, macrophage activation requires FASN activity (Wei et al., 2016). FASN promotes the differentiation of CD4⁺ T cells into the Th17 phenotype, characterized by pro-inflammatory Interleukin-17 (IL-17) production (Young et al., 2017). Eventually, FASN-mediated DNL is also essential for the maturation and function of dendritic cells. Indeed, mice treated with C75 - a FASN inhibitor (**Table 1**) - presented a significant reduction of CD11c⁺ dendritic cells in the liver, spleen and bone marrow. Inhibition of ACC also suppressed dendritic cells Major Histocompatibility Complex II (MHC II) expression (Rehman et al., 2013). These data highlight the importance of DNL in regulating immune system function.

Strengthening the link between FASN and autophagy, its activity has been reported as essential for adipocyte autophagosome and lysosome growing and activity (Rowland et al., 2023). Indeed, autophagosomes are double-membrane vesicles highly-demanding in phospholipids. For example, a 500-nanometers autophagosome requires 4,000 phospholipid molecules per second to form completely in 10 minutes (Prinz & Hurley, 2020). Thus, while FA produced by FASN are a critical source of lipids for these vesicles in adipocytes, exogenous FA appear to be the main source for lipid droplets formation. FASN-mediated autophagy has also been observed in HepG2 hepatic cancer cells (Rowland et al., 2023). These results support the idea that generating new membranes is more favorable than recycling pre-existing ones. It is less costly to directly use newly synthesized membranes than to remove embedded proteins from existing membranes prior to reuse (Girardi et al., 2011).

1. 3. 3. Some of the multiple functions of FASN at the molecular level

When in excess, carbohydrates are converted into FA. *De novo* synthesized FA are then assembled into triglycerides and stored in the form of lipid droplets (especially in adipocytes) or secreted by the way of very low-density lipoproteins (VLDL) by the liver (Fhu & Ali, 2020). Thanks to its key-role in FA synthesis, FASN participates in remodeling lipid rafts (**Figure 2**).

Lipid rafts ensure the correct location of receptors to the cell membrane and potentiate the activation of signaling pathways. FASN activity is mainly required for lipid rafts phospholipids synthesis compared to those in non-raft membranes (Swinnen et al., 2003).

In skeletal muscle, FASN is necessary for the proper sarcoplasmic *reticulum* membrane composition. Skeletal muscle-specific FASN knockout mice significantly reduces their running time and running distance (Funai et al., 2013). Plasma membrane cholesterol retention in macrophages relies on FASN (Wei et al., 2016) as in neural retina (Rajagopal et al., 2018), what is crucial for inflammation and vision, respectively. Indeed, FASN depletion in macrophages significantly reduces the number of incorporated lipids, including cholesterol, into lipid rafts. It also alters lipid rafts protein composition, decreases lipopolysaccharide (LPS)-mediated c-Jun N-terminal kinase (JNK) phosphorylation and therefore macrophage activation (Wei et al., 2016).

Likewise, deletion of *FASN* from the neural retina decreases membrane cholesterol content as well as neurotrophic signaling, leading to apoptosis (Rajagopal et al., 2018). Schwann cells myelination also depends on FASN activity (Montani et al., 2018). By producing palmitate, FASN is indirectly involved in protein palmitoylation as evidenced for the Wnt proteins (Proffitt & Virshup, 2012). Wnt proteins palmitoylation by Porcupine enables their transport, secretion and activity. Palmitoylation of the SARS-Cov2 Spike protein was recently described (Mekhail et al., 2022). The virus exploits host cell FASN activity, which is essential for Spike palmitoylation, proper trimerization and incorporation to ensure an efficient viral replication.

Thus, FASN is involved in numerous fundamental physiological functions, ranging from molecular processes to whole-organism development and homeostasis (**Figure 6**).

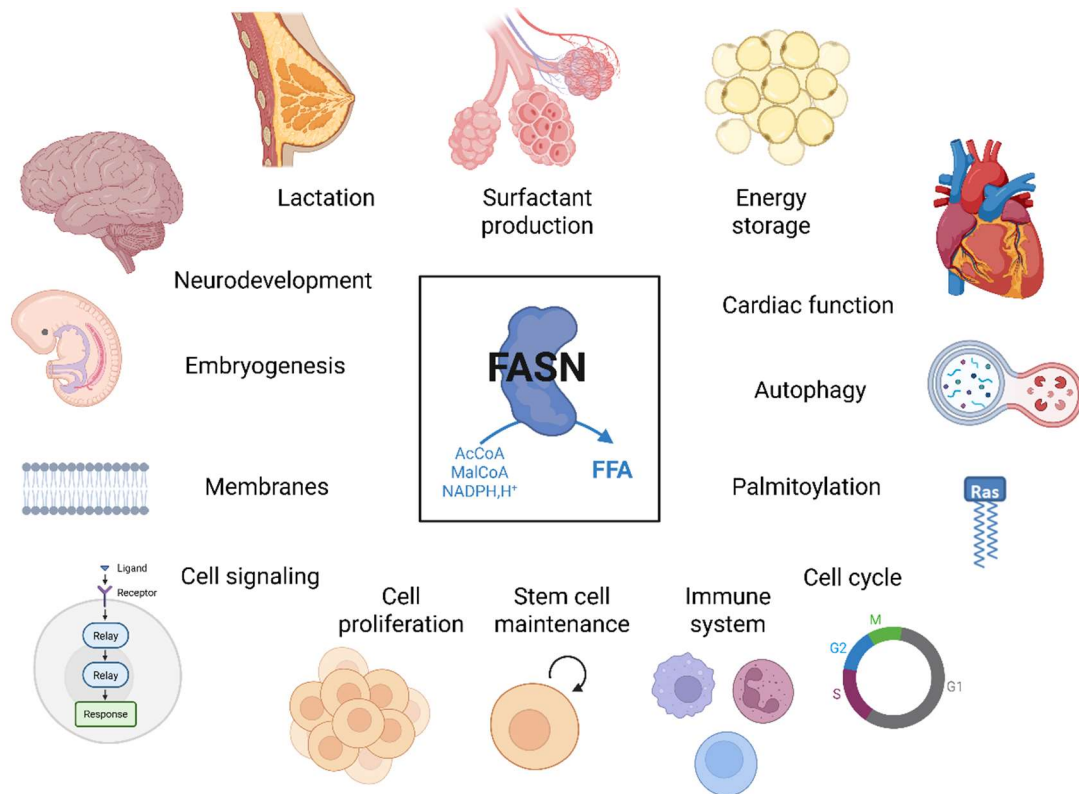


Figure 6: Summary of the multiple physiological functions of FASN. See text for details.

1. 4. FASN dysregulation in cancers

One of the characteristics of cancer cells is metabolic reprogramming (Hanahan & Weinberg, 2011). One of the most striking metabolic changes characteristic of cancer cells is an increased lipogenesis. Healthy cells, on the other hand, exhibit reduced lipogenesis, having enough FA with feeding. The acceleration of lipogenesis in cancer cells promotes cell proliferation, survival, invasion and resistance to chemotherapy (Vanauberg et al., 2023). An increased lipogenic flux has been reported in hepatocellular carcinoma (HCC) (Calvisi et al., 2011) as in breast cancer (Giudetti et al., 2019), glioblastoma (Geng et al., 2016), colorectal cancer (CRC) (Y. Zhu et al., 2021) and in hematologic malignancies like acute myelogenous leukemia and endemic Burkitt lymphoma (Southam et al., 2015). This increase in lipogenic flux may be linked to the Warburg effect but also to the overexpression of the second enzyme of the DNL pathway being FASN.

FASN was primarily named as Oncogenic Antigen-519 (OA-519), its overexpression has been first observed in human breast carcinoma cells in 1994 (Kuhajda et al., 1994). FASN overexpression was reported in many other kinds of cancers: prostate (Migita et al., 2009), colorectal (Rashid et al., 1997), esophageal (Orita et al., 2010), stomach (Kusakabe et al., 2002), pancreatic (Walter et al., 2009), lung (Visca et al., 2004), ovarian (Cai et al., 2015), melanoma (Innocenzi et al., 2003), hepatic (Raab et al., 2022), primary effusion lymphoma (Bhatt et al., 2012) and glioma (Zhou et al., 2016). FASN is usually associated with poor prognosis (Cigliano et al., 2024) but is not considered as an oncogene as its overexpression is not able to transform healthy hepatocytes into cancer cells (Che et al., 2017). In addition, tumors can be dependent or independent of DNL, so that of FASN, for their growth in function of the oncogenes involved (Che et al., 2017).

However, FASN promotes many hallmarks of cancer as reviewed by us recently (Vanauberg et al., 2023): FASN can therefore be qualified as a pro-oncogenic enzyme and is a promising therapeutic target. As a matter of fact, FASN promotes dysregulation of cell metabolism, proliferative signaling, resistance to cell death, angiogenesis induction, migration/invasion and inhibition of the immune system (**Figure 7**). Examples of FASN involvement in these cancer hallmarks are described below.

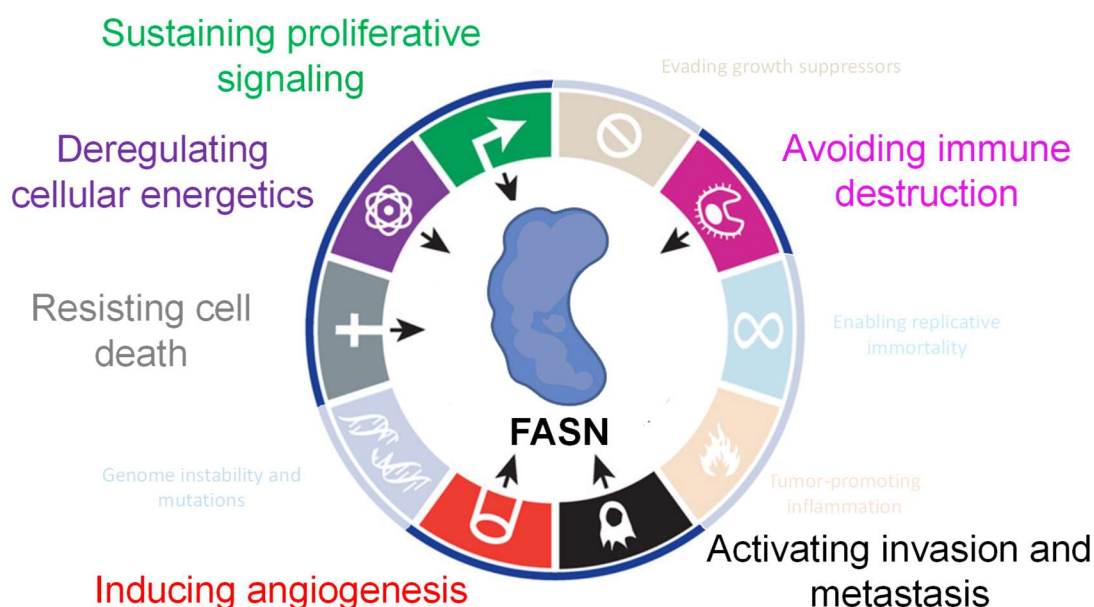


Figure 7: FASN involvement in hallmarks of cancer. FASN is at the center of many hallmarks of cancer by promoting proliferative signaling, dysregulation of cell metabolism, resistance to cell death, induction of angiogenesis, activation of invasion/metastasis and immune escape. From Vanauberg et al., 2023.

1. 4. 1. FASN prevents cancer cell death

Blocking FASN can induce prostatic (De Schrijver et al., 2003) or mammary (Fan et al., 2016) cancer cells apoptosis *via* small interfering ribonucleic acid (siRNA) transfection or curcumin treatment, respectively. Resistance to this cell death subtype being a major hallmark of cancer cells (Hanahan, 2022). Most recently, resistance to B-cell-lymphoma-2 (BCL2) homology-3 mimetics (BH3 mimetics)-induced mitochondrial apoptosis in pancreatic ductal adenocarcinoma (PDAC) has been linked to FASN (Steen et al., 2025). By concomitantly treating PDAC cell lines with BH3 mimetics and TVB-3166 - a FASN inhibitor (**Table 1**) -, the authors synergistically increased mitochondrial apoptosis. Same results were obtained from patient-derived xenografts (Steen et al., 2025).

1. 4. 2. FASN promotes cancer cell migration and metastasis

FASN would also promote epithelial-mesenchymal transition (EMT) and consequently cell migration. In the HER2+ breast cancer cell line BT-474, FASN fosters cell invasion *via* Matrix metalloproteinase 9 (MMP-9) activation (J. Li et al., 2014). Metastasis of primary solid tumors relies on EMT to shift from an epithelial to a mobile mesenchymal phenotype. Hence, FASN also promotes metastasis as recently demonstrated for CRC (Liu et al., 2025). By knocking down FASN, this study revealed a significant inhibition of tumor growth and CRC cells metastatic spread to the lungs. Likewise, cerulenin - a natural FASN inhibitor (**Table 1**) - prevented liver metastasis of CRC cells in mice (Murata et al., 2010).

1. 4. 3. FASN deregulates cancer cell metabolism

At the metabolic level, Ravi and collaborators (Ravi et al., 2021) established a positive link between FASN and the pentose-phosphate pathway (PPP) in Non-Hodgkin Lymphoma. By consuming NADPH, H^+ , FASN increases the pool of $NADP^+$, a co-substrate of the PPP enzyme 6-phosphogluconate dehydrogenase (PGDH) which promotes its activity and finally enhances deoxyribonucleic acid (DNA)/RNA synthesis. FASN overexpression directly disrupts cancer cell metabolism by increasing lipogenic fluxes to meet the elevated membrane biogenesis demands of cancer cells (Y. Zhu et al., 2021). At last, by positively regulating mTOR, FASN also promotes protein biosynthesis in colorectal and hepatic cancer cell lines (T. Lu et al., 2019; Raab et al., 2021).

1. 4. 4. FASN sustains proliferative signaling

FASN contributes to the resistance to tyrosine kinase inhibitors (TKIs) that target Epidermal growth factor receptor (EGFR) to reduce proliferative signaling. Indeed, FASN indirectly induces EGFR palmitoylation (Bollu et al., 2015). This modification is required for EGFR dimerization and kinase activation, promoting resistance to TKIs. In non-small cell lung cancer (NSCLC) cells with mutated EGFR and resistant to TKI, FASN inhibition with orlistat (**Table 1**) prevents EGFR palmitoylation and conversely induces EGFR K48-ubiquitination resulting in a reduction of tumor growth (Ali et al., 2018). Through its activity, FASN supplies lipid rafts with lipids. Consequently, FASN may indirectly promote the localization of oncogenic receptors (like EGFR) within these microdomains, thereby potentiating their proliferative signaling. This requires further investigation.

1. 4. 5. FASN and angiogenesis

Several studies show that FASN must be active for proper angiogenesis. Firstly, breast cancer cell lines treated with orlistat present a significant down-regulation of Vascular endothelial growth factor (VEGF) (Jovankić et al., 2023). Secondly, by knocking-down FASN in endothelial cells (ECs), excess malonyl-CoA not metabolized by FASN causes K1218 mTOR malonylation (Bruning et al., 2018). Malonylation impairs mTOR complex 1 (mTORC1) kinase activity, thereby reducing protein synthesis and subsequent ECs proliferation. By concomitantly knocking-down ACC1 in FASN depleted ECs, malonyl-CoA levels were normalized and mTOR reactivated. This reinforces the positive cross-regulation existing between FASN and mTOR.

1. 4. 6. FASN in immune escape

FASN involvement in immune escape has gained insights in the last past years. First finding was made by Cioccoloni and collaborators who described a positive regulation of Programmed death ligand 1 (PD-L1) protein expression by FASN in Jurkat leukemic cells (Cioccoloni et al., 2020). PD-L1 and its receptor PD-1 consists in an immune checkpoint. PD-L1 is highly expressed on cancer cell surface and interacts with PD-1 exposed on cytotoxic T lymphocytes. This interaction induces an inhibitor signal in the latter, attenuating the antitumor activity (Brahmer et al., 2012). As of 2025, other studies deciphered FASN-mediated immune escape (Liu et al., 2025). In addition to FASN contribution to CRC lung metastasis described above, Liu and collaborators show FASN ability to inhibit natural killer (NK) cells. Mechanistically,

phosphatidylcholine produced in part *via* FASN activity, inhibited NK anti-tumor effect against CRC cells.

1. 4. 7. FASN inhibits autophagy in cancer cells

As discussed before, FASN is involved in autophagy by promoting autophagosomes building and fusion with lysosomes in adipocytes and in HepG2 cells (Rowland et al., 2023). However, Zaytseva and collaborators were the first to suggest a FASN-mediated autophagy inhibition (Zaytseva et al., 2015). They observed significantly higher expression of FASN and p62 (a marker of autophagy inhibition) in CRC tissues compared to normal colon tissues. Under metabolic stress conditions, the SW480 colon cell line overexpresses FASN and accumulates non-degraded p62. In human breast cancer cells, inhibition of FASN triggers ER stress and autophagy as survival pathways to counteract its inhibition (Huang et al., 2019). Addition of exogenous palmitic acid blocks both ER stress and autophagy, demonstrating FASN activity involvement in autophagy inhibition.

mTORC1 inhibits autophagy by phosphorylation and inactivation of the autophagy initiators Unc-51-like autophagy activating kinase 1 (ULK1) and Atg13 (Rabanal-Ruiz et al., 2017). mTORC1 is a nutritional sensor which promotes biosynthetic pathways under nutrient-rich conditions and prevents catabolism through autophagy inhibition. Due to the positive cross-regulation between FASN and mTOR, we can speculate that FASN inhibits autophagy through mTOR activation in cancer. Humbert et al. highlighted this relationship in acute myeloid leukemia (AML) cells (Humbert et al., 2020, 2021). AML cells overexpress FASN compared to mature granulocytes. Their differentiation can be induced by all-*trans* retinoic acid (ATRA). During ATRA-induced differentiation, the authors observed FASN degradation by autophagy. A reduced FASN expression decreases mTOR-mediated inhibition of ULK1, promoting autophagy activation. This facilitates AML cells ATRA-induced differentiation. Finally, the tumor suppressor Fatty acid-binding protein 5 (FABP5) interacts with FASN and increases FASN ubiquitination and proteasomal degradation, suppressing mTOR signaling: thus, autophagy is facilitated. Interestingly, FABP5 is markedly down-regulated in CRC, its low expression being associated with shorter survival probability of patients (Ye et al., 2023). Altogether, these data point out FASN-mediated inhibition autophagy in cancer cells as a pro-survival mechanism. Inhibiting FASN could therefore induce autophagy-mediated cancer cell death.

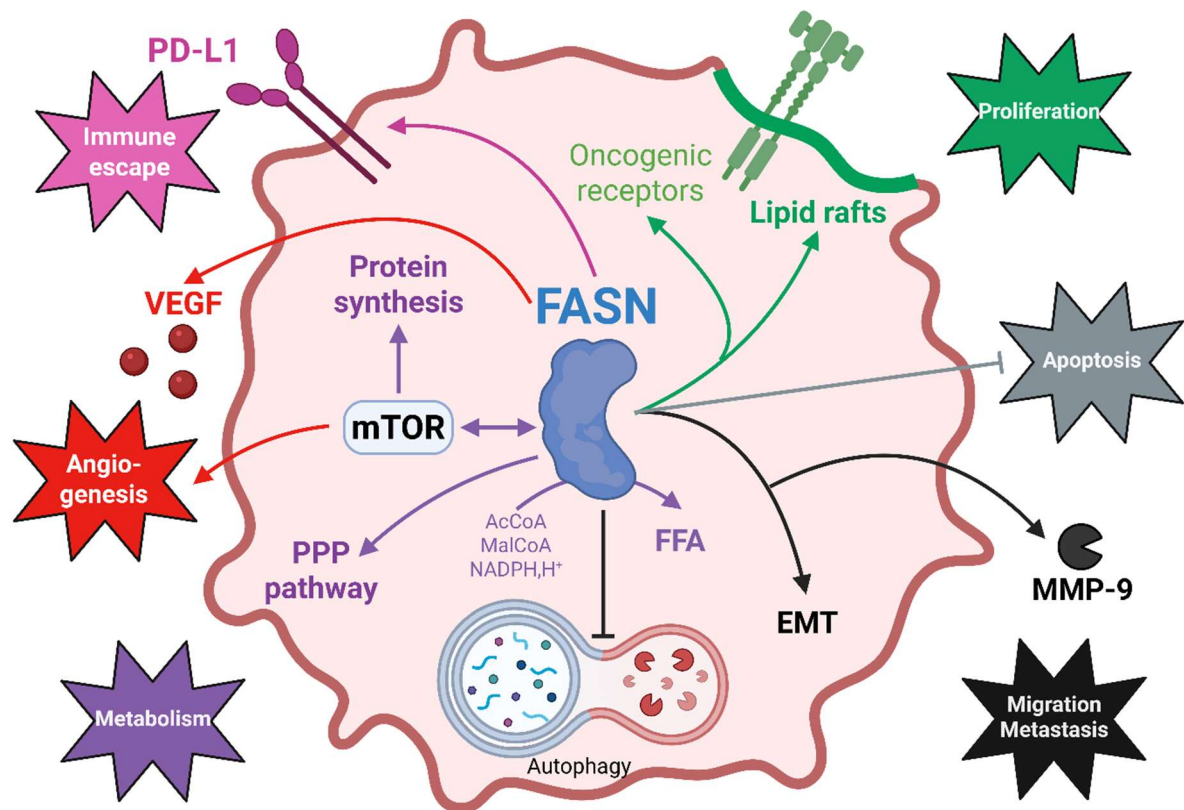
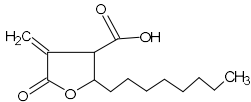
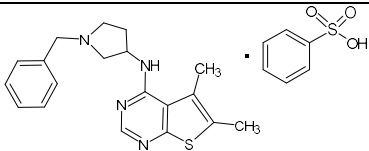
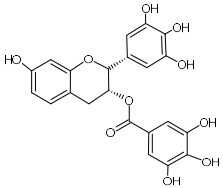
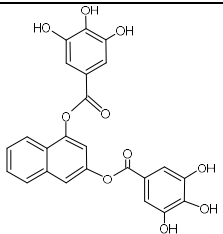
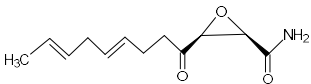
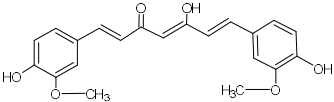
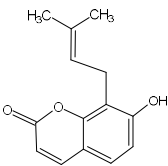
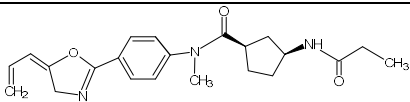
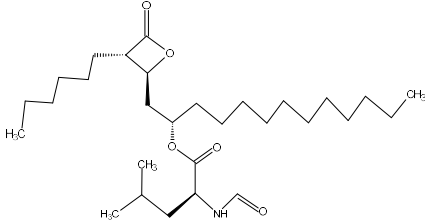


Figure 8: Summary of the different FASN pro-oncogenic features. Through its activity, FASN promotes lipogenesis as well as protein and DNA/RNA synthesis *via* mTOR and PPP pathways activation, respectively (purple). Angiogenesis induction is favored by FASN-mediated VEGF secretion and mTOR activation (red). By increasing PD-L1 protein expression, FASN is involved in immune escape (pink). Proliferative signaling is enhanced by FASN *via* lipid rafts building that recruits oncogenic receptors (green). In addition, FASN blocks cancer cell apoptosis (gray) and induces EMT and MMP-9 activation (black). Thus, FASN is involved in migration and metastasis and is also able to inhibit cancer cell autophagy.

Due to its pro-oncogenic features (**Figure 8**), FASN is certainly a compelling target for anti-cancer therapies. To this aim, many FASN inhibitors have emerged but few are at the pre-clinical stage as the synthetic drug TVB-2640 (Denifanstat) (**Table 1**). TVB-2640, targeting FASN KR domain, is in phase II clinical trial for different cancers like HER2 positive metastatic breast cancer (NCT03179904). Orlistat is the only approved drug used in clinic to treat obesity. Due to its very low systemic absorption, unlikely, orlistat cannot be used to treat cancers (Hao et al., 2023).

Compound	Structure	Origin	Targeted domain	Development stage
C75		Cerulenin synthetic derivate	β -ketoacyl synthase, thioesterase, enoyl reductase	Pre-clinic
C93		Synthetic	β -ketoacyl synthase	Pre-clinic
EGCG		Plant-derived polyphenol (natural component of green tea)	β -ketoacyl reductase	Pre-clinic
G28		EGCG synthetic derivate	Same as EGCG?	Pre-clinic
Cerulenin		Antibiotic produced by <i>Cephalosporium caerulens</i>	β -ketoacyl synthase	Pre-clinic
Curcumin		Antioxidant produced by <i>Curcuma longa</i>	Malonyl/acetyl transferase	Pre-clinic
Osthole		Botanical anti-fungal agent produced by <i>Cnidium monnieri</i>	?	Pre-clinic
BI99179		Synthetic	β -ketoacyl reductase	Pre-clinic
Orlistat		Derivative of lipstatin isolated from <i>Streptomyces toxytricini</i>	Thioesterase	FDA-approved anti-obesity drug

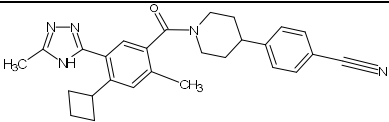
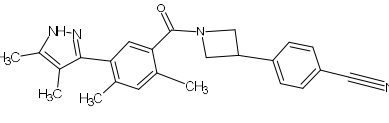
TVB-2640		Imidazopyridine-based inhibitor	β -ketoacyl reductase	In phase II trial
TVB-3166		Pre-clinical version of TVB-2640	Same as TVB-2640?	Pre-clinic

Table 1: Summary of FASN inhibitors. From Vanauberg, Schulz and Lefebvre, 2023.

Part 2: The nutrient-dependent modification O-GlcNAcylation

2. 1. The Hexosamine Biosynthetic Pathway

O-linked β -N-acetylglucosaminylation (O-GlcNAcylation) consists in the addition of a single N-acetyl-D-glucosamine (GlcNAc) residue on the hydroxyl group of a serine/threonine of a cytosolic, nuclear or mitochondrial protein (Vercoutter-Edouart et al., 2015). O-GlcNAcylation is a post-translational modification (PTM) akin to phosphorylation as it is reversible, its dynamism being orchestrated by two enzymes, the O-GlcNAc transferase (OGT) and the O-GlcNAcase (OGA). Both enzymes are well conserved along evolution, as indicated by the high sequence similarity between human OGT and OGA with their homologs in zebrafish (*Danio rerio*) and fruit fly (*Drosophila melanogaster*) (Y. Zhu & Hart, 2021). O-GlcNAc processing enzymes were also identified in the nematode *Caenorhabditis elegans* (Hanover et al., 2005). OGT-like enzymes were also identified in the model plant *Arabidopsis thaliana*: SPINDLY (SPY) and SECRET AGENT (SEC) (Hartweck et al., 2002). However, only SEC appears to be a real O-GlcNAc transferase (Zentella et al., 2017).

OGT transfers the GlcNAc residue on a target protein from UDP-GlcNAc. This substrate is synthesized by the Hexosamine Biosynthetic Pathway (HBP) which is supplied by several sources of nutrients, making this pathway an indicator of the nutritional status (**Figure 9**). UDP-GlcNAc also fuels complex glycosylations as it can be converted into UDP-GalNAc and CMP-NeuAc (Biwi et al., 2018). About 2 to 3% of glucose enters this metabolic pathway (Marshall et al., 1991). Contrary to other types of glycosylation or to many phosphorylation sites, no strict consensus sequence has yet been described for O-GlcNAcylation (Mauri et al., 2021). Wulff-Fuentes et al. (Wulff-Fuentes et al., 2021) analyzed 7,002 O-GlcNAc sites reported on 1,803 proteins in order to refine the preferred sequence for O-GlcNAcylation. They identified “semi-consensus” sequences, characterized by an abundance of serine or threonine residues around the O-GlcNAc site: P-P-(V/T)-**g**(S)-(S/T)-A / (P/T)-P-(V/T)-**g**(T)-(S/T)-(A/T). Also, the presence of glutamic acid or cysteine was shown to be unfavorable to O-GlcNAcylation. In 2025, more than 22,000 O-GlcNAcylated proteins among 48 species were reported in the O-GlcNAc database. Over 9,000 intracellular human proteins belong to the “O-GlcNAcome” (Malard et al., 2025).

The GlcNAc released by OGA can re-enter HBP in order to constantly fuel the pool of available UDP-GlcNAc for OGT. Free GlcNAc can also be recycled from lysosomal degradation of glycoproteins, glycolipids and proteoglycans. This “salvage pathway” involves the N-acetylglucosamine kinase (NAGK) enzyme which phosphorylates GlcNAc to generate GlcNAc-6-phosphate. NAGK-dependent HBP salvage can be triggered by glutamine deprivation, allowing a continue synthesis of UDP-GlcNAc (Campbell et al., 2021). However, HBP must be finely regulated for a correct *O*-GlcNAc homeostasis. This regulation implies the rate-limiting enzyme Glutamine:fructose-6-phosphate amidotransferase (GFAT).

There are two isoforms of GFAT encoded by distinct genes: GFAT-1 and GFAT-2, sharing 75.6% of sequence homology. Both enzymes catalyze the conversion of fructose-6-phosphate into glucosamine-6-phosphate consuming glutamine. While GFAT-1 is ubiquitous, GFAT-2 is mainly expressed in the central nervous system, explaining why studies primarily focus on GFAT-1 (Oki et al., 1999). Ruegenberg and collaborators identified G451 residue of GFAT-1 as a major site mediating its sensitivity to UDP-GlcNAc inhibition (Ruegenberg et al., 2020). GFAT-2, in contrast, is less sensitive to UDP-GlcNAc feedback inhibition. In addition to the pathway end product, GFAT-1 may also be inhibited by its direct product glucosamine-6-phosphate by competing with fructose-6-phosphate binding (Broschat et al., 2002).

At last, GFAT-1 is also regulated transcriptionally and post-translationally. Different transcription factors induce *GFAT-1* gene expression like Activating transcription factor 4 (ATF4) during nutritional deprivation (Chaveroux et al., 2016). Post-translationally, Protein kinase A (PKA) phosphorylates GFAT-1 at S250, what decreases basal activity of the kinase and abolishes UDP-GlcNAc feedback inhibition (Ruegenberg et al., 2021). Also, AMP-activated protein kinase (AMPK) catalyzes GFAT-1 S243 phosphorylation diminishing its activity (Zibrova et al., 2017). GFAT regulation by PTMs deserves further investigation.

Thus, several studies demonstrate that GFAT is the most heavily regulated enzyme of HBP (Paneque et al., 2023).

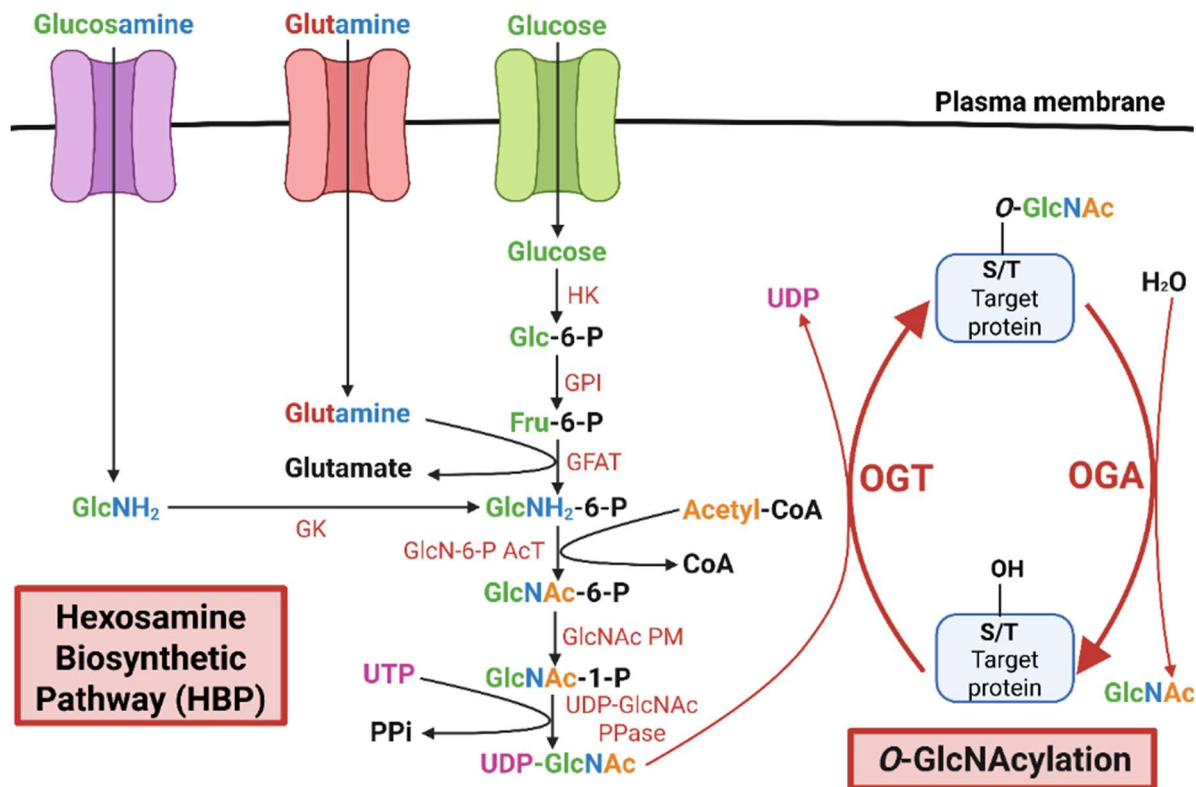


Figure 9: Hexosamine Biosynthetic Pathway (HBP) and O-GlcNAcylation. 2 to 3% of glucose fuels the HBP pathway which leads to the synthesis of UDP-GlcNAc. UDP-GlcNAc is a nutritional sensor, its biosynthesis depending on **glucose**, **amino acids**, **fatty acids** and **nucleotides** metabolism. From this nucleotide-sugar, OGT adds GlcNAc on the hydroxyl group of a serine or threonine of a target protein. The antagonist enzyme, OGA, hydrolyzes the GlcNAc residue. HK: Hexokinase; GPI: Glc-6-P isomerase; GK: Glucosamine kinase; GFAT: Glutamine:fructose-6-phosphate amidotransferase; GlcN-6-P AcT: GlcN-6-P acetyltransferase; GlcNAc PM: GlcNAc phosphomutase; UDP-GlcNAc PPase: UDP-GlcNAc pyrophosphorylase.

2. 2. O-GlcNAcylation enzymes: structure, activity and regulation

2. 2. 1. The O-GlcNAc transferase (OGT)

There are three OGT isoforms: nucleocytoplasmic OGT (ncOGT, 116kDa), mitochondrial OGT (mOGT, 103kDa) and short OGT (sOGT, 78kDa). These three isoforms are differentiated by their number of 34 amino acids repeats, constituting the N-terminal domain, called TPRs (Tetratricopeptide Repeats): 13.5 for ncOGT, 9.5 for mOGT and 3.5 for sOGT (Hanover et al., 2003) (**Figure 12**). Their TPR constitute an interaction surface with substrates (Ramirez et al., 2021). Some studies suggest that other proteins (qualified as partners or adapters) could assist in targeting the TPRs to specific substrates. For example, OGT interacts with the co-repressor mSin3a *via* TPRs 1-6 to induce Histone deacetylase 1 (HDAC1) interaction and subsequent O-GlcNAcylation (X. Yang et al., 2002). Function of this modification has later been highlighted,

in HCC HDAC1 *O*-GlcNAcylation promotes its phosphorylation and finally its activity (G. Zhu et al., 2016).

Each OGT isoform possesses two catalytic domains named CDI (Catalytic Domain I) and CDII (Catalytic Domain II) in addition to their phosphatidylinositol-3,4,5-triphosphate (PIP3) binding domain named PPO (PIP3-binding domain of OGT) (**Figure 12**). While sOGT is very poorly studied, mOGT is resident of the mitochondria; of particular interest, Pr. Daan van Aalten's group has demonstrated that ncOGT would be sufficient for *O*-GlcNAcylation of mitochondrial proteins (Trapannone et al., 2016). Thus, ncOGT is the most studied and appears to be the main isoform, the reason why it is simply named OGT.

OGT follows a bi-bi ordered mechanism. The enzyme first binds UDP-GlcNAc in its catalytic site and then the target protein at the TPR motifs. The Ser or Thr acceptor residue of the substrate protein then carries out a nucleophilic attack on the anomeric carbon of UDP-GlcNAc to form a β -glycosidic bond. The *O*-GlcNAcylated protein and UDP are then sequentially released (Lazarus et al., 2011) (**Figure 10**). In addition, OGT is active in homodimers. Mutating specific sites at the dimer interface of OGT, implicating TPRs 6 and 7, prevents OGT from dimerizing and reduces its enzymatic activity (Meek et al., 2021).

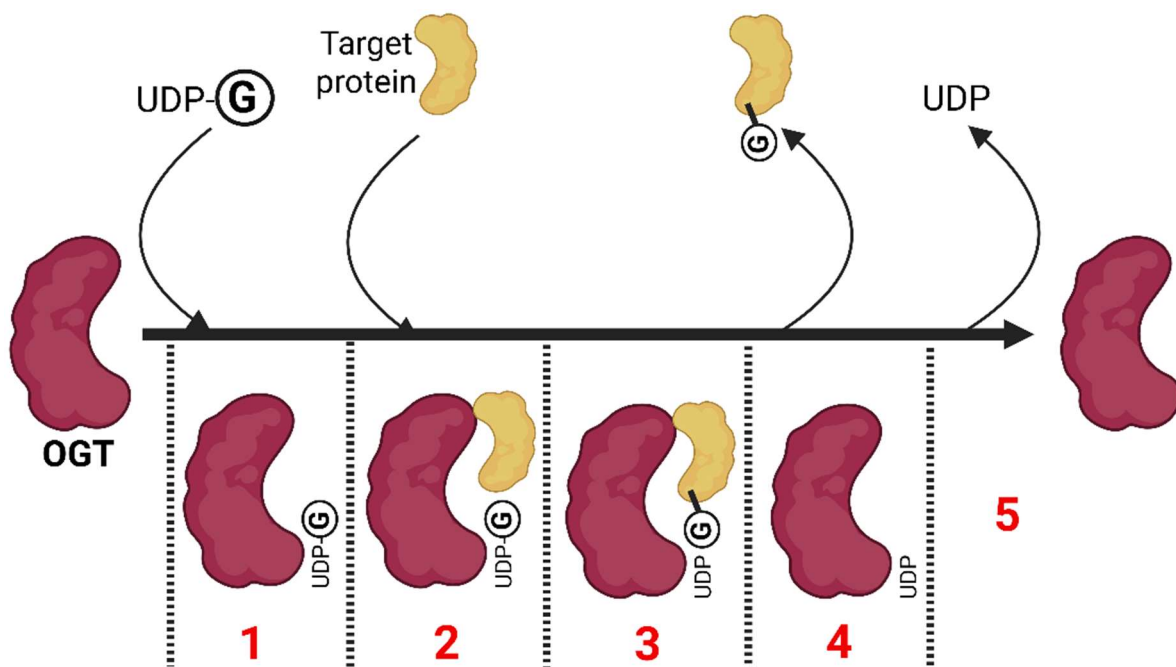


Figure 10: Simplified mechanism of protein *O*-GlcNAcylation. (1) OGT binds UDP-GlcNAc. (2) OGT interacts with the target protein. (3) Transfer of GlcNAc from UDP-GlcNAc to the target protein. (4) Release of the target protein. (5) Release of UDP.

Surprisingly, Seo and collaborators (Seo et al., 2016) highlighted OGT auto-*O*-GlcNAcylation at S389 which is required for its nuclear translocation. The authors also characterized the nuclear localization sequence (NLS) corresponding to the tripeptide DFP (residues 451-453). This DFP motif is required for OGT interaction with Importin α 5, allowing its subsequent nuclear translocation. In addition, OGT is not only a glycosyltransferase. OGT is also able to catalyze Host Cell Factor-1 (HCF-1) cleavage (Capotosti et al., 2011): OGT recognizes and cleaves an extended 21 amino acid sequence, resulting in two *N*-(HCF-1_N) and *C*-(HCF-1_C) terminal subunits. Then, both subunits still interact together and regulate cell cycle. The HCF-1_N subunit is involved in S-phase entry, while the HCF-1_C subunit contributes to M-phase exit (Julien & Herr, 2003).

2. 2. 2. The *O*-GlcNAcase (OGA)

OGA exists into two isoforms: the long form, OGA-L (130 kDa), mainly cytosolic, and the short form, OGA-S (75 kDa), mainly nuclear and associated to lipid droplets (Comtesse et al., 2001; Keembiyehetty et al., 2011). These two isoforms are distinct by the presence (OGA-L) or absence (OGA-S) of a Histone acetyltransferase (HAT)-like domain. Both isoforms possess an OGT-binding domain and a Glycoside Hydrolase (GH) domain (**Figure 12**) (Vocadlo, 2012).

The HAT activity of OGA is controverted. In fact, this activity has been first observed *in vitro* by Toleman et al. who also discovered a zinc-finger like motif in OGA that may be responsible for its interaction with histone tails (C. Toleman et al., 2004; C. A. Toleman et al., 2006). However, Butkinaree et al. were later unable to show that OGA possessed such an activity (Butkinaree et al., 2008). In addition, this C-terminal domain lacks key residues normally required to bind AcCoA (Rao et al., 2013). Considering these controversial results, this domain is now qualified as a HAT-like domain.

Specifically, and without a consensus sequence, OGA recognizes and binds in its catalytic pocket the GlcNAc residue whose C₁ carbon is involved in the β -bond with the target protein. OGA then catalyzes the formation of a bi-cyclic oxazoline intermediate, which is then hydrolyzed to release the target protein and the GlcNAc residue (**Figure 11**). In humans, the catalytic site of OGA is characterized by the conserved residues Asp 174 and Asp 175 (Cetinbaş

et al., 2006). As OGT, OGA is active in homodimers: deletion of OGA helical domain Q676-P694 compromises dimer formation and abolishes its activity (Roth et al., 2017).

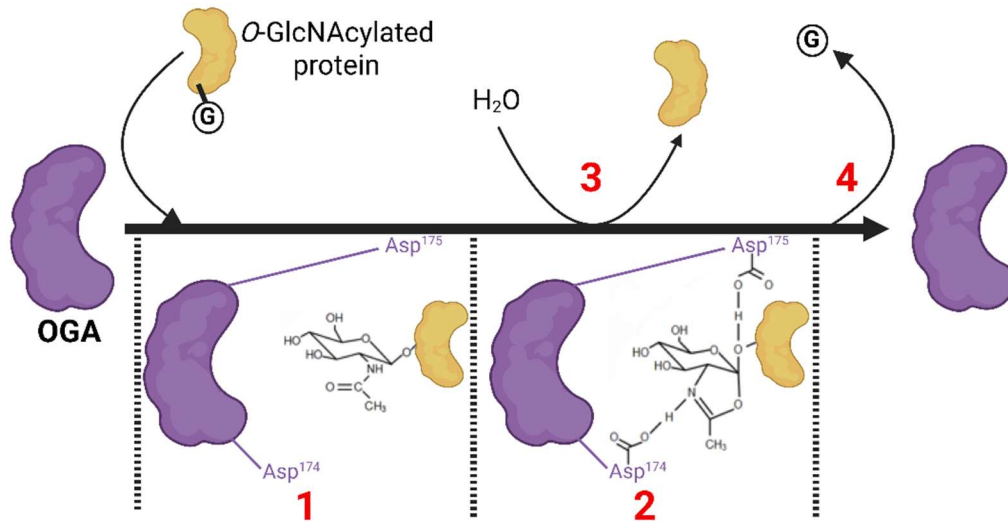


Figure 11: Simplified mechanism of OGA-mediated removal of *O*-GlcNAc modification. (1) OGA recognizes the *O*-GlcNAcylated protein. (2) OGA binds the GlcNAc residue of the target protein in its catalytic pocket through residues Asp 174 and 175, forming an oxazoline intermediate. (3) OGA hydrolyzes the GlcNAc residue from the target protein, releasing it. (4) OGA releases free GlcNAc.

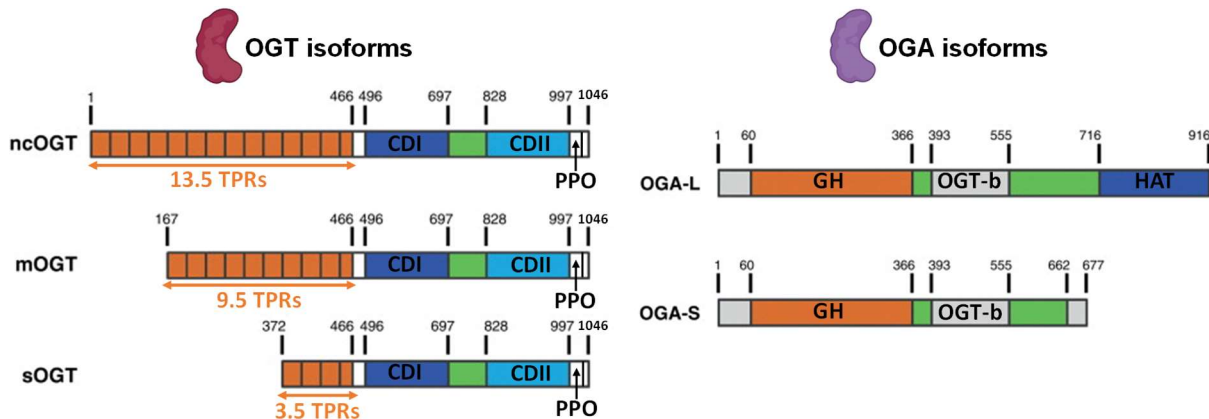


Figure 12: OGT and OGA isoforms. OGT exists as three isoforms: ncOGT (13.5 TPRs), mOGT (9.5 TPRs) and sOGT (3.5 TPRs). Each isoform has two catalytic domains: CDI and CDII, with a PIP3-binding domain (PPO). OGA exists as two isoforms: OGA-L (long form) and OGA-S (short form). Each has an OGT-binding domain (OGT-b) and a GH domain. Only OGA-L possesses a HAT-like domain. Adapted from Vocadlo, 2012.

2. 2. 3. OGA and OGT (cross-)regulation

OGA and OGT are both regulated at different levels. At the transcriptional level, E2F1, a transcription factor involved in the G1/S transition, regulates their gene expression. By binding to their respective promoters, E2F1 significantly reduces the expression of *OGT* and *MGEA5* (Muthusamy et al., 2015). Using *Drosophila* as an animal model, Liu et al. remarked daily oscillations of OGT and OGA messenger RNA (mRNA)/protein levels. They show that nutrient availability can disturb these rhythms, emphasizing the critical interplay between circadian and metabolic signals (Liu et al., 2021). Two nutrient-sensing pathways are known to regulate *O*-GlcNAcylation levels: mTOR and insulin signaling as glucose itself. In HepG2 cells, induced autophagy by mTOR inhibition decreases OGT and conversely increases OGA (S. Park et al., 2014). The same authors demonstrate that OGT stability is regulated by both autophagy and proteasomal degradation (S. Park et al., 2014). In the same cell type, Perez-Cervera et al. observed a time-dependent increase in OGT protein level under insulin stimulation (Perez-Cervera et al., 2013). Consistent with this, another study highlighted the feedback mechanism triggered by glucose deprivation of HepG2 cells (Taylor et al., 2009). This nutritional stress quickly decreases HBP flux, what induces increased OGT and decreased OGA protein level. At the post-translational level, different PTMs were reported on OGT and OGA.

2. 2. 3. 1. Post-translational modifications of OGT

Indeed, OGT TPRs present several phosphorylation sites (**Figure 13**). For instance, Glycogen synthase kinase 3 β (GSK3 β) phosphorylates OGT at S3 or S4 to increase its enzymatic activity. Interestingly, these sites can also be *O*-GlcNAcylated (Kaasik et al., 2013). AMPK also phosphorylates OGT at T444, preventing OGT-chromatin association, histone H2B *O*-GlcNAcylation and gene expression (Bullen et al., 2014; Xu et al., 2014). OGT S20 phosphorylation by Calcium/calmodulin-dependent kinase II (CaMKII) upon starvation promotes its activity (Ruan et al., 2017). In turn, OGT *O*-GlcNAcylates and activates ULK1, promoting autophagy activation. OGT S20 can also be targeted by Checkpoint kinase 1 (Chk1), increasing OGT stability. In addition to promote global *O*-GlcNAcylation, OGT S20 phosphorylation indirectly increases Vimentin S71 phosphorylation. This modification being critical for severing Vimentin filaments, it allows a proper reorganization of the intermediate filament network during cytokinesis (Z. Li et al., 2017). As discussed previously, OGT is also able of auto-*O*-GlcNAcylation at S389 which is required for its nuclear translocation (Seo et al.,

2016). Other *O*-GlcNAcylated sites were identified by mass spectrometry: T1043, one between 396-409 residues, and one between 826-832 residues (Trinidad et al., 2012). Recently, Meng and collaborators described ubiquitination-mediated regulation of OGT during mitosis (Meng et al., 2024). The Anaphase promoting complex/cyclosome (APC/C)-Cell division cycle 20 (CDC20), APC/C^{CDC20}, recognizes OGT K352 ubiquitination allowing its binding to the enzyme. Then, the complex induces OGT poly-ubiquitination and subsequent degradation. Finally, a study from Ryu and Do suggests OGT S-nitrosylation as a PTM significantly reducing its enzymatic activity (Ryu & Do, 2011). Three S-nitrosylated residues were later reported in OGT: C845, C921 and C965 (Ryu et al., 2016).

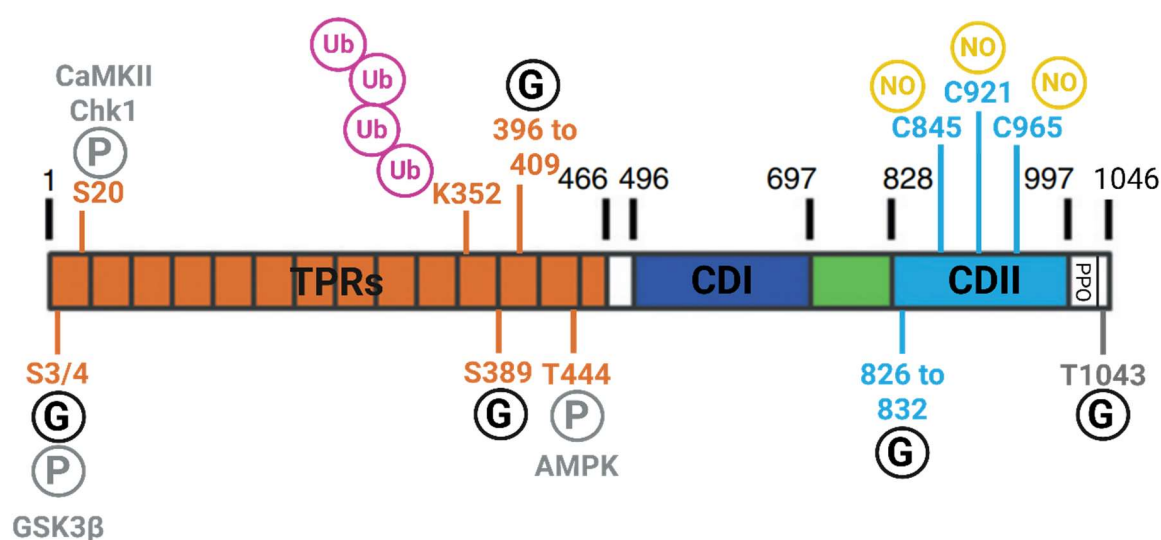


Figure 13: Summary of OGT PTMs. OGT is depicted with its PTMs: phosphorylation (P), ubiquitination, S-nitrosylation and *O*-GlcNAcylation (G). Structure of OGT is adapted from Vocadlo, 2012. Ub: Ubiquitin. NO: Nitric oxide.

2. 2. 3. 2. Post-translational modifications of OGA

OGA is *O*-GlcNAcylated at S405 (Gorelik et al., 2019), what reduces its stability. This modification could be involved in the regulation of *O*-GlcNAc homeostasis, but further investigations are still required. Many phosphorylation sites have also been reported on OGA (**Figure 14**) which function is still unknown (Nagel & Ball, 2014). During apoptosis, OGA can be cleaved by Caspase-3 at Asp 413 into two fragments: an *N*-terminal fragment containing the GH domain and a *C*-terminal fragment containing the pseudo-HAT domain. However, OGA remains active as both fragments re-associate after cleavage (Butkinaree et al., 2008).

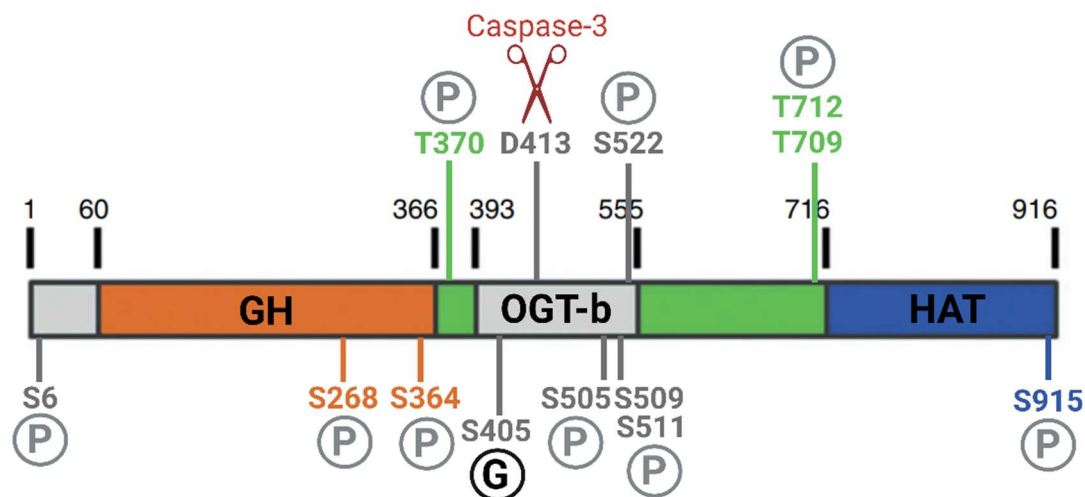


Figure 14: Summary of OGA PTMs. OGA is depicted with its PTMs on identified residues: phosphorylation (P), O-GlcNAcylation (G) and truncation by Caspase-3. Structure of OGA is adapted from Vocadlo, 2012.

2. 2. 3. 3. Regulation of O-GlcNAc homeostasis by OGA/OGT cross-regulation

Interestingly, there is a cross-regulation between OGA and OGT. As recently resolved by cryo-electron microscopy (cryo-EM) (P. Lu et al., 2023), both enzymes form a complex in which they regulate each other. It consists of a mutual inhibition that may maintain O-GlcNAc homeostasis. In response to disruption of O-GlcNAc homeostasis, HCT 116 cells modulate their OGT and OGA protein amounts (Decourcelle et al., 2020). Increased HBP flux, resulting in increased O-GlcNAcylation, induces concomitant reduced and increased expression of OGT and OGA, respectively. Likewise, the authors prevented expression or activity of OGA using siOGA or Thiamet-G (TG), respectively. Both approaches lead to a significant reduced amount of OGT.

In the same way, a compensatory mechanism was deciphered in the case of a X-linked intellectual disability (XLID) related mutation of OGT (L254F). Despite this mutation apparently reduces OGT protein levels, OGT is still active and global O-GlcNAc levels remain unaltered. The authors discovered an enrichment of the transcriptional repressor complex mSin3a-HDAC1-OGT at the proximal promoter region of *OGA* that decreases gene expression (Vaidyanathan et al., 2017). Conversely, ERK signaling has been shown to promote OGA activity which subsequently increases *OGT* expression at the transcriptional level (Qian et al., 2018). These data underline the ability of cells to maintain steady-state O-GlcNAc levels in response to different perturbations (**Figure 15**).

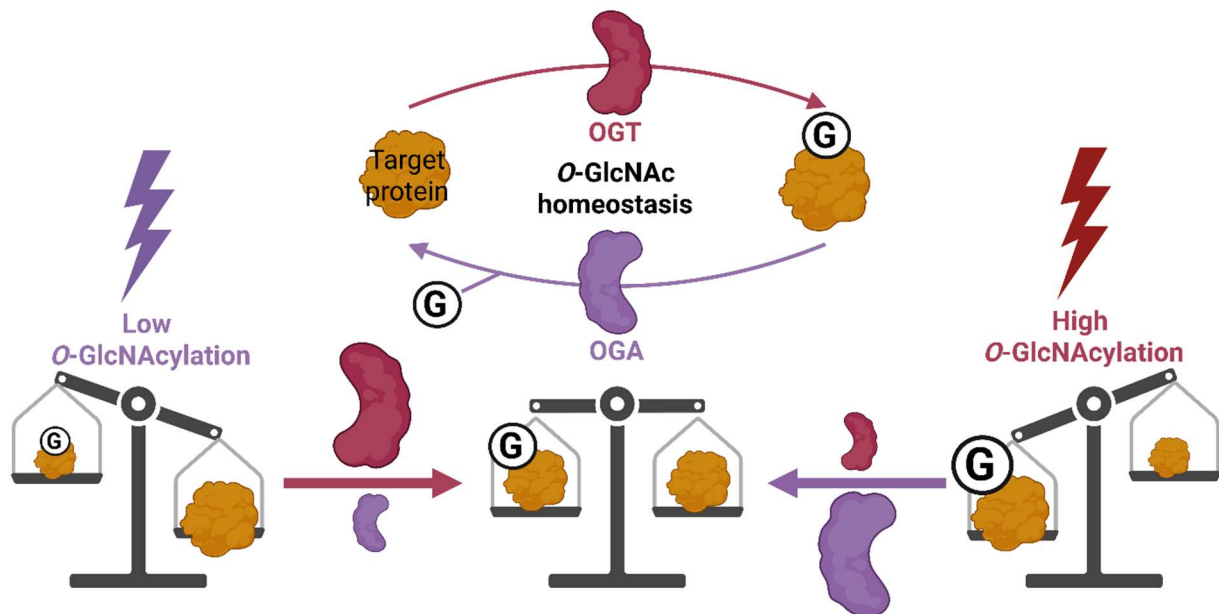


Figure 15: OGT and OGA cross-regulation. When *O*-GlcNAc homeostasis is disrupted, cells are able to adapt: a higher *O*-GlcNAcylation reduces OGT and increases OGA expression, respectively. Conversely, the opposite mechanism is triggered when *O*-GlcNAcylation levels are reduced.

2. 3. Physiologic *O*-GlcNAcylation

2. 3. 1. Systemic roles of *O*-GlcNAcylation

O-GlcNAcylation plays a crucial role in embryonic development. Indeed, knock-out of *Ogt* in mouse embryonic stem cells induces their death at an advanced stage (Shafi et al., 2000). *O*-GlcNAcylation is also involved in aging as deletion of *oga-1* or *ogt-1* in *C. elegans* increases or decreases lifespan, respectively (Rahman et al., 2010).

Long term memory may also be regulated by *O*-GlcNAcylation of the transcription factor Cyclic AMP-response element binding protein (CREB) at S40. This modification represses its transcriptional activity, resulting in decreased expression of neuronal target genes involved in brain development and memory consolidation (Rexach et al., 2012).

Moreover, OGT is involved in anti-viral immunity. Kaposi's sarcoma-associated herpesvirus (KSHV) Replication and transcription activator (RTA), simplified as K-RTA protein, is *O*-GlcNAcylated at T366 and T367. Modification of these sites by host OGT is involved in K-RTA interaction with Poly[ADP-ribose] polymerase 1 (PARP1), a negative regulator of this viral protein. Hence, OGT is able to prevent viral production (Ko et al., 2012).

At last, circadian rhythms could also be regulated by *O*-GlcNAcylation. A study from Pr. Xiaoyong Yang's laboratory highlighted Brain and muscle ARNT-Like 1 (BMAL1) and Circadian locomotor output cycles kaput (CLOCK) *O*-GlcNAcylation, two components of the mammalian circadian clock. The authors show that their *O*-GlcNAcylation prevents their ubiquitination, avoiding aberrant circadian rhythms of glucose homeostasis (M.-D. Li et al., 2013).

2. 3. 2. O-GlcNAcylation and cellular functions

At the cellular level, *O*-GlcNAcylation is involved in the different phases of the cell cycle. First, expression and catalytic activity of OGT are essential to the G0/G1 transition: OGT induces serum-stimulated cyclin D1 synthesis as well as Phosphatidylinositol 3-kinase (PI3K) and Mitogen-activated protein kinase (MAPK) pathways activation (Olivier-Van Stichelen et al., 2012). During S-phase, *O*-GlcNAcylation of subunits belonging to the Minichromosome maintenance proteins 2-7 complex (MCM 2-7 complex) is required for their binding to DNA. This complex is crucial for the initiation of DNA replication through its DNA helicase activity (Leturcq et al., 2018). At last, OGT speeds up M-phase promoting factor (MPF) and Erk2 activation, therefore promoting the G2/M transition (Dehennaut et al., 2008). Hence, OGT is involved in cell proliferation.

In addition to cell cycle and cell proliferation, OGT mediates cell differentiation. For instance, OSMI-1 - a specific and potent OGT inhibitor - alters the differentiation and function of monocytes into dendritic cells (Weiss et al., 2021). *O*-GlcNAcylation is also involved in keratinocyte differentiation. During calcium-induced keratinocyte differentiation, OGA protein level is significantly increased (Sohn et al., 2014). This is associated to an overall decreased *O*-GlcNAcylation, including that of the transcription factor Sp-1. During differentiation, decreased *O*-GlcNAcylation of Sp-1 appears to be important for its transcriptional activity. In that sense, it promotes Sp-1 activity towards *IVL*, a gene encoding the keratinocyte marker Involucrin.

Cell respiration also involves protein *O*-GlcNAcylation. Increased *O*-GlcNAcylation with the OGA inhibitor NButGT in isolated cardiac mitochondria promotes oxidative phosphorylation (OXPHOS) (Dontaine et al., 2022). Mechanistically, increased *O*-GlcNAcylation is associated with increased respiration in presence of pyruvate-malate, enhanced complex I activity, and reduction of mitochondrial reactive oxygen species (ROS) release. Thus, this PTM also plays a role in cardiac physiology where it must be finely regulated. Indeed, excessive

O-GlcNAcylation causes heart failure as shown in a mouse model with myocardial overexpression of OGT. These mice have increased calcium sparks and impaired mitochondrial energetics (Umapathi et al., 2021).

O-GlcNAcylation is gaining recognition for its involvement in the regulation of autophagy. As described before, OGT *O*-GlcNAcyates and activates the autophagy initiator ULK1 in mice livers (Ruan et al., 2017). This occurs in adaptation to starvation, highlighting the ability of OGT to sense and respond to cellular stress. Additionally, colon cells with reduced OGT activity accumulate the autophagy inhibitor p62 and display an impaired autophagosomes and lysosomes fusion (Ben Ahmed et al., 2024). These data suggest that OGT promotes autophagy.

2. 3. 3. Functions of O-GlcNAcylation at the molecular level

At the molecular level, *O*-GlcNAcylation is involved in transcription, translation and epigenetics as in the reformation of misfolded proteins. For example, *O*-GlcNAcylation of the TATA-box binding protein (TBP) at T114 is critical for the expression of 408 genes, especially genes involved in lipid metabolism (Hardivillé et al., 2020).

In addition to transcription, translation is also regulated by *O*-GlcNAcylation as demonstrated for the eukaryotic translation initiation factor 2 α (eIF2 α). While eIF2 α S51 phosphorylation inhibits its activity, therefore altering global translation initiation, its *O*-GlcNAcylation prevents S51 phosphorylation under ER stress (I. Jang et al., 2015). This example highlights the existing crosstalk between *O*-GlcNAcylation and phosphorylation that will be discussed below.

Concerning epigenetics, the mSin3a-HDAC1-OGT complex previously described clearly shows how this modification can modulate chromatin compaction and, consequently, gene expression (Vaidyanathan et al., 2017).

O-GlcNAcylation ensures proper protein synthesis and maturation by regulating protein fate from gene expression to folding control. Indeed, OGT is required for the expression of many chaperones like Heat shock protein 72 (HSP72) for the re-conformation of stress-induced misfolded proteins (Kazemi et al., 2010).

O-GlcNAcylation can regulate many properties of proteins: stability, subcellular location, protein-protein interactions and enzymatic activities. As described before, OGT auto-*O*-GlcNAcylation induces its nuclear translocation (Seo et al., 2016) while modification of OGA reduces its stability (Gorelik et al., 2019). *O*-GlcNAcylation of TBP at T114 blocks its interaction

with BTA1, thereby preventing the formation of the B-TFIID complex regulating gene expression (Hardivillé et al., 2020). Enzymatic activities can be regulated by *O*-GlcNAcylation as seen before with eIF2 α whose activity is promoted by this modification (I. Jang et al., 2015). *O*-GlcNAcylation has also an emerging role in the regulation of protein trafficking as showed for Rab7 (Pekkurnaz et al., 2014): OGT depletion in cultured rat hippocampal neurons reduces retrograde trafficking of Rab7.

Thus, *O*-GlcNAcylation is involved in numerous fundamental physiological functions, ranging from molecular processes to whole-organism development and homeostasis (**Figure 16**).

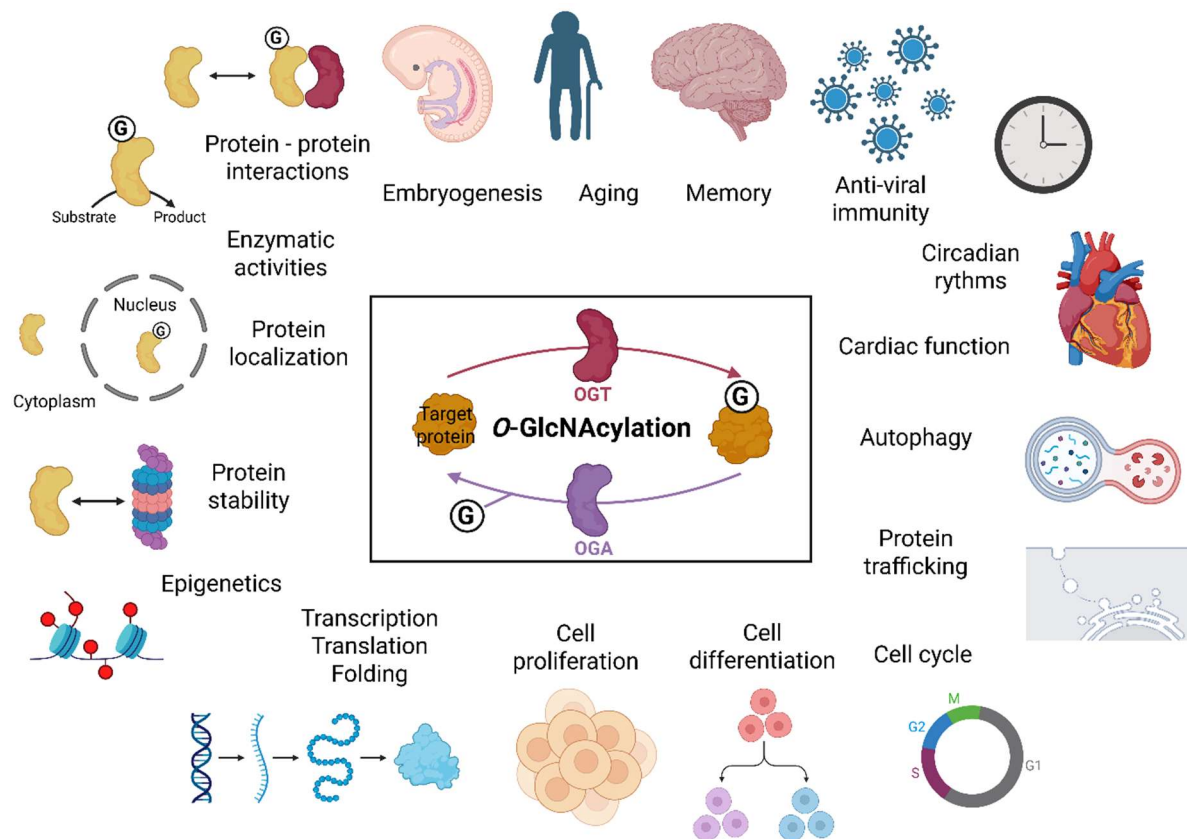


Figure 16: Summary of the multiple physiological functions of *O*-GlcNAcylation. See text for details.

2. 3. 4. *O*-GlcNAcylation and phosphorylation cross-talk

Altogether these different studies demonstrate the pivotal role of *O*-GlcNAcylation in different physiological contexts. However, *O*-GlcNAcylation can compete or promote phosphorylation, emphasizing a cross-talk between the two PTMs (Butkinaree et al., 2010). A reciprocal occupancy can occur at the same serine or threonine residues but also at different sites (**Figure**

17). For example, while T41 phosphorylation of β -catenin induces its degradation, *O*-GlcNAcylation of this residue prevents β -catenin ubiquitination and subsequent proteasomal degradation (Olivier-Van Stichelen et al., 2014). For eIF2 α , point mutation of distant *O*-GlcNAc sites (S219, T239 and T241) increases S51 phosphorylation (I. Jang et al., 2015). Despite this classical “yin-yang” rule, proteins can also bear the two PTMs at the same time (**Figure 17**) as shown for Insulin receptor substrate-1 (IRS-1) (Ball et al., 2006). In addition, a positive cross-talk has been observed for HDAC1 as its *O*-GlcNAcylation promotes its phosphorylation and, consequently, its activity in HCC (G. Zhu et al., 2016).

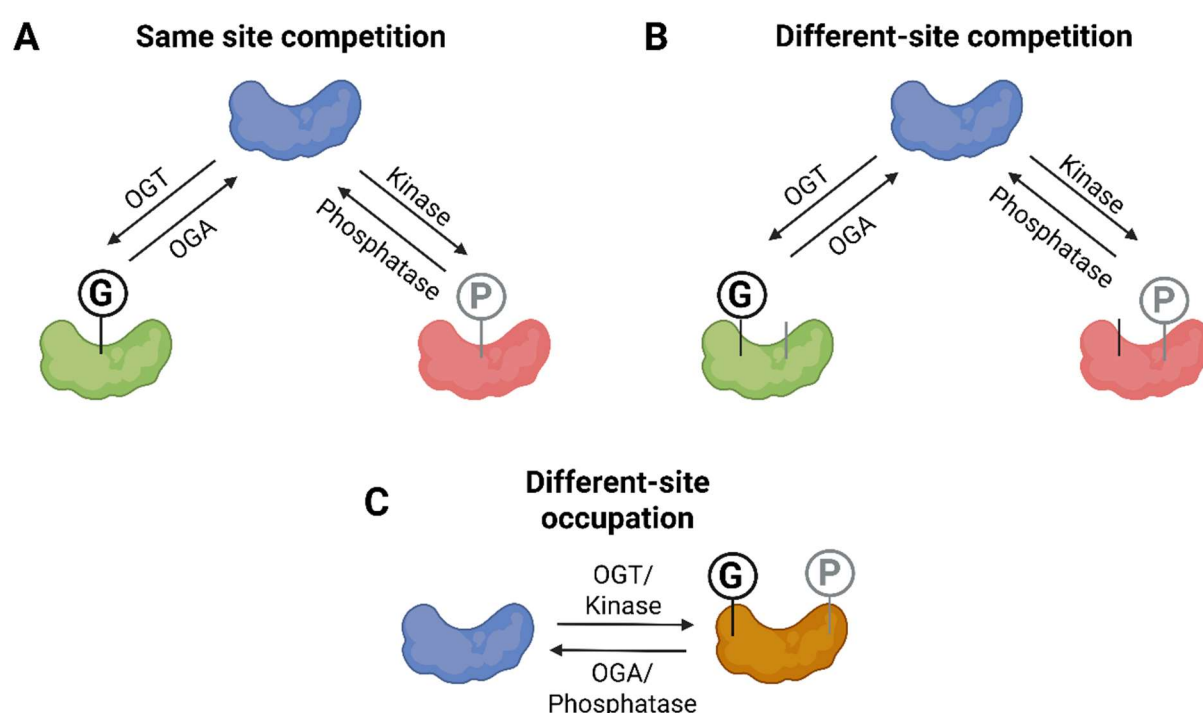


Figure 17: Dynamic interplay between *O*-GlcNAcylation and phosphorylation. The two PTMs can compete for the same site (**A**) or for different sites (**B**). Both can also be present at the same time on the protein but on distinct amino acids (**C**). Adapted from Butkinaree et al., 2010.

2. 4. *O*-GlcNAcylation and cancer

Reports indicate that dysregulations of *O*-GlcNAcylation homeostasis are involved in diverse pathologies including cancer (Ferrer et al., 2016). In cancer cells, an increased *O*-GlcNAcylation is often correlated to an overexpression of OGT. Indeed, OGT and *O*-GlcNAcylation levels are elevated in HCC (Raab et al., 2022), prostate cancer (Lynch et al., 2012), breast cancer (Champattanachai et al., 2013), lung cancer (Y.-C. Lin et al., 2018), bladder cancer (Rozanski et

al., 2012), CRC (Steenackers et al., 2016) as well as in chronic lymphocytic leukemia (Shi et al., 2010). This increase can be linked to the glucose avidity of cancer cells (Warburg effect) and to glutamine, two essential metabolites of HBP (**Figure 9**). Hence, OGT may be involved in all hallmarks of cancer (**Figure 18**) as highlighted by different studies described below.

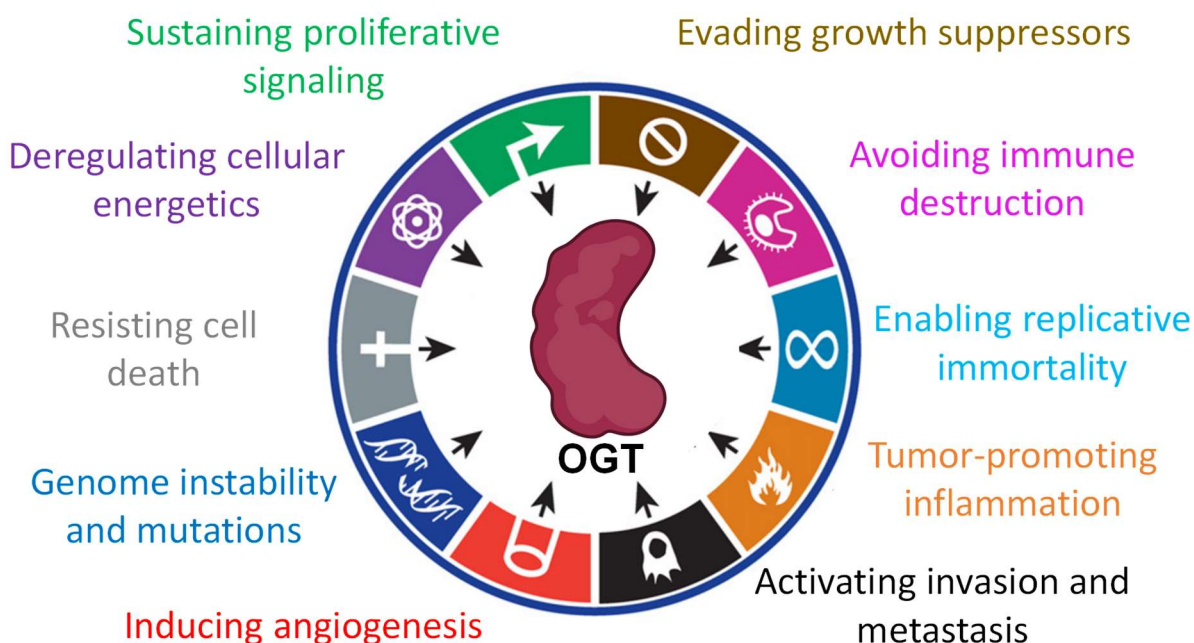


Figure 18: OGT may be involved in all hallmarks of cancer. Due to its pro-oncogenic character, OGT could promote any hallmarks of cancer. Adapted from Hanahan and Weinberg, 2011.

2. 4. 1. OGT sustains proliferative signaling

In breast cancer, OGT is overexpressed *via* the mTOR/c-Myc/HSP90A pathway (Sodi et al., 2015). The oncogenic transcription factor c-Myc promotes *HSP90A* expression. HSP90A stabilizes OGT and, conversely, OGT stabilizes c-Myc by *O*-GlcNAcylation at T58. Thus, a positive feedback loop is engaged, sustaining a proliferative signaling (**Figure 19**).

As described before, OGT *O*-GlcNAcyates β -catenin at T41, promoting its stability (Olivier-Van Stichelen et al., 2014). As an oncogene, β -catenin is therefore able to sustain proliferative signals. On the other hand, siOGT-transfected HepG2 cells display a significant reduction of p70 ribosomal protein S6 kinase (p70S6K) phosphorylation along cell cycle (Raab et al., 2021). p70S6K being the downstream target of mTOR, these data show that OGT is required to activate mTOR signaling in hepatic cancer cells.

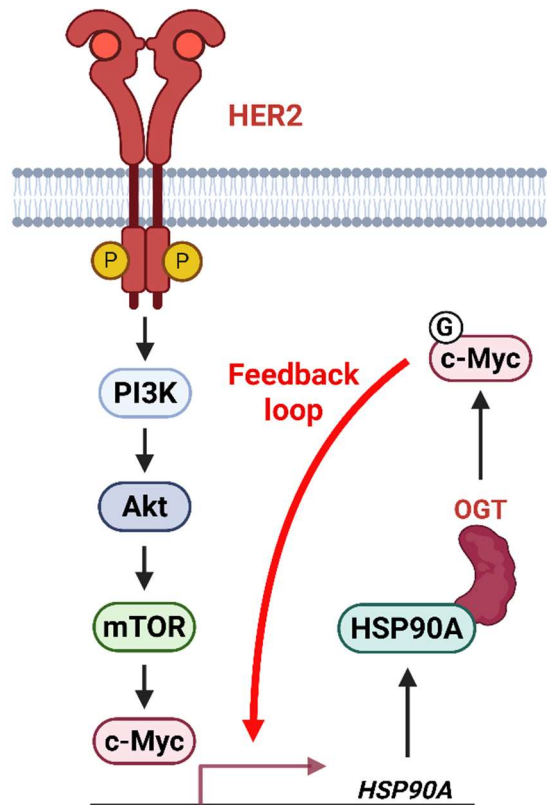


Figure 19: mTOR/c-Myc/HSP90A/OGT positive feedback loop. Adapted from Sodi et al., 2015.

2. 4. 2. OGT reprograms cancer cell metabolism

OGT deregulates cellular energetics as shown for Phosphofructokinase 1 (PFK1) in lung cancer (Yi et al., 2012). In response to hypoxia, O-GlcNAcylation of S529 sterically occludes binding of the allosteric activator Fructose-2,6-Bisphosphate (F-2,6-BP). This prevents PFK1 oligomerization and activity (**Figure 20**), redirecting glucose to the PPP and subsequently increasing cancer cell proliferation.

Interestingly, OGA pseudo-acetyltransferase activity is apparently able to reprogram cancer cell metabolism (Singh et al., 2020). Indeed, OGA increases Pyruvate kinase M2 (PKM2) acetylation *in vitro* and *in vivo*. PKM2 acetylation promotes O-GlcNAcylation at S362 and T365 which inhibits PKM2 tetramerization and catalytic activity. This promotes aerobic glycolysis and tumor growth.

Recently, PI3K β has been shown to phosphorylate OGT at T985, enhancing its activity towards ACLY in glioblastoma cells with high glucose supplementation (He et al., 2025). O-GlcNAcylation of this DNL enzyme at T639 and S667 increases its activity and, therefore, FA levels. This cascade sustains glioblastoma tumor growth.

2. 4. 3. *OGT prevents cancer cell death*

In breast cancer-derived MCF-7 cells treated with tamoxifen, *O*-GlcNAcylation levels influence tamoxifen sensitivity (Kanwal et al., 2013). Increased *O*-GlcNAcylation by OGA inhibition or glucosamine supplementation protects MCF-7 cells from tamoxifen-induced cell death. On the contrary, knocking down *OGT* by siRNA increases cell death induced by tamoxifen. Remarkably, increased *O*-GlcNAcylation reduces Estrogen Receptor α (ER α) mRNAs and protein levels. This observation may explain the reduced sensitivity to tamoxifen when *O*-GlcNAcylation is more elevated. Counter-intuitively, increasing *O*-GlcNAcylation levels in CRC tissues with Thiamet-G has a synergistic inhibitory effect when combined to 5-fluorouracil (5-FU) (Very et al., 2022). In fact, 5-FU targets and inhibits Thymidylate synthase (TS) whose *O*-GlcNAcylation promotes both stability and activity. However, increased TS *O*-GlcNAcylation enhances its sensitivity to 5-FU, increasing tumor growth inhibition.

2. 4. 4. *OGT and genomic instability*

Although no study has yet highlighted a role of *OGT* in genomic instability, proteins involved in genomic stability and chromatin remodeling interact with *OGT*. This observation suggests that dysregulation of the *O*-GlcNAc homeostasis in cancers could disturb these mechanisms and, therefore, induce genomic instability. Indeed, a stable OGA knockdown HeLa cell line displays aberrant mitotic spindles and aneuploidy. These cells have disturbed Cyclin expressions and increased Rb phosphorylation, showing the crucial role of *O*-GlcNAc rheostat in cell cycle and, consequently, in genomic stability (Lanza et al., 2016). In addition, the master regulator of genomic stability, p53, is *O*-GlcNAcylated at S149 (W. H. Yang et al., 2006). This modification decreases p53 T155 phosphorylation, resulting in decreased p53 ubiquitination (**Figure 20**). Ten-eleven translocation 1/2/3 (TET1/2/3) proteins involved in DNA demethylation promote chromatin decompaction (Mahfoudhi et al., 2015) and can interact with *OGT* (Vella et al., 2013). TET proteins present loss-of-function mutations in cancers and can recruit *OGT* to chromatin. Thus, changes in TET-*OGT* complexes at specific genomic regions could result in chromatin alterations, promoting cancer phenotypes (Ferrer et al., 2016). Very recently, Chen et al. demonstrated that *Ogt* knockout results in cytosolic double-stranded DNA accumulation in different murine colorectal carcinoma models (Chen et al., 2024). As a result, *OGT* could actually directly or indirectly regulate genomic stability.

2. 4. 5. OGT and angiogenesis

OGT promotes angiogenesis in prostate cancer (Lynch et al., 2012). In fact, by stabilizing the transcription factor Forkhead Box M1 (FoxM1), a key regulator of invasion and angiogenesis, OGT indirectly promotes the expression of Vascular Endothelial Growth Factor (VEGF) mRNA. Indeed, a prostatic cancer cell line with a stable knocked down *OGT* presents a significant reduction of VEGF mRNA expression as well as decreased expression of MMP-2/9 (**Figure 20**). Incubation of human umbilical vein endothelial cells (HUVECs) with conditioned medium from these prostatic cancer cells significantly impedes tubulogenesis. Additionally, reduced OGT levels inhibit prostate cancer metastasis to bone in xenografted mice.

2. 4. 6. OGT promotes cancer cell migration and metastasis

As discussed previously, OGT promotes prostate cancer cell invasion and bone metastasis (Lynch et al., 2012). Metastatic dissemination of cancer cells relies on different processes including EMT. EMT enables cell migration and is regulated by the repressive transcription factor of E-cadherin, Snail1. OGT glycosylates Snail1 at S112, increasing its stability and therefore promoting EMT of MCF-7 cells (S. Y. Park et al., 2010) (**Figure 20**). siOGT-transfected HT29 and HCT116 cells, two CRC cell lines, present a significant reduced migration compared to their siControl-transfected counterparts (Steenackers et al., 2016).

2. 4. 7. OGT and tumor-promoting inflammation

In human colitis-associated cancer (CAC) tissues, *O*-GlcNAcylation is increased compared to normal surrounding tissues (Y. R. Yang et al., 2015). At the molecular level, the authors found T322 and T352 *O*-GlcNAcylation sites on p65 nuclear factor-kappa B (NF-κB) subunit. These glycosylations of p65 are critical for its promoter binding, increasing NF-κB signaling that promotes the development of CAC.

OGT could also be involved in tumor-promoting inflammation by modifying Signal transducer and activator of transcription 3 (STAT3). In colon macrophages, *O*-GlcNAcylation of STAT3 at T717 negatively regulates its phosphorylation. STAT3 transcriptional activity is reduced, decreasing anti-inflammatory Interleukin-10 (IL-10) production (**Figure 20**). As a result, tumor-promoting inflammation is exacerbated in a CAC mouse model (X. Li et al., 2017).

2. 4. 8. OGT and replicative immortality

O-GlcNAcylation stabilizes c-Myc (Sodi et al., 2015) and c-Myc has been shown to activate telomerase (Wang et al., 1998). While no evidence has yet been determined on OGT ability to promote replicative immortality, we can hypothesize that the activation of telomerase may at least partially depend on OGT expression (**Figure 20**). Supporting this idea, *O*-GlcNAcylation can avoid replication arrest after genotoxic stress (Efimova et al., 2019). Therapy-induced senescence of MCF-7 cells triggered by radiation depends on OGT activity (**Figure 20**). Indeed, silencing OGT leads to delayed double-strand break repair, reduced cell proliferation, and increased cell senescence *in vivo*. Thus, OGT could promote replicative immortality of cancer cells but further investigation is required to gain insights into underlying mechanisms.

2. 4. 9. OGT and immune escape

OGT involvement in avoiding immune destruction has recently gained insights. *O*-GlcNAcylation promotes tumor immune escape by preventing PD-L1 intracellular trafficking from early endosomes to lysosomes for degradation (Q. Zhu et al., 2023). Precisely, Hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), a key component of the endosomal sorting machinery, is *O*-GlcNAcyated. HGS glycosylation inhibits its interaction with PD-L1, preventing lysosomal degradation of the latter (**Figure 20**). An anti-tumor immune response is synergistically induced when anti-PD-L1 antibodies are combined with OSMI-4, a specific inhibitor of OGT. A very recent study also added evidence of OGT-mediated inhibition of anti-tumor immunity (Chen et al., 2024). This study shows that OGT inactivates the cGAS-STING-IFN-I [Cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase - Stimulator of interferon genes - Type I interferons] pathway (**Figure 20**), preventing CD8⁺ T-cell-dependent antitumor immunity.

2. 4. 10. OGT and evasion of growth suppressors

As described before, the oncogenic transcription factor FoxM1 is stabilized by *O*-GlcNAcylation in prostate cancer cells (Lynch et al., 2012). Another study demonstrated FoxM1 destabilization by decreased breast cancer cells *O*-GlcNAcylation levels, reducing multiple FOXM1-specific targets, including S-phase kinase-associated protein 2 (SKP2) (Caldwell et al.,

2010). SKP2 targets the growth suppressors CDKI (CDK inhibitors), like $p27^{Kip1}$, for degradation during the G1/S transition. Thus, SKP2 downregulation leads to increased level of $p27^{Kip1}$, inducing a G1 arrest of breast cancer cells. Regulation of cell proliferation through the OGT-FoxM1-SKP2 axis (**Figure 20**) also involves another CDKI, p21, encoded by the *CDKN1A* gene (de Queiroz et al., 2022). Protein levels of p21 can also be regulated by the transcriptional activity of the tumor suppressor p53. In this study, the authors highlighted a p53-dependent as a p53-independent regulation of *CDKN1A* expression, both involving OGT. Indeed, OGT inhibition with OSMI-1 increased p21 levels in both HT1080 p53 WT and HT1080 p53 KO fibrosarcoma cells. Stabilization of p21 was mediated by downregulation of the FoxM1-SKP2 pathway. Subsequently, p21 induced cell cycle arrest and decreased cell proliferation.

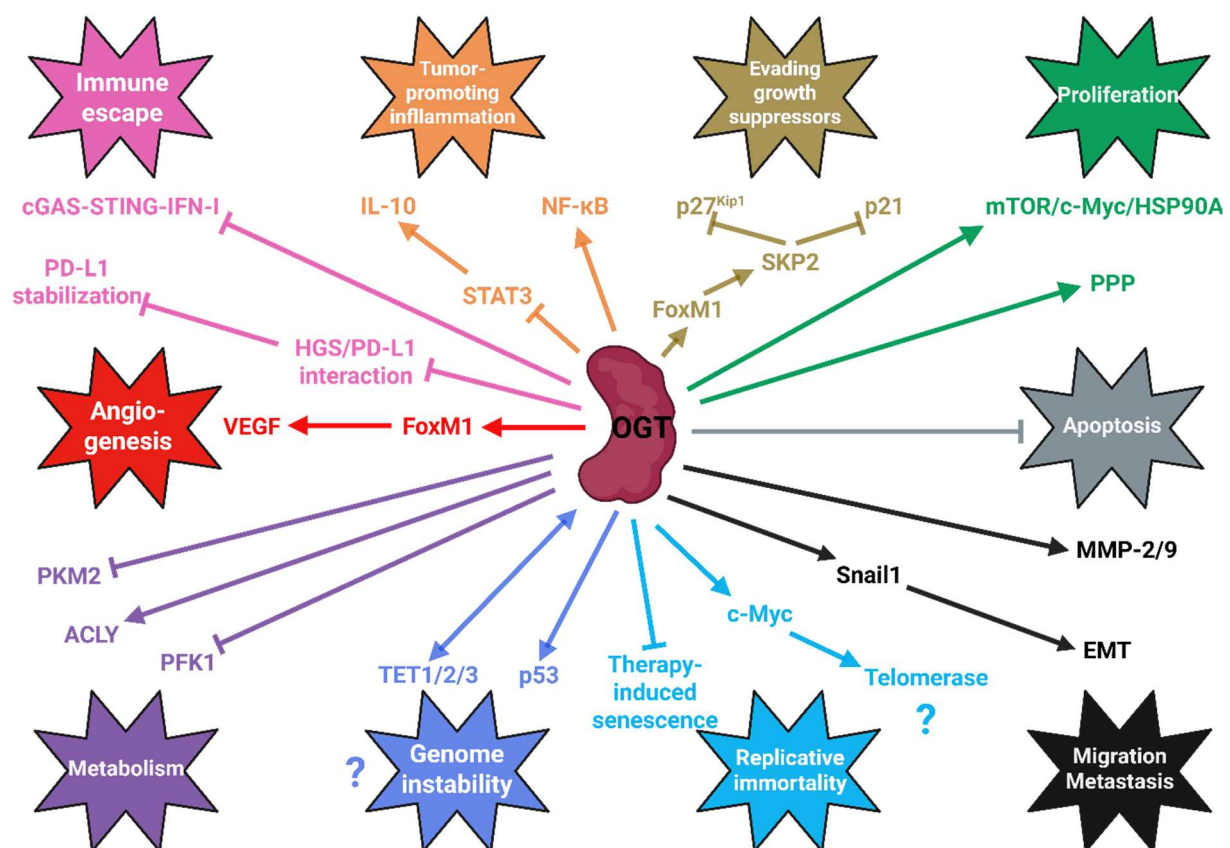


Figure 20: Summary of the different OGT pro-oncogenic features. OGT O-GlcNAcyates and inhibits PKM2 and PFK1 while activating ACLY (purple). Angiogenesis induction is favored by FoxM1-induced VEGF mRNA expression level (red). By increasing PD-L1 stabilization and inhibiting the cGAS-STING-IFN-I pathway, OGT is involved in immune escape (pink). OGT promotes tumor-inflammation via STAT3 inhibition and NF- κ B activation (orange). By stabilizing FoxM1, OGT also promotes degradation of CDKIs (brown). Proliferative signaling is enhanced by OGT via mTOR/c-Myc/HSP90A and PPP pathways activation (green). In addition, OGT blocks cancer cell apoptosis (gray) and induces EMT and MMP-2/9 expression (black). Thus, OGT is involved in migration and metastasis. At last, OGT is also involved in replicative immortality (cyan) and in genome instability (blue).

PhD PROJECT

Thesis objectives

Although ubiquitous, physiologic lipogenesis is more elevated in the liver, a central organ in the regulation of the glucido-lipidic metabolism (Che et al., 2017). At the pathological level, we observe an overactivation of lipogenesis in hepatocellular carcinoma (HCC), the main common form of primary hepatic tumors (Chidambaranathan-Reghupaty et al., 2021). Hepatic cancer is the sixth most common cancer and the third cause of death by cancer worldwide (Sung et al., 2021). Diagnosis is often delayed and treatments inefficient for advanced stages. Several risk factors were identified: alcohol, tobacco, aflatoxin B1, infection by hepatitis B and C viruses, or diabetes and obesity (Janevska et al., 2015). The two latter are directly linked to the nutritional status and consequently to *O*-GlcNAcylation and lipogenesis, two processes involved in oncogenesis in part *via* FASN.

In that sense, the team highlighted FASN *O*-GlcNAcylation in HepG2 hepatic cancer cells and in mice livers (Baldini et al., 2016). This PTM promotes FASN interaction with the de-ubiquitinase Ubiquitin-specific peptidase 2a (USP2a). Consequently, this decreases FASN proteasomal degradation and increases its stability and subsequent activity. Conversely, FASN promotes OGT expression and activity in HepG2 cells (Raab et al., 2021). In mouse hepatocytes, *O*-GlcNAcylation also promotes FASN expression by modifying ChREBP transcription factor. ChREBP *O*-GlcNAcylation increases its stability and transcriptional activity towards *FASN* (Guinez et al., 2011). In addition, FASN is able to inhibit OGA in order to increase global *O*-GlcNAcylation during oxidative stress (Groves et al., 2017). This has been described as a pro-survival mechanism.

FASN *O*-GlcNAcylation also promotes cancer cell survival during starvation (Wong et al., 2022). Lastly, Li and collaborators (X. Li et al., 2025) recently highlighted OGT interaction at FASN S1483 site in mouse hepatocytes (AML-12 cells). They suggest that *O*-GlcNAcylation at S1483 reduces FASN K48-ubiquitination and degradation, increasing its activity and subsequent hepatic lipid accumulation. Thus, deciphering the link between these two pro-oncogenic enzymes in HCC is of particular interest, both promoting many hallmarks of cancer and being overexpressed in this cancer subtype (Raab et al., 2022).

My PhD project was carried out on HepG2 and Hep3B hepatic cancer cells. By combining cell culture, mass spectrometry, molecular biology, protein biochemistry, lipidomics, cellular assays, confocal microscopy and bioinformatics, my PhD followed two aims (**Figure 21**):

1) Studying the regulation of FASN properties by *O*-GlcNAcylation at the molecular and cellular levels.

2) Determining FASN and OGT respective interaction regions.

The rationale for this project was that finding key *O*-GlcNAcylation site(s) and how both enzymes interact together should shed the light to new therapeutic targets for patients suffering from cancer.

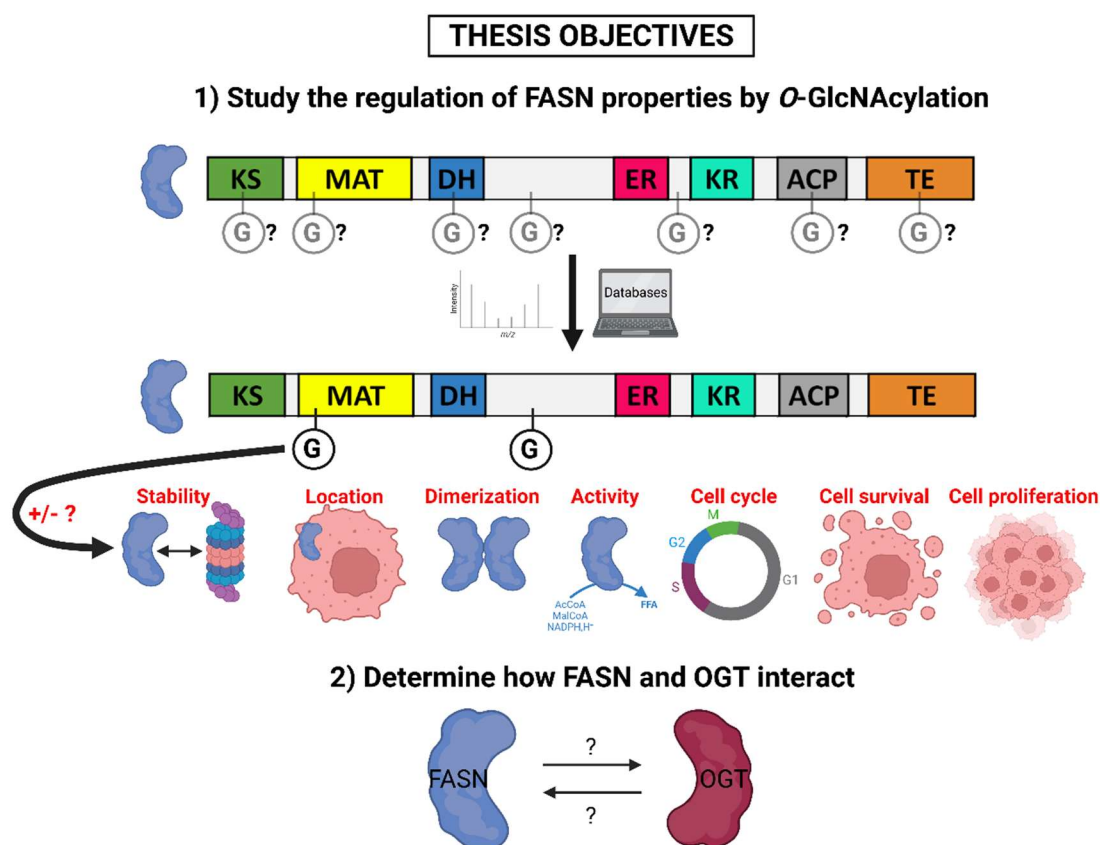


Figure 21: Summary of thesis objectives. By mass spectrometry and databases exploration, we identified FASN *O*-GlcNAcylated sites in order to study how these modifications regulate its properties. Thus, the effect of *O*-GlcNAcylation was analyzed on FASN stability, location, dimerization and activity. The impact on cell cycle progression, cell survival and cell proliferation were also investigated in hepatic cancer cells **(1)**. At last, we aimed to determine how FASN and OGT interact in these cells **(2)**.

PERSONAL WORKS

Part 1: *O*-GlcNAcylation of fatty acid synthase is required for its proper subcellular localization, expression level and activity

1. Context

As described before, involvement of *O*-GlcNAcylation and FASN in tumorigenesis has largely been investigated. However, direct regulation of FASN by *O*-GlcNAcylation still required further studies. My team demonstrated that this PTM prevents FASN proteasomal degradation in HepG2 cells (Baldini et al., 2016). A positive cross-regulation has also been highlighted in this hepatic cancer cell line (Raab et al., 2021). Hence, we aimed to decipher the role of this PTM by identifying *O*-GlcNAcylated sites of FASN involved in the regulation of its pro-oncogenic properties in hepatic cancer cells.

This work was the subject of an original article published in the *Journal of Biological Chemistry*.

Dimitri Vanauberg, Céline Schulz, Sadia Raab, Gabriela Fuentes-García, Emilie Cadart, Quentin Lemaire, Stéphanie Olivier-Van Stichelen, Fabrice Bray, Guillaume Brysbaert, Yannick Rossez, Stéphan Hardivillé, and Tony Lefebvre (2025). *O*-GlcNAcylation of fatty acid synthase is required for its proper subcellular localization, expression level and activity. *Journal of Biological Chemistry*. <https://doi.org/10.1016/j.jbc.2025.110497>

Major results of this study are described below.

2. Results

2. 1. Regulation of fatty acids synthesis by O-GlcNAcylation

Firstly, we investigated the impact of *O*-GlcNAcylation levels modulation on FA synthesis in HepG2 cells measured by gas phase chromatography with a downstream flame ionization detector (GC-FID). We showed that decreased *O*-GlcNAcylation induced by siOGT results in a global decrease of FA, particularly of palmitic acid (C16:0), the main and final product of FASN (**Publication I - Figure 1**). Conversely, increased *O*-GlcNAcylation induced by Thiamet-G upregulated FA synthesis, including C16:0 (**Publication I - Figure 2**).

These experiments were also conducted in another hepatic cancer cell line, the Hep3B cell line (**Figure 22**, unpublished data). After checking of the efficiency of the different treatments (**Supp A**), FA were measured individually (**Supp B**) and globally (**Supp C** and **D**) as previously described. Although not always significant, the same trends were observed as for HepG2 cells. FA synthesis is therefore positively regulated by *O*-GlcNAcylation.

At last, cells were also counted (**Supp E**) to determine the effect of *O*-GlcNAcylation levels on cell proliferation. Consistent with the literature, it appears that FASN inhibition (C75) and decreased *O*-GlcNAcylation (siOGT) reduce cell proliferation.

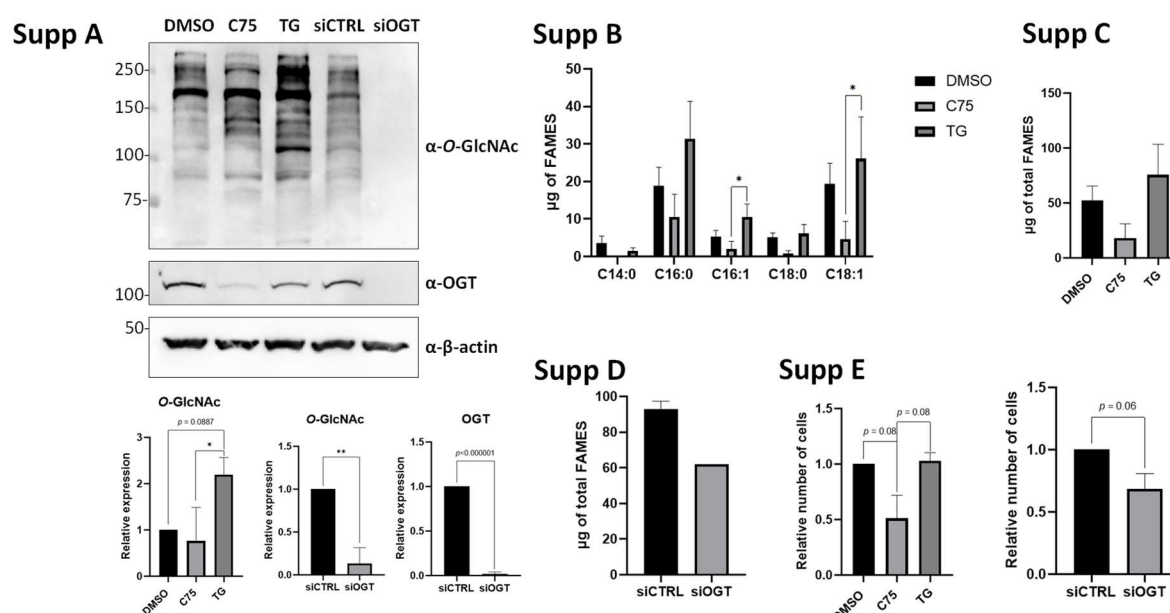


Figure 22: Effect of *O*-GlcNAcylation levels modulation on fatty acids synthesis of Hep3B cells.

Hep3B cells were treated with the FASN inhibitor C75 or OGA inhibitor Thiamet-G (TG), or transfected with siRNA targeting OGT mRNA. Cell lysates were analyzed by Western blot according to their OGT and *O*-GlcNAc contents (**Supp A**). Molecular mass markers are indicated on the left (kDa). Optical densities were measured and normalized with β -actin expression. For cells treated with C75 or TG, individual (**Supp B**) or global (**Supp C**) amounts of FAMES were measured by GC-FID. FAMES from Hep3B transfected cells were measured globally in two independent experiments (**Supp D**). To assay cell proliferation, cells were counted after treatment/transfection (**Supp E**). Data are presented with means $\pm$ SEM ($n = 3$) and were compared using one-way or two-way ANOVA and Student's *t*-test (two-tailed and unpaired *t*-test). $p < 0.05$ *; $p < 0.01$ **.

2. 2. Identification of FASN O-GlcNAcylated sites and functional analysis

Secondly, we reported two O-GlcNAcylated sites on FASN: the S595 and T980 residues. By single point mutations, we identified T980 O-GlcNAcylation as a major FASN regulating PTM. By Western blotting and cycloheximide chase assay, we observed that FASN T980A mutant was significantly less stable. As showed by native-PAGE and BODIPY assay, dimerization and subsequent activity were reduced, respectively, as well as reduced membrane association (**Publication I - Figures 3 and 4**). Using flow cytometry, clonogenicity/MTS assays and cell counting, we demonstrated a subsequent blockade of cell cycle progression associated to decreased cell survival and proliferation of Hep3B hepatic cancer cells (**Publication I - Figure 5**). Despite several assays, no differences were observed in their migration (data not shown).

2. 3. FASN T980 phosphorylation

Due to the apparent importance of FASN T980 site and to the existing “yin-yang” rule (**Figure 17**), we wondered whether this O-GlcNAcylation site could also be phosphorylated (**Figure 23**, unpublished data). By re-exploring our liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) data, we effectively observed phosphorylation of FASN at T980 (**Supp A**). To investigate the impact of FASN T980 phosphorylation on its protein amount, the 3xFlag-FASN T980D mutant was constructed and expressed in Hep3B cells in parallel to empty vector (negative control) (**Supp B**).

Interestingly, while absence of O-GlcNAcylation at T980 constantly reduces FASN protein amount (FASN T980A), mimicked phosphorylation resulted in almost complete loss of the enzyme (FASN T980D). In addition, the T980D mutation totally abrogated FASN ability to promote cell cycle progression (**Supp C**). FASN T980D expression even led to the disappearance of cells in G2-M. Therefore, these preliminary data suggest that a “same site competition” would occur at FASN T980 with opposite effects between both PTMs. Further investigations are necessary to complete this observation.

Reference Spectrum

Intensity (counts) (10³)

m/z

274.15692
VSENGNLV⁺, SENGALV⁺
86.09702
VY⁺, NLVY⁺
72.08154
b₁⁺
54.04301
SP
1.2
VSEN⁺
303.14270
GR⁺, LVVS⁺, LVVSG⁺
186.08711
ENG⁺, KYVQWDDPDRFL⁺, NLVVSQKVVQWDDPDR⁺
301.11399
a₁⁺-NH₃, SENGNLVVSQKVVQWDDPDRFLFDHPESPTPN⁺
773.37122
a⁺-NH₃, KYVQWDD⁺, VYQWDDPDRFLFDHP⁺, EYSENGNLVVSQKVVQWDDPDR⁺
686.21970

— Precursor, Precursor-H, Precursor-H₂O, Precursor-NH₃ — v, y-H₂O, y-NH₃ — a, a-H₂O, a-NH₃, b, b-H₂O, b-NH₃ — va, vb

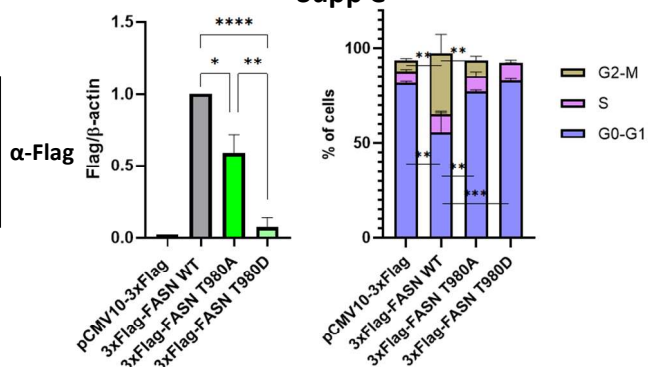
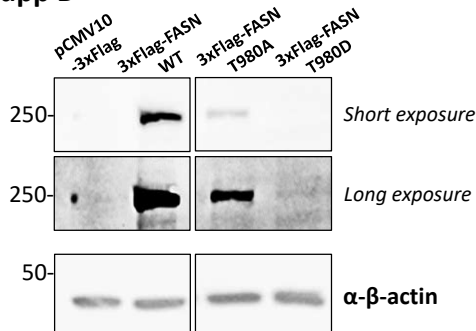


Figure 23: FASN T980 phosphorylation. LC-MS/MS analyses of immunopurified FASN revealed threonine 980 as a FASN phosphorylated site (A). Hep3B cells were transfected with an empty vector or 3xFlag-FASN (wild type and mutants) expressing vectors. Cell lysates were analyzed by Western blot (n=3) by using an anti-Flag antibody (B). Molecular mass markers are indicated on the left (kDa). Optical densities were measured and normalized with β -actin expression. (C) Cell cycle was analyzed by flow cytometry (n=3). Data are presented with means $\pm$ SEM and were compared using one-way or two-way ANOVA. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Altogether, these experiments highlight the pivotal role of FASN T980 *O*-GlcNAcylation site in regulating the enzyme's properties. This work further elucidates the involvement of this nutrient-dependent modification in the regulation of lipogenesis in hepatic cancer cells.



O-GlcNAcylation of fatty acid synthase is required for its proper subcellular localization, expression level, and activity

Received for publication, June 11, 2025, and in revised form, July 5, 2025 Published, Papers in Press, July 18, 2025

<https://doi.org/10.1016/j.jbc.2025.110497>

Dimitri Vanauberg¹, Céline Schulz¹, Sadia Raab¹, Gabriela Fuentes-García¹, Emilie Cadart¹, Quentin Lemaire¹, Stéphanie Olivier-Van Stichelen¹, Fabrice Bray², Guillaume Brysbaert¹, Yannick Rossez¹, Stéphan Hardivillé¹, and Tony Lefebvre^{1,*}

From the ¹University of Lille, CNRS, UMR 8576 - UGSF - Unité de Glycobiologie Structurale et Fonctionnelle, Lille, France; ²Medical College of Wisconsin, Department of Biochemistry, Wisconsin, Milwaukee, USA; ³University of Lille, CNRS, UAR 3290-MSAP-Miniaturisation pour la Synthèse, l'Analyse et la Protéomique, Lille, France

Reviewed by members of the JBC Editorial Board. Edited by Robert Haltiwanger

Fatty Acid Synthase (FASN) is involved in various fundamental cellular processes through its pivotal role in producing fatty acids through the *de novo* lipogenesis pathway. FASN is frequently overexpressed in tumors and participates in cancer cell proliferation. Little has been documented regarding post-translational modifications of FASN. We previously demonstrated that O-GlcNAcylation regulates FASN in mice livers and in the HepG2 hepatic cancer cell line. In the present study, we show that modulation of global O-GlcNAcylation levels impacts fatty acids production in HepG2 cells. We identified serine 595 and threonine 980 as major O-GlcNAcylation sites. While mutation of S595 moderately affects FASN behavior, T980 is crucial for FASN expression, membrane localization, homodimerization, stability, and activity in Hep3B cells. This residue is necessary for FASN properties, promoting cell survival, cell proliferation, and cell cycle progression. Our results suggest that targeting FASN at T980 may open an interesting path for controlling its catalytic activity.

Hepatic cancer is an emerging major public health concern, ranking as the sixth most common cancer worldwide and the third leading cause of cancer-related mortality (1). Hepatocellular carcinoma (HCC), the most represented subtype of hepatic cancer, is characterized by a dysregulation of lipid metabolism and storage, with an accumulation of fat in diseased hepatocytes (2). This phenomenon results in part from the overexpression of enzymes involved in the *de novo* lipogenesis pathway, including fatty acid synthase (FASN), whose expression is associated with a poor prognosis in HCC (3, 4).

FASN is a ubiquitous and high-molecular-weight enzyme (273 kDa) that functions as a homodimer (4, 5). The enzyme is mainly located in the cytosolic compartment but also

interacts with lipid microdomains (6). FASN produces fatty acids using acetyl-CoA, malonyl-CoA and NADPH, H⁺ via 7 different catalytic activities. Fatty acids can be stored following esterification in the form of lipid droplets for energy storage, be used for membranes building, protein palmitoylation or to generate second messengers (7). Therefore, FASN is involved in cell proliferation and contributes to many hallmarks of cancers (8). Often overlooked, FASN is regulated by post-translational modifications (PTM) among which O-β-N-acetyl-D-glucosaminylation or O-GlcNAcylation (9).

O-GlcNAcylation consists of the addition of a single N-acetyl-D-glucosamine (GlcNAc) onto the hydroxyl group of serine and threonine residues of thousands of proteins by O-GlcNAc transferase (OGT) (10, 11). Like phosphorylation, this modification is dynamic, as O-GlcNAcase (OGA) removes the GlcNAc group from proteins. O-GlcNAcylation is a nutrient-dependent modification since the level of the nucleotide-sugar UDP-GlcNAc, the donor of the GlcNAc group, depends on the metabolism of glucose, fatty acids, amino acids, and nucleotides (11). Like FASN, OGT is overexpressed in cancers, including HCC, and is thought to participate in many hallmarks of cancer by regulating protein expression, activity, subcellular location, and interactions (12).

Due to their respective pro-oncogenic properties, the investigation of the relationship between FASN and OGT in HCC is of particular interest. First, O-GlcNAcylation enhances lipid accumulation in HepG2 hepatic cancer cells (13). Furthermore, we demonstrated that FASN and OGT physically interact in the liver (9). Indeed, O-GlcNAcylation of FASN, in mice livers and in HepG2 cells, promotes its interaction with the ubiquitinase Ubiquitin Specific Peptidase 2a (USP2a). This results in reduced FASN ubiquitination, decreasing its proteasomal degradation and, therefore, increasing its activity. In mouse hepatocytes, O-GlcNAcylation also promotes FASN expression by modifying the carbohydrate-responsive element-binding protein (ChREBP) transcription factor (14). ChREBP O-GlcNAcylation increases its stability and its transcriptional activity towards FASN. In addition, FASN inhibition reduces OGT protein amount and conversely OGT inhibition decreases FASN content, disturbing cell cycle progression and

* For correspondence: Tony Lefebvre, tony.lefebvre@univ-lille.fr.

Present addresses for: Sadia Raab, University of Lille, Inserm, CHU Lille, Institut Pasteur de Lille, U1011 – EGID – European Genomic Institute for Diabetes, Lille, France; Emilie Cadart, University of Lille, Inserm, CHU Lille, Institut Pasteur de Lille, U1011 – EGID – European Genomic Institute for Diabetes, Lille, France



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reducing cancer cell viability (15). FASN can also inhibit OGA in U2OS cells under oxidative stress to promote cancer cell survival (16). Finally, FASN O-GlcNAcylation promotes HeLa cell survival during starvation (17). Altogether, these data highlight the complex relationship between FASN and O-GlcNAcylation in cancer cells.

By combining fatty acid analyses, mass spectrometry-based O-GlcNAc site-mapping, expression of FASN O-GlcNAcylation mutants, Western blotting, and cellular assays, this study highlights the role of O-GlcNAcylation on FASN in HCC cells. We first showed that O-GlcNAcylation regulates fatty acid synthesis in HepG2 cells. We then identified serine 595 (S595) and threonine 980 (T980) of FASN as O-GlcNAcylation sites. Site-directed mutagenesis showed that T980 is essential for FASN expression level, homodimerization, membrane residence, stability, and enzymatic activity in Hep3B cells. Furthermore, T980 sustains FASN activity, therefore promoting Hep3B cells' survival and proliferation as cell cycle progression.

Results

Fatty acid synthesis is regulated by O-GlcNAcylation levels

To expand on our previous data indicating a positive cross-regulation between FASN and OGT (9, 15), we performed fatty acid analyses on HepG2 cells (Figs. 1 and 2). First, OGT was silenced by incubating two different sets of siRNA on HepG2 cells for 96 h, resulting in a significant reduction of both OGT and O-GlcNAcylation levels (Fig. 1A). The measurement of global fatty acid methyl esters (FAMES) by GC-FID indicated that down-regulating O-GlcNAcylation significantly decreased fatty acids production (Fig. 1B), particularly palmitic acid (C16:0) - the primary and final product of FASN - and oleic acid (C18:1) (Fig. 1C). Conversely, increased O-GlcNAcylation level by inhibiting OGA with Thiamet-G (TG) (Fig. 2A) significantly increased global FAMES (Fig. 2B), and more particularly palmitic acid (C16:0), palmitoleic acid (C16:1), and oleic acid (C18:1) (Fig. 2C). Silencing of OGA by siRNA transfection also

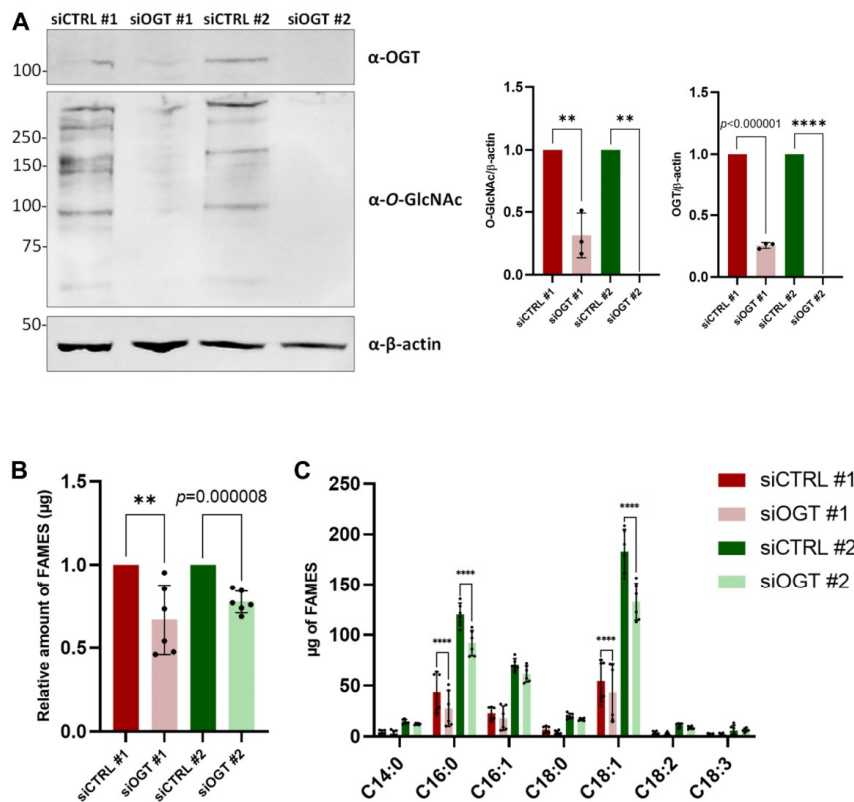


Figure 1. OGT knockdown negatively impacts fatty acids synthesis. HepG2 cells were transfected with two different sets of siRNA targeting OGT mRNA. Cell lysates were analyzed by Western blot ($n = 3$ for siOGT#1 and $n = 6$ for siOGT#2) according to their OGT and O-GlcNAc contents (A). Molecular mass markers are indicated on the left (kDa). Optical densities were measured and normalized with β -actin expression. Global (B) and individual (C) amounts of FAMES from HepG2 transfected cells were measured by GC-FID ($n = 6$). Data are presented with means $\pm$ SD. ** $p < 0.01$; **** $p < 0.0001$.

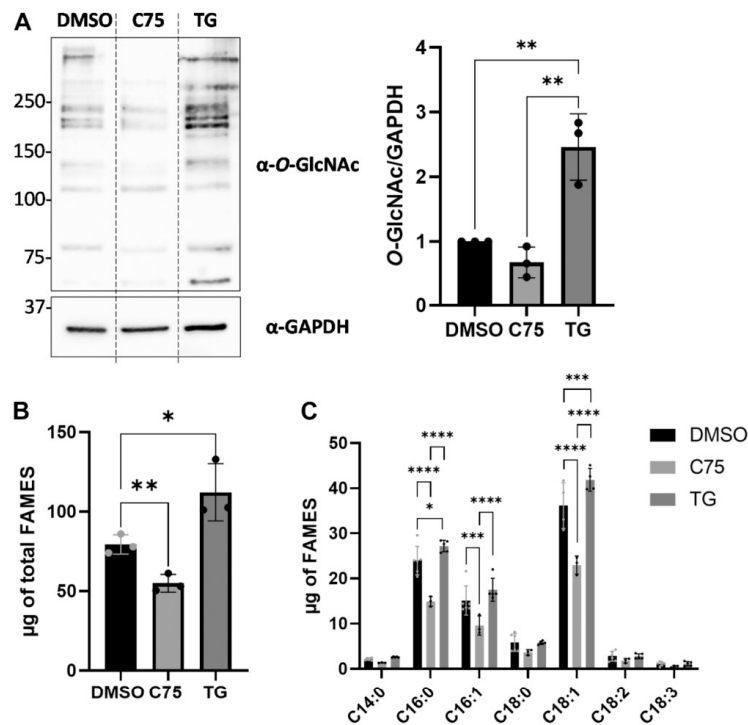


Figure 2. OGA inhibition positively impacts fatty acids synthesis. Cell lysates from HepG2 treated with the FASN inhibitor C75 or OGA inhibitor Thiamet-G (TG) were analyzed by Western blot (n = 3) according to their O-GlcNAc content (A). Optical densities were measured and normalized with GAPDH expression. Global (B) and individual (C) amounts of FAMES from HepG2-treated cells were measured by GC-FID (n = 6). Data are presented with means $\pm$ SD. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

significantly increased global FAMES (Fig. S1). In contrast, C75, an inhibitor of FASN, significantly reduced global FAMES, palmitic acid (C16:0), palmitoleic acid (C16:1), and oleic acid (C18:1). Taken together, these experiments demonstrate that fatty acid production is positively regulated by O-GlcNAcylation in hepatic cancer cells.

FASN is O-GlcNAcylated at S595 and T980, two sites conserved along evolution

To map FASN O-GlcNAcylation sites, LC-MS/MS analyses were performed on the enzyme immunopurified from HepG2 protein lysate. Mass spectrometry revealed two major sites of O-GlcNAcylation at threonine 980 (Fig. S2A) and serine 595 (Fig. S2B). Of particular interest, O-GlcNAcylation at T980 was previously reported by Woo and collaborators in 2018 (18).

Mutation of FASN at T980 O-GlcNAcylation site impairs protein level, membrane localization, dimerization, and stability of the enzyme

To investigate the impact of S595 and T980 O-GlcNAcylation sites on FASN regulation, the 3xFlag-FASN S595A and

T980A mutants were constructed and expressed in Hep3B cells in parallel to empty vector (negative control) (Fig. 3). The glycosylation status of the different forms of FASN and their ability to interact with OGT were evaluated respectively by enrichment onto WGA-coupled beads (Fig. S3A) and by co-immunoprecipitation experiments (Fig. S3B). The results seem to indicate that T980 is a major O-GlcNAcylation site but that mutation of FASN does not affect its interaction with OGT. A decrease in FASN level was observed for the T980A isoform whereas only a slight decrease was noticed for the S595A isoform compared to wild type FASN (Fig. 3A). While FASN is mainly localized in the cytosolic compartment, a fraction of the enzyme is resident of membranes (6). Therefore, the distribution of the mutants *versus* the wild-type isoforms was evaluated by cell fractionation (Fig. 3B). As a result, FASN T980A was significantly less located in the membrane fraction compared to FASN wild type and FASN S595A isoforms. The efficiency of cell fractionation was assessed by using antibodies directed against GAPDH and E-cadherin (respectively markers of cytosolic and membranes fractions). FASN is essentially active as a homodimer (4, 5, 7). Then, we performed native-PAGE to examine the impact of both mutations on the dimerization of the enzyme (Fig. 3C).

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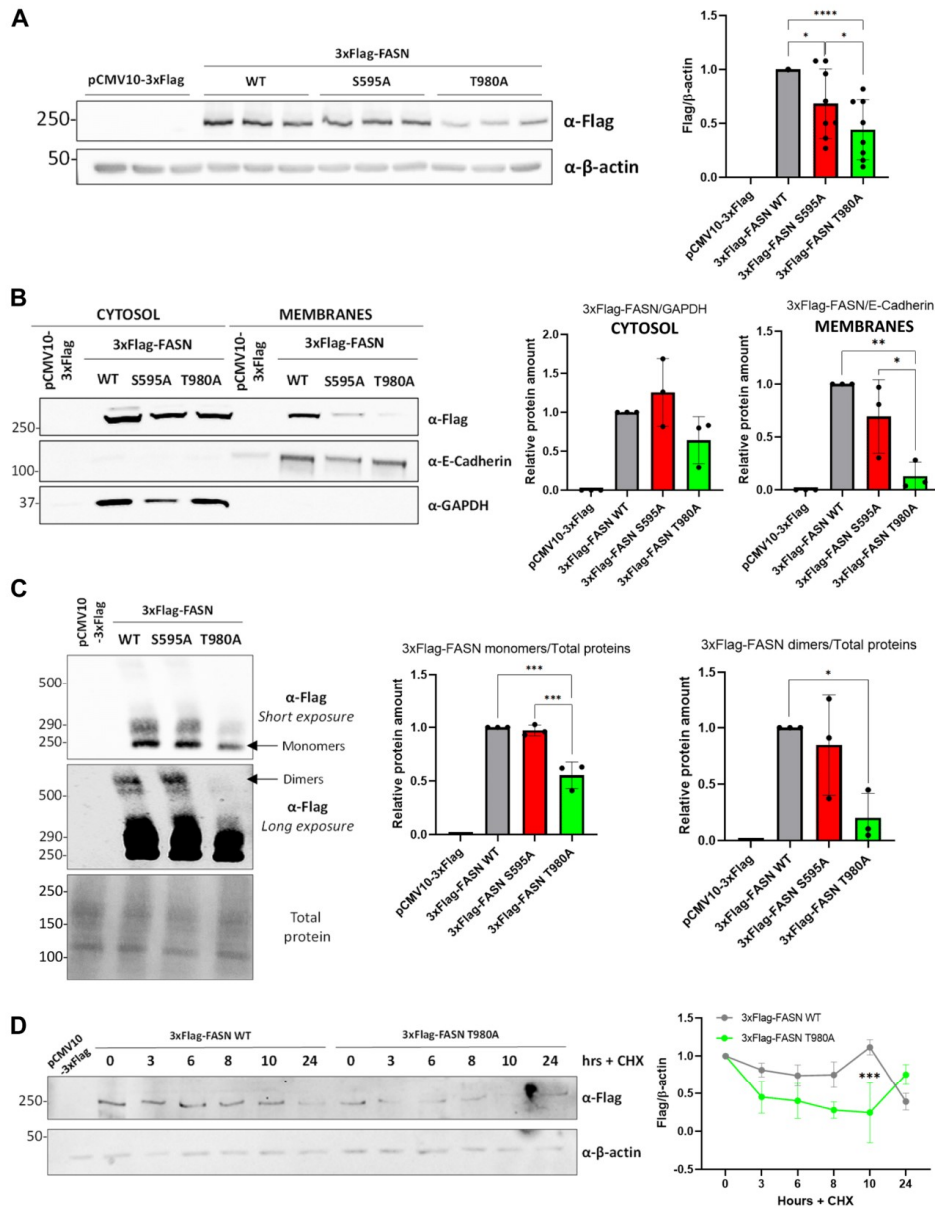


Figure 3. Mutation of the T980 O-GlcNAcylation site reduces FASN expression, membrane association, homodimerization, and stability. Hep3B cells were transfected with an empty vector or 3xFlag-FASN (wild type and mutants) expressing vectors. Cell lysates were analyzed by Western blot ($n = 8$) by using an anti-Flag antibody (A). Molecular mass markers are indicated on the left (kDa). Optical densities were measured and normalized with β -actin expression. B, cytosolic and membrane-associated 3xFlag-FASN contents were prepared by cell fractionation and analyzed by Western blot ($n = 3$). Fractionation efficiency was assessed using anti-GAPDH and anti-E-Cadherin antibodies for cytosolic and membrane fractions, respectively. Optical densities were measured and normalized with these markers. C, cell lysates were analyzed by Native-PAGE ($n = 3$) according to their 3xFlag-FASN monomers (~250 kDa) and dimers (~500 kDa) contents. Optical densities were measured and normalized with total protein. D, CHX chase assay was performed by treating cells with 40 μ g/ml CHX for the indicated time periods (0–24 h). Data are presented with means $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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Native-PAGE allowed the identification of the monomeric (~250 kDa) and dimeric (~500 kDa) forms of the enzyme (Fig. 3C). Of particular interest, a significant decrease in both forms was observed for FASN T980A but with an even more significant reduction for dimeric forms than monomers. Regarding the impact of mutation of FASN at T980, we finally wondered whether this site could be involved in protein stability. For that purpose, we used cycloheximide (CHX), an inhibitor of protein synthesis (Fig. 3D). While we observe a slight decrease in the wild-type version of FASN along the time-course experiment, the T980A mutant form decreases much more rapidly, from 3 h after the application of CHX, indicating that the T980 site plays a preponderant role in the expression of the enzyme. We have in a last time used MG132, a proteasome inhibitor, to test its capability to reduce the degradation of FASN mutants, which was partly observed (Fig. S4). Thus, the mutation of FASN at T980 suggests that O-GlcNAcylation of this residue is key for regulating the properties of the enzyme.

Mutation of FASN at T980 reduces lipid droplets number

Since T980 O-GlcNAcylation site seems essential for FASN subcellular localization and dimerization, we then investigated its enzymatic activity by labeling lipid droplets (LDs) with the green fluorescent dye BODIPY 493/503 in Hep3B cells transfected with FASN expressing vectors (red staining) (Fig. 4A). LDs were then counted (Fig. 4B). When wild type FASN was overexpressed, LDs were increased compared to control cells (transfected with empty vector). However, when

the T980A FASN-encoding vector was transfected, Hep3B cells displayed a significantly reduced number of LDs compared to wild type and FASN S595A, suggesting that O-GlcNAcylation at T980 is required for FASN activity.

The FASN T980 O-GlcNAcylation site is crucial for cell survival, proliferation, and cell cycle progression of Hep3B cells

FASN is involved in different hallmarks of cancer, which strongly emphasizes its pro-oncogenic nature (8). Thus, we next focused on the involvement of S595 and T980 O-GlcNAcylation sites on FASN's ability to promote Hep3B cells' survival, proliferation, and cell cycle progression. Using clonogenicity assays (Fig. 5A), we observed an increase in colony formation for cells overexpressing wild-type FASN compared to empty vector-transfected cells. Although no noticeable difference was observed for the S595A mutant, an apparent decrease in cell survival occurred for the T980A mutant. MTS assays were performed to validate these observations and showed significant differences for the T980A mutant compared to the wild type FASN (Fig. 5B). Then, cell counting, 24 hours after transfection, highlighted the ability for wild-type and S595A FASN isoforms to increase cell proliferation in contrast to the T980A mutant (Fig. 5C). At last, flow cytometry analysis showed a significant increase in cells in G2/M phase following overexpression of wild-type FASN (and of S595A mutant to a lesser extent), while the T980A mutant exhibited a profile similar to empty vector (Fig. 5D). Taken together, our data strongly suggest that

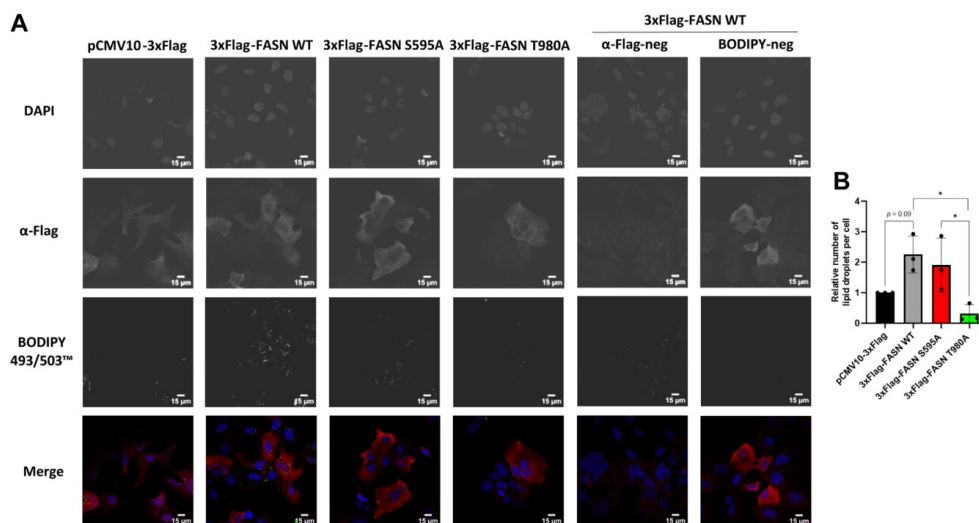


Figure 4. FASN T980 is crucial for FASN activity. Hep3B cells were transfected with an empty vector or 3xFlag-FASN (wild type and mutants) expressing vectors and incubated with the lipid droplet marker BODIPY 493/503 (n = 3) (A). Lipid droplets were counted using the ImageJ software (B). Confocal microscopy analysis indicates that FASN T980A mutation significantly impacts lipid droplets formation (bar size: 15 μm). Data are presented with means ± SD. *p < 0.05.

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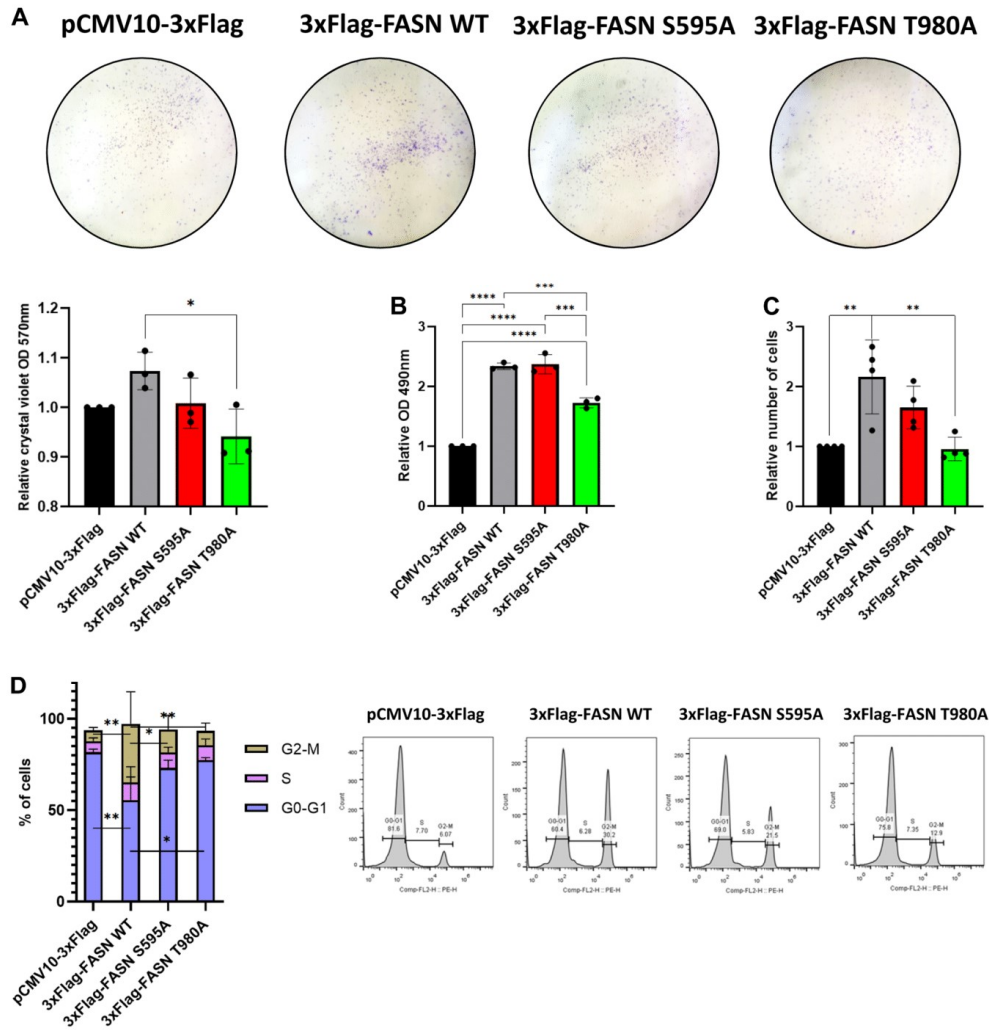


Figure 5. FASN O-GlcNAcylation at T980 is pivotal for Hep3B cells survival, proliferation, and cell cycle progression. Low-density Hep3B cells were transfected with an empty vector or 3xFlag-FASN (wild type and mutants) expressing vectors. Six days later, colonies were fixed and stained with crystal violet ($n = 3$) (A). Using MTS assay (B), cell survival was evaluated according to densitometry measured at $\lambda = 490$ nm ($n = 6$). Cell proliferation was determined by cell counting ($n = 4$) (C). Cell cycle was analyzed by flow cytometry ($n = 3$) (D). Data are presented with means $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

FASN O-GlcNAcylation at T980 is essential to sustain Hep3B cell survival, proliferation, and cell cycle progression.

Discussion

FASN is a major pro-oncogenic enzyme in the liver as its expression can play a crucial role in the initiation and progression of hepatic tumorigenesis (19). Increased *de novo* lipogenesis, promoted by the Warburg effect, is a recurrent

cancer hallmark with FASN as the pivotal enzyme involved in deregulating this metabolism (8). Thus, investigation of FASN regulation in HCC is of particular importance. Here, we show that the nutrient-dependent modification O-GlcNAcylation positively regulates fatty acids synthesis. This observation correlates with previous reports showing that O-GlcNAcylation promotes FASN expression and stability in mice livers, HepG2 cells (9, 15), and HeLa cells (17). Due to its pivotal

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position in cell homeostasis, we wondered which O-GlcNAc sites of FASN could be involved in this positive regulation.

Thus, we identified FASN S595 and T980 residues as major O-GlcNAcylation sites. T980 was already reported by Woo and collaborators (18). Protein sequences alignment indicated that S595 is well conserved among various species, with some discrepancies for T980 suggesting that this site has a major influence on lipogenic flow from one species to another (Fig. S5). Indeed, mutation of the two sites demonstrated that T980 has a key role in the behavior of FASN, notably altering the ability of the enzyme to dimerize, limiting the formation of lipid droplets (Fig. 6). However, mutation at T980 does not impact FASN folding as shown by prediction done with AlphaFold3 (Fig. S6). Since it has been reported that a pool of FASN protein is associated with lipid microdomains in prostate cancer cells (6), our observation also suggests that mutation at T980 reduces this interaction. While we did not look at the partitioning of the enzyme in either its wild-type form or mutated form, we showed that a mutation at T980 might reduce the interaction of FASN with membrane components. Further experiments are necessary to determine whether the reduction of FASN localization at the cell membrane is associated with decreased residence in lipid microdomains.

FASN activity is increased in tumor cells, and an elevation of O-GlcNAcylation is usually observed during carcinogenesis; we thus suggested that O-GlcNAcylation at T980 can be required to assist the pro-oncogenic features of FASN. We therefore evaluated the incidence of mutations at S595 and T980 on cell survival, proliferation, and cell cycle progression. As expected, mutation at T980 disturbed these processes when compared to

the wild-type enzyme, but further investigations are nevertheless necessary to check this hypothesis.

However, our observations are strengthened by a recent study by Köberlin and collaborators (20). Indeed, while the authors highlighted an anticipatory lipid checkpoint in G1-phase mediated by FASN, our flow cytometry experiments (Fig. 5D) also support the ability of FASN to promote cell cycle progression.

Owing to the role of nutrient-sensing of O-GlcNAcylation, our findings align with previous reports highlighting FASN O-GlcNAcylation as a modification that promotes cancer cell survival during starvation (17). Similar to key mutations in oncoproteins (G12 in KRAS, V600 in BRAF, or T41 in β -catenin), we investigated whether mutations at T980 occur in FASN across various cancers. To our knowledge, no hotspot has been reported for FASN in cancer. Nevertheless, due to the close relationship between O-GlcNAcylation processes and nutritional status (the donor of the GlcNAc group being UDP-GlcNAc positioned at the intersection of different metabolisms), it will be interesting to evaluate in the near future the activity of FASN in pathologies associated with nutritional disorders or for which increased OGT activity has been reported.

The FASN interactome could also be a focus of attention to providing valuable insights, advancing our understanding of the functional role of T980 site-specific function. Deng and collaborators recently identified Heat shock protein 90 α (Hsp90 α) as a crucial partner of FASN, enhancing its stability in HCC (21). While it is known that FASN O-GlcNAcylation plays a role in promoting its expression and activity, our work

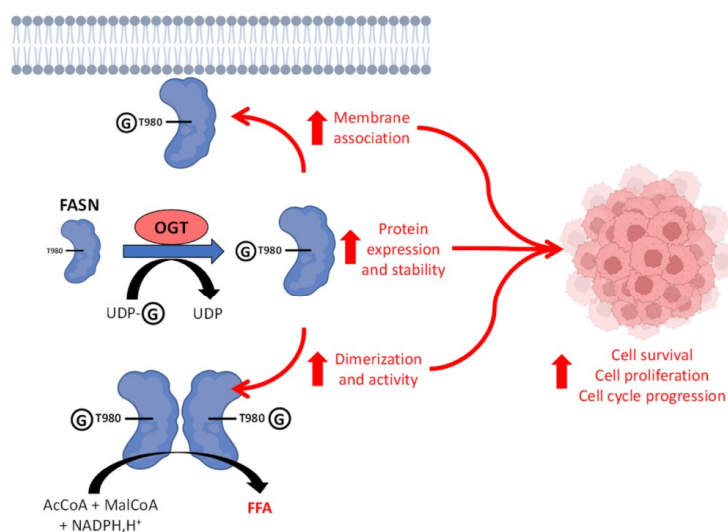


Figure 6. FASN T980 is crucial for various properties of Hep3B cells. The O-GlcNAcylation at T980 is crucial for FASN expression, stability, membrane residence, homodimerization, and activity, promoting Hep3B cells survival, proliferation, and cell cycle progression. This highlights the O-GlcNAcylation of FASN at T980 as a potential key modification that supports hepatic carcinogenesis.

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sheds additional light by identifying the T980 residue as a promising pivotal element for the etiology of HCC. It can be hypothesized that modification of FASN by OGT at T980 provides the protein with an interacting surface enhancing Hsp90 α binding to the enzyme, resulting in increased stability and, consequently, activity.

Taken together, our results pave the way for future exploration of targeting FASN at T980, offering potential therapeutic avenues for patients with hepatic cancer.

Experimental procedures

Reagents, siRNA, and antibodies

C75, MG132 (MedChemExpress), and Thiamet-G (generously provided by Pr. David Voadlo, Simon Fraser University, Burnaby, CA) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM, 50 mM and 1 mM as stock solutions, respectively. Control siRNA (siCTRL#1 - MISSION siRNA universal negative control) and siRNA targeting human OGT (siOGT#1: GGAGGCUAUUCGAAUCAGU[dT][dT] forward, ACUGAUUCGAAUAGCCUCC [dT][dT] reverse) were purchased from Sigma-Aldrich or from Dharmacon for siCTRL#2 (ON-TARGETplus Non-targeting Pool), siOGT#2 (ON-TARGETplus Human OGT (8473) siRNA), and siOGA (siGENOME Human MGEA5 (10,724) siRNA). The antibodies used are listed in Table S1.

Cell culture

The human hepatoblastoma HepG2 and hepatocarcinoma Hep3B cell lines were respectively provided by the EGID (European Genomic Institute for Diabetes) and ONCOLille institutes in Lille. HepG2 and Hep3B cell lines are ATCC certified. Both cell lines, tested and free from *mycoplasma*, were cultured in Minimal Essential Medium (MEM, Biowest) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% (v/v) fetal calf serum (FCS) (Dominique Dutscher), and penicillin/streptomycin at 1:100 dilution (Gibco). Cells were incubated at 37 °C, in a 5% (v/v) CO₂-enriched, humidified atmosphere.

Cell treatment and cell transfection

HepG2 cells were treated 24 h with C75 or Thiamet-G at a final concentration of 100 μ M and 1 μ M, respectively, in cell culture medium. DMSO was used as a negative control (vehicle). For RNA silencing, HepG2 cells were reverse-transfected with small interfering RNA (siRNA)—siCTRL#1/#2, siOGT#1/#2 or siOGA at 10 nM—using Lipofectamine RNAiMAX (Invitrogen) as described in (22). Ninety-six hours later, cells were harvested in Phosphate-Buffered Saline (PBS).

Hep3B cells were transiently transfected when 70% of confluence was reached for 24 h with empty vector (negative control) or plasmids encoding 3xFlag-FASN (wild type or mutant isoforms; 1 μ g/well in 6-well plate) using jet-OPTIMUS according to the manufacturer's instructions (Polyplus).

Cycloheximide chase assay

CHX chase assays were performed to assess FASN stability. Cells were seeded in 6-well plates and grown to ~70% confluence. 24 h after transfection, protein synthesis was inhibited by treating cells with 40 μ g/ml CHX (Sigma) for the indicated time periods.

Flow cytometry

Cell cycle progression of Hep3B transfected cells was monitored by propidium iodide (PI) staining and analysis by flow cytometry, as previously described (22).

Cell proliferation assay

Hep3B cells transfected with 3xFlag-FASN for 24 h in T25 flasks were washed with PBS and harvested by trypsinization. After trypsin neutralization by complete medium addition, cells were collected and a fraction was mixed (50:50) with 0.04% (v/v) trypan blue in PBS for cell counting using an automatic cell counter.

Cell viability assays

For MTS assays, 1000 Hep3B cells were seeded in 96-well plates and transfected with 3xFlag-FASN for 24 h. Cell viability was then measured using the MTS reagent (Promega) according to the manufacturer's instructions.

For clonogenicity assays, 8000 Hep3B cells were seeded in 6-well plates and transfected with 3xFlag-FASN for 24 h, followed by a medium change. Six days after transfection, cells were washed with ice-cold PBS before fixation with 4% (w/v) paraformaldehyde (PAF) in PBS at room temperature (RT) for 30 min. After three washes, surviving colonies were stained with 0.05% (v/v) Crystal violet in MilliQ water for 15 min with slight shaking and in the dark. Cells were finally washed with MilliQ water and colonies counted with ImageJ.

Immunofluorescence and confocal microscopy

Hep3B cells transfected with 3xFlag-FASN on coverslips were incubated for 1 h with BODIPY (493/503) (Invitrogen) diluted at 1:1000 in the cell culture medium. Then cells were washed in ice-cold PBS before fixation with 4% (w/v) PAF in PBS at RT for 30 min. After three washes, cells were permeabilized with 0.5% (v/v) Triton X-100 in PBS for 20 min. After three new washes, coverslips were incubated with blocking buffer (2% (v/v) FCS, 2% (w/v) bovine serum albumin and 0.2% (w/v) gelatin in PBS) for 1 h at RT and subsequently incubated with the primary antibodies (α -Flag M2, 1:100) diluted in the blocking buffer, overnight at 4 °C. Then coverslips were washed three times with PBS and incubated with Alexa Fluor 546-conjugated secondary antibodies (1:600) diluted in blocking buffer (Table S1) for 1 h in the dark, at RT. After three new washes, nuclei were labeled with DAPI solution at 1 μ g/ml for 10 min at RT. The coverslips were rinsed with ultrapure water and mounted on slides using Mowiol medium (Calbiochem, Merck chemicals). Negative controls were done using only the secondary antibodies. The

fluorescence was detected with an inverted Zeiss LSM780 confocal microscope with a 63 × oil immersion lens at room temperature and data were collected with the ZEN software (Zeiss, Oberkochen, Germany). Images were analyzed with ImageJ.

Cell fractionation

Transfected Hep3B cells in T25 flasks were fractionated to separate membrane and cytosolic fractions using the commercial Subcellular Proteome Extraction Kit (Merck) according to the manufacturer's instructions.

Cell lysis, SDS-PAGE, and Western blotting

Cells were first washed with ice-cold PBS and then incubated for 10 min with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% (w/v) sodium dodecyl sulfate, 1% (v/v) Triton-X100, 0.25% (w/v) sodium deoxycholate, pH 7.5) containing protease inhibitors (protease cocktail inhibitors, Sigma-Aldrich), 50 mM sodium fluoride (Sigma-Aldrich) and 1 mM sodium orthovanadate (Sigma-Aldrich). The cell lysates were then centrifuged at 20,000g for 15 min at 4 °C. The supernatants were collected and protein concentrations were measured using the micro BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Proteins were mixed with 5X Laemmli buffer (250 mM Tris-HCl, 5% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 40% (v/v) glycerol, pH 6.8) and heated at 95 °C for 7 min. Then, proteins (20 µg per lane) were resolved by 8% SDS-PAGE in electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.8) and transferred onto nitrocellulose membranes (HybondTM-C EXTRA, GE Healthcare) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, pH 8.8). Membranes were stained with a Ponceau red solution (5% (v/v) acetic acid and 0.1% (w/v) Ponceau red) to assess equal loading. Membranes were destained with TBS (Tris-Buffered Saline) - Tween20 (20 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween20 (Sigma-Aldrich), pH 7.5) (TBS-T), subsequently blocked in 5% (w/v) nonfat dry milk in TBS-T, and probed overnight at 4 °C with primary antibodies (Table S1). After three TBS-T washes, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit IgG-HRP linked, Table S1) for 1 h at RT. After three TBS-T washes, blots were revealed using enhanced chemiluminescence (West Pico Plus or Femto, ThermoScientific). The images were acquired using a CCD camera (Fusion Solo, Vilbert Lourmat). For additional probing, the membranes were stripped with the antibody stripping solution (Gene Bio-Application L.T.D.) for 15 min at RT, then washed in TBS-T and re-probed with primary antibodies. Densitometry analyses of Western blot images were done using Image J software.

Native-PAGE

Native-PAGE followed the same steps as the Western blotting section, but with the following modifications. Cells

were lysed in 10 mM Tris/HCl, 1 mM EDTA, 1 mM EGTA, 0.5% (v/v) Triton X-100 lysis buffer, protease inhibitors, pH 7.5. Proteins were mixed with loading buffer (20 mM Tris-HCl, 50% (v/v) glycerol, bromophenol blue, pH 8.8) and run on a 4-to-10% acrylamide/bisacrylamide gel gradient (for note, 25% (v/v) glycerol was added in the 10% acrylamide/bisacrylamide gel solution). Finally, the migration step was done in electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.8) at 50 V overnight at 4 °C.

Immunoprecipitation and mass spectrometry analysis

Data were generated from four individual experiments. HepG2 cells were lysed in lysis buffer as in the Western blotting section. For the pre-clearing step, 700 µg of proteins were incubated with 20 µl of agarose-beads coupled to A/G proteins (50:50) (Cytiva) for 1 h at 4 °C. After centrifugation (2 min, 200 g, 4 °C), the supernatant was incubated overnight with 5 µg of anti-FASN antibody (Abcam, rabbit polyclonal, ab99359) or 5 µg of rabbit IgG-UNLB (Southern Biotech) for negative control. Then, 40 µl and 20 µl of agarose-beads coupled to A and G proteins were respectively added to samples and incubated 1 h at 4 °C. After three washes with lysis buffer, 25 µl of Laemmli 5X buffer were added and samples were heated 7 min at 95 °C. Proteins were resolved by 8% SDS-PAGE. The gel was stained with commercial Quick Coomassie Stain (NeoBiotech) according to the manufacturer's instructions.

The protein band of interest was digested with trypsin based on the protocol described by Helle *et al.* (23). The peptides were then analyzed by LC-MS/MS using an Orbitrap Q-exactive plus. Briefly, 1 µl was injected with solvent A (5% (v/v) acetonitrile and 0.1% (v/v) formic acid) for 3 min at a flow rate of 5 µl.min⁻¹ on an Acclaim PepMap100 C18 pre-column (5 µm, 300 µm i.d. × 5 mm) from ThermoFisher Scientific. The peptides were then separated on a C18 Acclaim PepMap100 C18 reversed phase column (3 µm, 75 µm i.d. × 500 mm), using a linear gradient (1–40%) of solution B (75% (v/v) acetonitrile and 0.1% (v/v) formic acid) at a rate of 250 nL.min⁻¹ during 60 min. The column and the pre-column were placed in an oven at a temperature of 45 °C. The LC runs were acquired in positive ion mode with MS scans from *m/z* 350 to 1500 in the Orbitrap mass analyzer at 70,000 resolution at *m/z* 200. The automatic gain control was set at 1e6, and the maximum ion time was set to 90 ms. Sequentially, MS/MS scans were acquired in the high-energy collision dissociation cell for the 15 most intense ions detected in the full MS survey scan. Automatic gain control was set at 5e105, and the normalized collision energy was set to 28 eV. The resolution was set at 17,500 at *m/z* 200. The automatic gain control was set at 5e5, and the maximum ion time was set as 140 ms. The isolation windows for MSMS were set at 2 *m/z*. Dynamic exclusion was set at 90 s, and ions with 1 or more than 8 charges were excluded.

The acquired raw files were analyzed with Proteome Discoverer 2.4 (ThermoScientific) using a custom-made database including FASN sequence. The peptide mass

The pivotal status of FASN T980 O-GlcNAcylated site

tolerance was set to 10 ppm and 0.05 Da for MS/MS. Variable modifications included were as follows: oxidation of Met and Pro, deamidation of Asn and Gln, phosphorylation of Tyr, Ser, and Thr, pyro-Glu, and O-GlcNAcylation of Ser and Thr. Fixed modifications included carbamidomethylation of Cys. For high-confidence, the peptide threshold was FDR 1% and proteins were identified with two peptides (24).

Co-immunoprecipitation

Hep3B cells were lysed in co-IP buffer (50 mM Tris/HCl, 150 mM NaCl, 0.5% NP-40 (v/v), and protease inhibitors, pH 8.0). 20 µg of proteins were set aside for control (input). For the pre-clearing step, 500 µg of proteins were incubated with 50 µl of agarose-beads coupled to A/G proteins (50:50) (Cytiva) for 1 h at 4 °C. After centrifugation (3 min, 300 g, 4 °C), the supernatant was incubated overnight with 2.5 µg of DM17 antibody or 2.5 µg of rabbit IgG-UNLB (Southern Biotech) for negative control. Then, 20 µl and 10 µl of agarose-beads coupled to A and G proteins were, respectively, added to samples and incubated 1 h at 4 °C. After three washes with co-IP buffer, 20 µl of Laemmli 5X buffer were added and samples were heated 7 min at 95 °C. Proteins were resolved by 8% SDS-PAGE followed by Western blotting.

WGA-beads enrichment

Protein samples from Hep3B-transfected cells were diluted into co-IP buffer until reaching a final dilution of 500 µg of protein per mL. Then, samples were incubated with 20 µl of WGA-agarose beads (VectorLabs) with or without 0.5 M GlcNAc overnight at 4 °C to check specificity of WGA. WGA-bound proteins were collected, washed four times with washing buffer (10 mM Tris/HCl, 100 mM NaCl, 0.4% (w/v) sodium deoxycholate, 0.3% (w/v) SDS, and 0.2% (v/v) NP-40, pH 7.5), resuspended in Laemmli buffer, boiled, resolved by SDS-PAGE, and analyzed by Western blot.

Fatty acids analysis by GC-FID

After treatment with Thiamet-G or C75, or siRNA transfection, HepG2 cells were scraped in PBS and lyophilized in a Teflon-lined screw-capped glass tube. Then, 1 ml of freshly prepared 5% H₂SO₄ (v/v) in methanol was added and completed with 300 µl of toluene. The mix was vortexed vigorously for 30 s before heating for 1 h at 90 °C. Fatty acid methyl esters (FAMES) were extracted with 1.5 ml of 0.9% (w/v) NaCl and 1 ml of heptane. The tube was vortexed and then centrifuged briefly to facilitate phase separation. The heptane extracts (upper organic phase) were transferred to a new glass tube and evaporated under a stream of N₂, then dissolved in 50 µl heptane. FAME composition was determined by Gas phase Chromatography with a downstream Flame Ionization Detector (GC-FID). The GC-FID analyses were carried out on a Thermo Scientific Trace GC Ultra chromatography, and chromatographic separation was performed on a BPX70 column (30 m × 0.25 mm id, film

thickness 0.25 µm) with helium as carrier gas and according to the following program: 150 °C for 3 min, then temperature gradient from 150 °C to 240 °C at a rate of 10 °C/min, and finally 240 °C for 5 min. One µl of sample was injected in a 260 °C inlet with a 40:1 split ratio. Methyl ester derivatives were detected using a FID at 260 °C.

FASN cloning

To clone the FASN gene into the pCMV10-3xFlag expression vector, the Hind III - FASN sense (5' → 3') and FASN - Xba I antisense (3' → 5') primers (Eurogentec) (Table S2) were used to amplify the FASN sequence by PCR from the vCMVp-mCherry-FASN-V5Tag vector (eZyvec). After preparing the reaction mixture (1X GC buffer, 3% (v/v) DMSO, deoxyNucleotide TriPhosphates at 200 µM each, sense and antisense primers (0.5 µM), Phusion polymerase (0.02 U/µl; NEB) and 25 ng of vCMVp-mCherry-FASN-V5Tag plasmid), PCR was carried out under the following conditions: 2 min at 95 °C followed by 30 cycles of denaturation (30 s, 95 °C)—primer hybridization (1 min, 60 °C)—elongation (4 min, 72 °C) and 10 min at 72 °C. The amplicons and pCMV10-3xFlag vector were then incubated with Hind III and Xba I restriction enzymes (NEB) to generate cohesive ends at the insert and vector level. The addition of T4 DNA ligase enabled ligation.

Site-directed mutagenesis by PCR

Site-directed mutagenesis was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). The mix used for the PCRs was as follows: 10X buffer (5 µl), vector (20 ng), sense and antisense primers (2.5 µl; 5 µM) (Table S2), dNTPs (1 µl), Quick Solution (3 µl), Pfu turbo ultra (1 µl; 2.5 U/µl), *qs* 50 µl Milli Q water. PCR conditions were carried out as follows: 60 s at 95 °C followed by 18 cycles of denaturation (95 °C, 50 s)—hybridization (60 °C, 50 s)—elongation (68 °C, 14 min) followed by 7 min at 68 °C. After PCR, the unmutated parent vector was digested by incubating the PCR mix with the kit's Dpn I enzyme (1 µl) for 1 h at 37 °C after centrifugation for a few seconds.

Bacteria transformation

XL10 Gold or DH5α bacteria (*Escherichia coli*) were transformed with plasmids by incubating 45 µl of bacteria with 2 µl of β-mercaptoethanol and incubating for 10 min on ice. Next, 2 µl of PCR product was added before incubation for 30 min on ice followed by 30 s at 42 °C and then 2 min on ice. Bacteria were then incubated for 1 h at 37 °C in 500 µl of LB (Lysogeny Broth) medium with constant agitation before being plated on an LB agar with Carbenicillin (50 µg/ml final) Petri dish.

Plasmid extraction and amplification

Plasmids were extracted from bacteria using the Plasmid DNA purification—nucleospin Plasmid kit (Macherey Nagel).

A colony was seeded in 5 ml LB (+antibiotic) at 37 °C overnight. The following day, 450 µl of bacteria were mixed with 50 µl of DMSO for storage at –80 °C. The remaining bacteria were centrifuged at 2,400g for 15 min at room temperature and plasmids extracted according to the manufacturer's instructions. DNA amounts were assayed with NanoDrop by measuring absorbance at 260 nm, and plasmids were sequenced by Eurofins Genomics. Plasmids were amplified using the NucleoBond Xtra-Midi kit (Macherey Nagel) according to the manufacturer's instructions. DNA amounts were assayed with NanoDrop.

FASN dimer structure prediction

All FASN dimer structures were predicted with AlphaFold3 (25) with default parameters. ipTM and pTM scores are respectively 0.68 and 0.7 for the wild type structure, 0.67 and 0.69 for T980A and 0.67 and 0.69 for O-GlcNAcylated T980. The respective Predicted Aligned Error matrices are provided below each structure in Fig. S6. The rendering and Root Mean Square Deviation computations were performed with PyMol 2.3.0 (The PyMOL Molecular Graphics System, Version 2.3.0 Schrödinger, LLC.).

Statistical analysis

Data are represented with means ± SD and were compared using one-way or two-way ANOVA and Student's *t* test (two-tailed and unpaired *t* test). Statistical analyses were performed using Graph-Pad Prism 8.0.2 (GraphPad Software, Inc.) software.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Supporting information—This article contains supporting information.

Acknowledgments—The authors are grateful to the University of Lille, and the "Centre National de la Recherche Scientifique (CNRS)" for the funding of the work. DV is a recipient of a fellow from the "Ministère de l'Enseignement Supérieur et de la Recherche". We also thank Ms Cécile Mahieu for technical assistance.

Author contributions—D. V. and T. L. conceptualization; D. V., C. S., S. R., G. F. G., E. C., G. B., Q. L., and S. H. methodology; D. V., C. S., S. R., G. F. G., E. C., G. B., Q. L., S. R., S. O. V. S., F. B., S. H., D. V. and Y. R. investigation; D. V., Y. R., S. H., and T. L. formal analysis; D. V. and T. L. writing—original draft; D. V., C. S., S. R., G. F. G., E. C., G. B., Q. L., S. R., S. O. V. S., F. B., S. H., D. V., and Y. R. writing—review and editing; T. L. funding; T. L. supervision.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ChREBP, carbohydrate-responsive element-binding protein; CHX, cycloheximide; FAMES, fatty acid methyl esters; FASN, Fatty Acid Synthase; HCC,

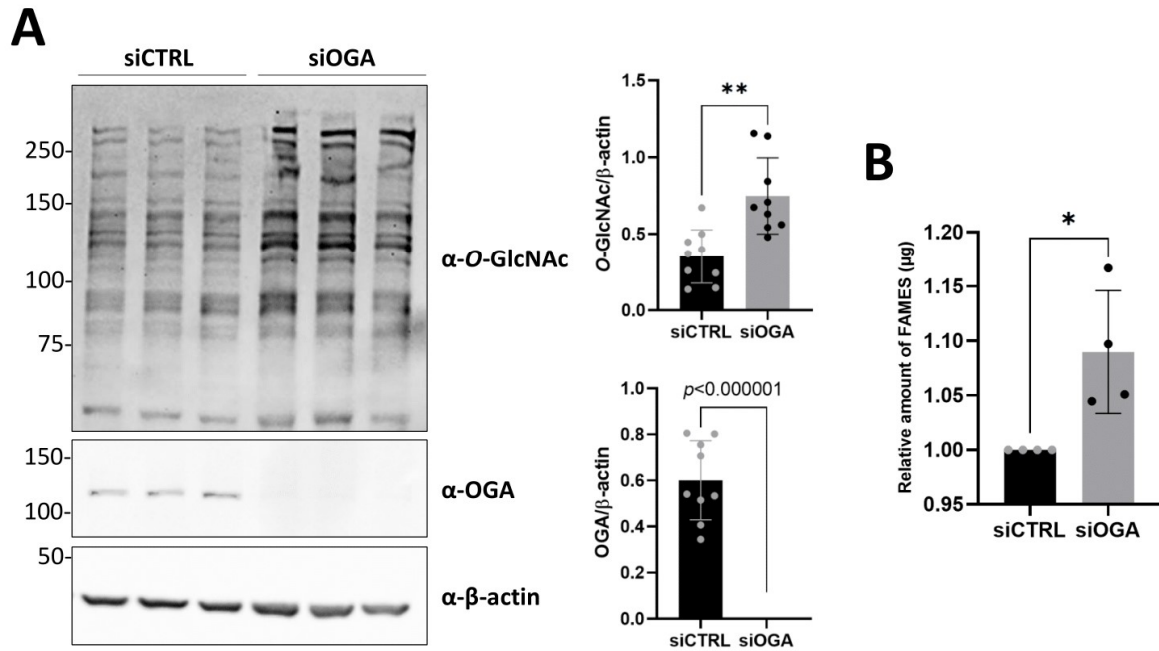
Hepatocellular carcinoma; LDs, lipid droplets; OGT, O-GlcNAc transferase; PTM, post-translational modifications.

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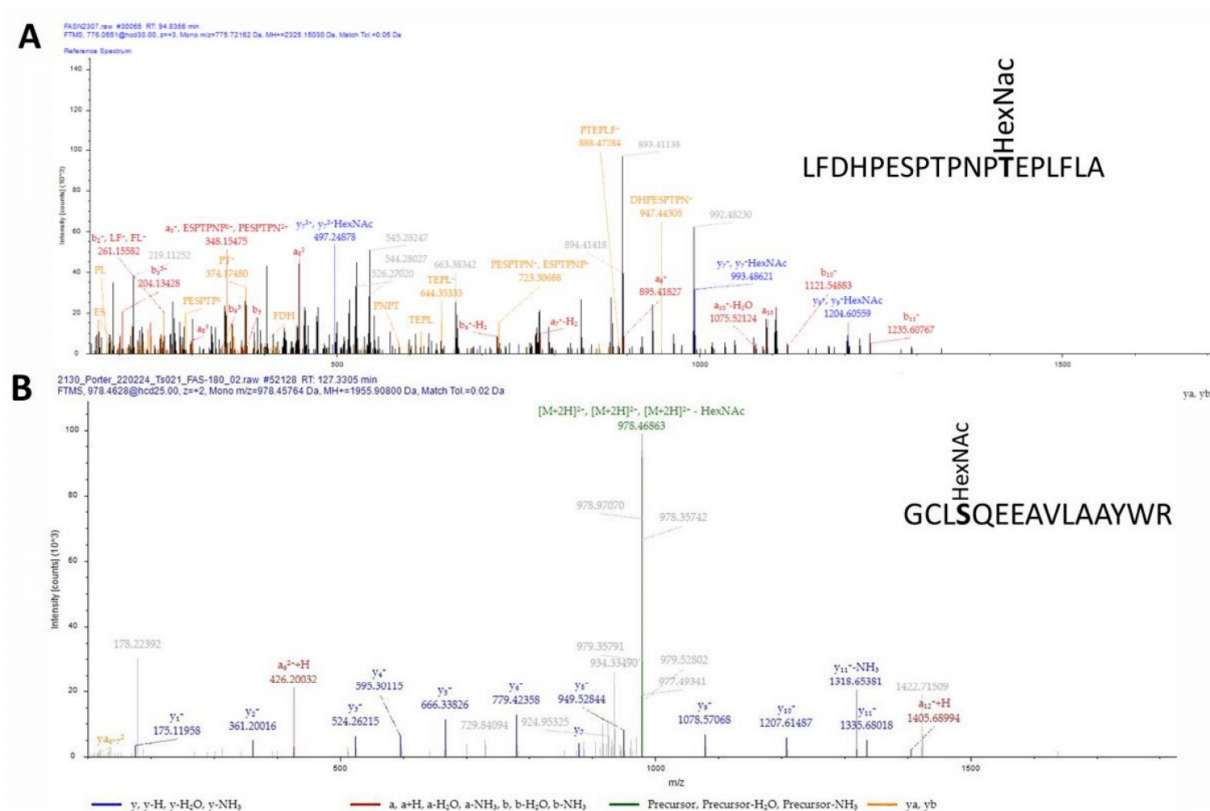
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Supplementary Figure 1: OGA knockdown positively impacts fatty acids synthesis.

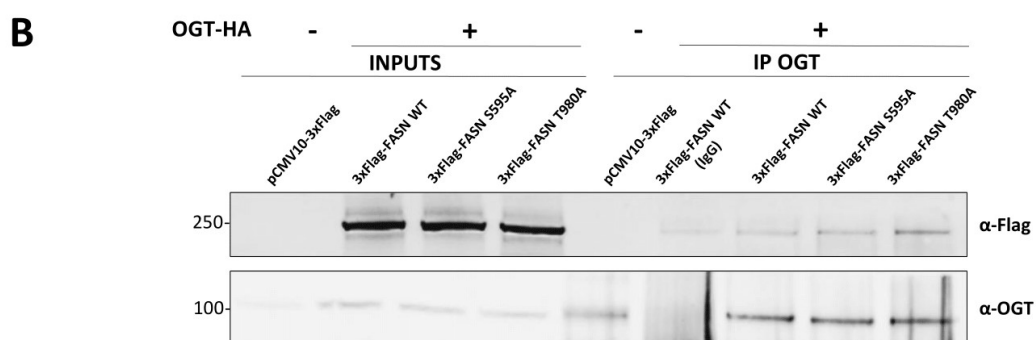
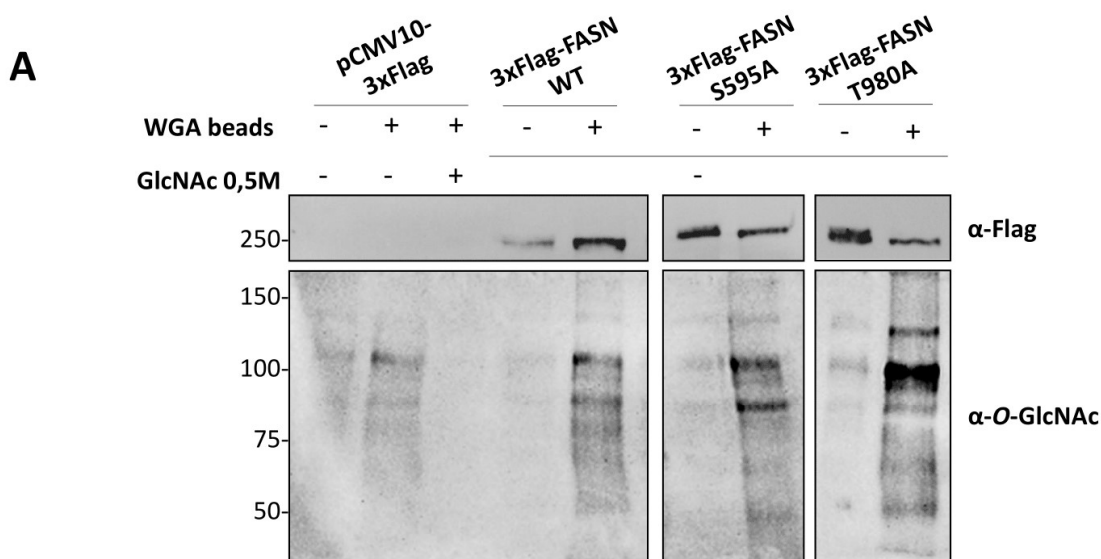
HepG2 cells were transfected with siRNA targeting OGA mRNA. Cell lysates were analyzed by Western blot ($n=9$) according to their OGA and O-GlcNAc contents (A). Molecular mass markers are indicated on the left (kDa). Optical densities were measured and normalized with β -actin expression. Global amounts of FAMES (B) from HepG2 transfected cells were measured by GC-FID ($n=4$). Data are presented with means $\pm$ SD. * $p < 0.05$; ** $p < 0.01$.



Supplementary Figure 2: FASN is O-GlcNAcylated at S595 and T980.

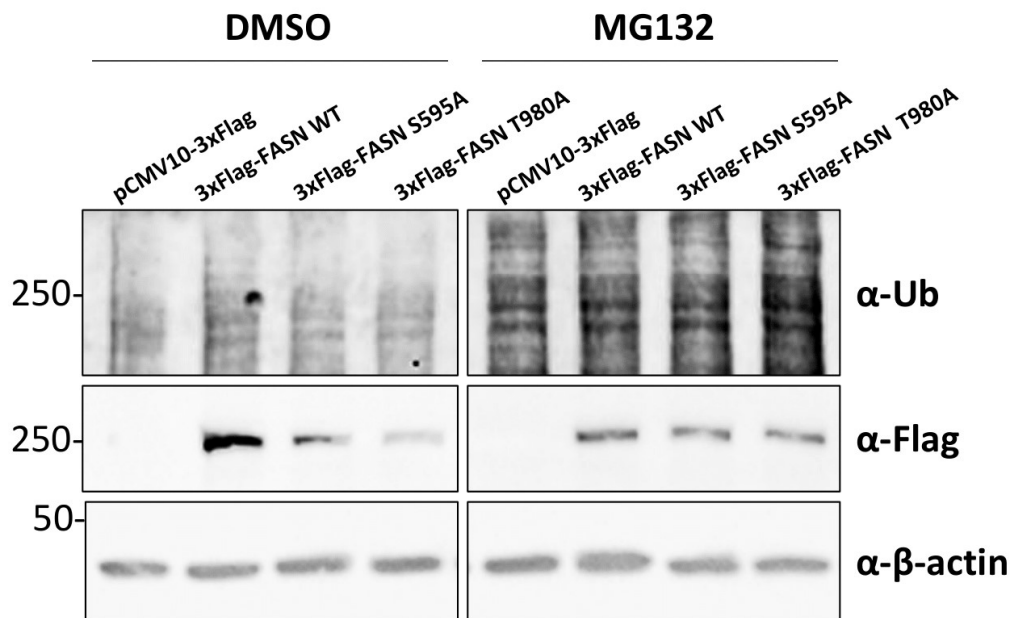
LC-MS/MS analyses of immunopurified FASN revealed threonine 980 (A) and serine 595 (B) as FASN O-GlcNAcylated sites.

Vanauberg et al. 2025 – supp fig. 2



Supplementary Figure 3: Evaluation of wild type and mutant FASN forms glycosylation status and interaction with OGT.

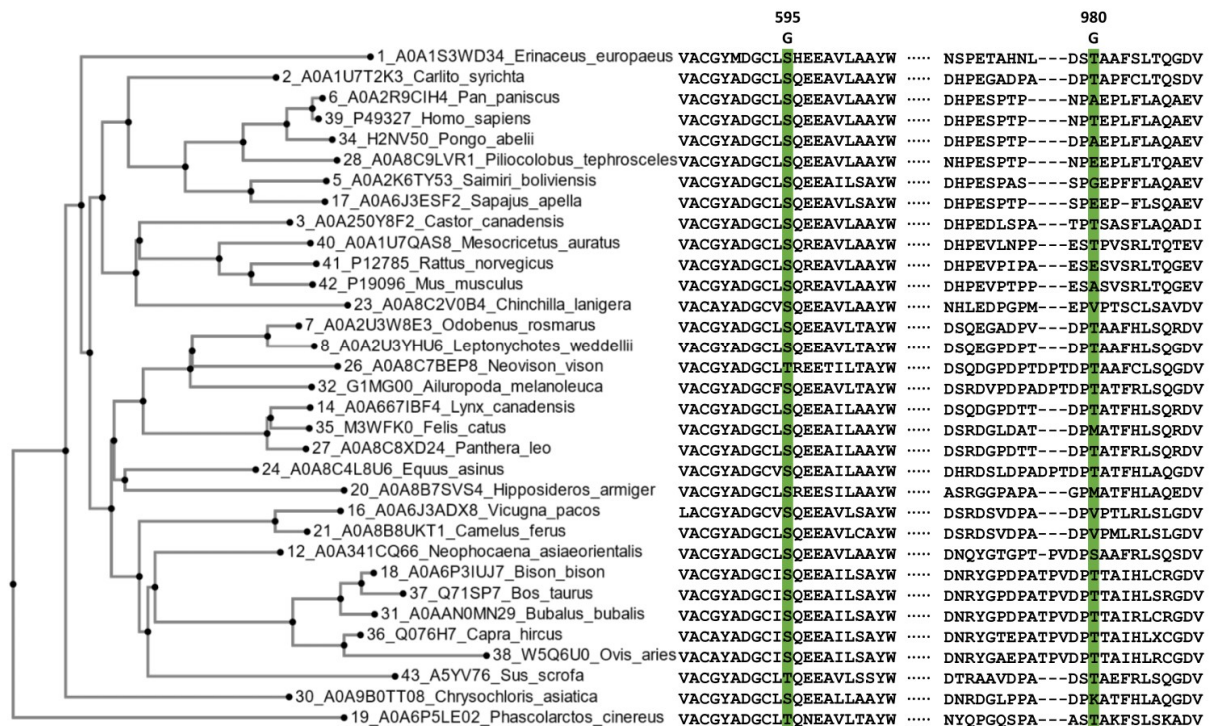
The different forms of FASN were enriched on WGA-coupled beads. Bound proteins were resolved by SDS-PAGE, electroblotted onto nitrocellulose and probed with anti-Flag or anti-O-GlcNAc antibodies (**A**). Co-immunoprecipitation of OGT-HA followed by anti-Flag (FASN) and anti-OGT immunoblotting was performed with the different forms of FASN (**B**).



Supplementary Figure 4: Inhibiting proteasome restores in part expression of mutant FASN proteins.

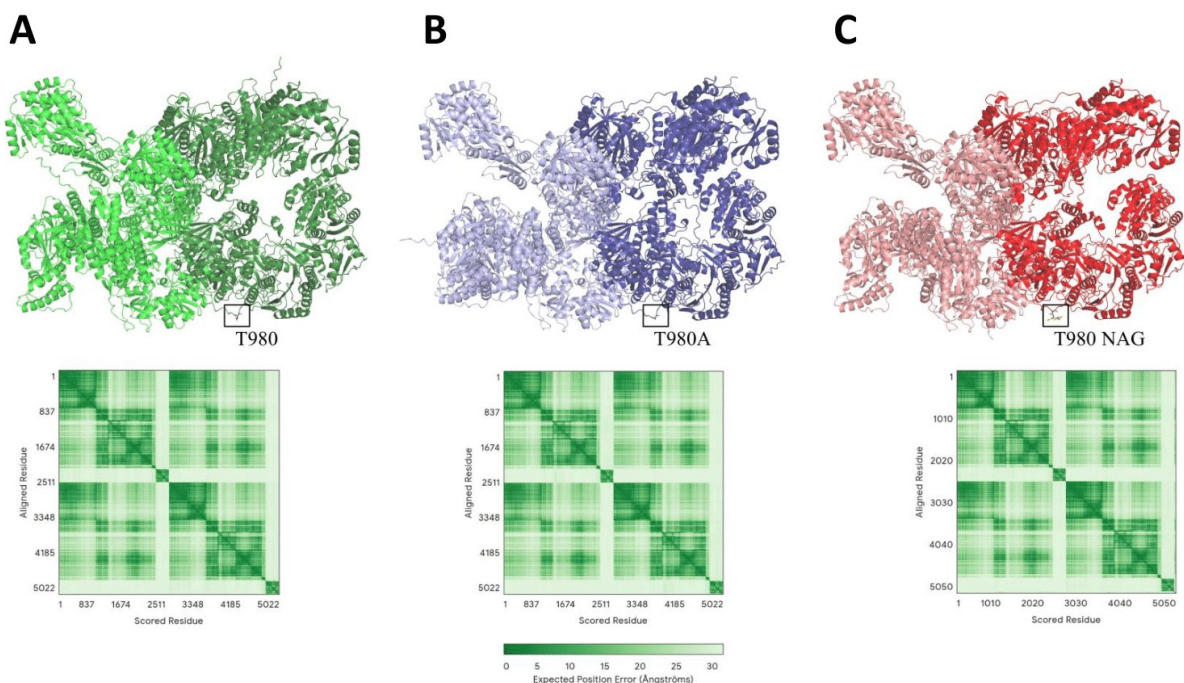
Hep3B cells were transiently transfected with the various plasmids encoding either wild type or mutant FASN, or with an empty vector. Cells were then treated with the proteasome inhibitor MG132 (4h, 5 μ M) and expression of the different forms of FASN was evaluated by Western blot. The efficiency of MG132 was verified by probing whole proteins with an anti-ubiquitin antibody.

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Supplementary Figure 5: Mammalian FASN protein sequence alignments.

Various species FASN protein sequences were retrieved from UniProt database and aligned using MAFFT V7 with default settings. Sequences with more than 97% similarity were removed for visualization purposes. S595 and T980 are conserved among mammals at 88% and 58%, respectively.



Supplementary Figure 6: FASN dimer structures.

FASN WT (**A**), T980A (**B**) and *O*-GlcNAcylated at T980 (**C**) dimer structures were predicted by the AlphaFold3 server; each dimer is coded in two shades of green (WT), blue (T980A) and red (*O*-GlcNAcylated at T980), one shade per chain, in cartoon representation; T980 and T980A are represented in grey in stick mode; the N-AcetylGlucosamine (NAG) is shown in yellow, in stick mode. Below each structure is depicted the corresponding Predicted Aligned Error matrix. The Root Mean Square Deviation (RMSD) calculated between T980A and WT is 1.911 Å; the RMSD between *O*-GlcNAcylated T980 and WT is 1.690 Å.

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Supplementary table 1: Antibodies used in this study.

Antibodies	Dilution for WB	Dilution for IF
Primary antibodies (supplier, reference)		
Mouse monoclonal anti-O-GlcNAc (Thermo Scientific, RL2)	1 : 1,000	
Rabbit polyclonal anti-OGT (Sigma-Aldrich, DM-17)	1 : 1,000	
Mouse monoclonal anti-Flag M2 (Sigma-Aldrich, F1804)	1 : 1,000	1 : 100
Mouse monoclonal anti-β-actin (Merck, A1978)	1 : 5,000	
Mouse monoclonal anti-GAPDH (Santa Cruz, 6C5)	1 : 1,000	
Mouse monoclonal anti-E-Cadherin (Santa Cruz, G-10)	1 : 1,000	
Rabbit monoclonal anti-OGA (Abcam, ab124807)	1 : 10,000	
Rabbit polyclonal anti-Ubiquitin (Enzo Life Sciences, ADI-SPA-200)	1 : 5,000	
Secondary antibodies (supplier)		
Sheep anti-mouse IgG/HRP conjugated (GE Healthcare)	1 : 10,000	
Donkey anti-rabbit IgG/HRP conjugated (GE Healthcare)	1 : 10,000	
Goat anti-mouse AlexaFluor546 (Invitrogen)		1 : 600

Supplementary table 2: Primers used in this study.

Primer type	Name	Sequence (5' → 3')
Cloning	Hind III_FASN_Fw	CCCAAGCTTATGGAGGAGGTGGTGATTGCCGGC
	FASN_Xba I_Rv	GCTCTAGATTACGTAGAATCGAGTCCGAGGAGAGGGTTAGGGATAG
Mutagenesis	FASN S595A_Fw	CGACGGCTGCCTGGCCCAGGAGGAGGC
	FASN S595A_Rv	GCCTCCTCCTGGGCCAGGCAGCCGTCC
	FASN T980A_Fw	CCCAACCCCGCGGAGCCCCTCTTCCTG
	FASN T980A_Rv	CAGGAAGAGGGGCTCCGCGGGGTTGGG
Sequencing	FASN_Fw1	CGCCACCATCCTGAACG
	FASN_Fw2	TGATGACATCGTCCATTC
	FASN_Fw3	CTTCCCCAACGGTTCAG
	FASN_Fw4	GCTGAAGATGGTGGTGC
	FASN_Fw5	CTTGGTGAAGTGTCTCC
	FASN_Fw6	GTCGCTTCCTGGAAATTG
	FASN_Fw7	GTGGAGACGATGAGCAC
	hGH_PA_Rv	CAGCTTGGTTCCCAATAG

Part 2: Fatty Acid Synthase and O-GlcNAc Transferase preferentially interact *via* their respective N-terminal region

1. Context

My team demonstrated that FASN and OGT physically interact in mice livers and in HepG2 cells (Baldini et al., 2016; Raab et al., 2021). This interaction has also been described in other models (X. Li et al., 2025; Wong et al., 2022). As OGT TPRs, constituting the N-terminal region, mainly recognize substrates, we hypothesized this region being OGT's interaction surface with FASN. On the other hand, little is known about FASN interactions with protein partners. Nevertheless, nothing is known about how both enzymes interact. Hence, we aimed to determine FASN and OGT respective interaction regions in Hep3B cells.

This work was the subject of an original article submitted to *Biochemistry and Biophysics Reports*.

Dimitri Vanauberg, Céline Schulz, Guillaume Brysbaert, Nessim Raouraoua, Peggy Mistarz-Gruau, Marc Lensink, Anne-Sophie Vercoutter-Edouart, and Tony Lefebvre. Fatty Acid Synthase and O-GlcNAc Transferase preferentially interact *via* their respective N-terminal region (2025). *Biochemistry and Biophysics Reports*. Submitted for publication.

Major results of this study are described below.

2. Results

Using a series of engineered 3xFlag-FASN deletants (**Publication II - Figure 1**) as well as supplied GST-OGT deletants, we respectively performed co-immunoprecipitations and GST pull-down assays. Thus, we determined that both enzymes preferentially interact *via* their respective N-terminal region (**Publication II - Figure 2**). This observation was strengthened by modeling FASN-OGT interaction with the AlphaFold 3 software (**Publication II - Figure 3**). In addition, by Wheat Germ Agglutinin (WGA)-beads enrichment we show that the more OGT interacts with the different FASN deletants, the more these deletants are glycosylated (**Publication II - Figure 2B**).

In this study we highlight an innovative interaction between the pro-oncogenic enzymes FASN and OGT. Our results pave the way for future exploration of targeting this interaction, offering potential therapeutic avenues for patients with hepatic cancer.

3. Publication II

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Vanauberg et al. 2025

Fatty Acid Synthase and O-GlcNAc Transferase preferentially interact *via* their respective N-terminal region

Dimitri Vanauberg¹, Céline Schulz¹, Guillaume Brysbaert¹, Nessim Raouraoua¹, Peggy Mistarz-Gruau¹, Marc Lensink¹, Anne-Sophie Vercoutter-Edouart¹, Tony Lefebvre^{1,*}

¹ University of Lille, CNRS, UMR 8576 - UGSF - Unité de Glycobiologie Structurale et Fonctionnelle, F-59000, Lille, France.

* **Corresponding authors** : Tony Lefebvre, Université de Lille, CNRS, UMR 8576 - UGSF - Unité de Glycobiologie Structurale et Fonctionnelle, Avenue Mendeleïev, bâtiment C9, F-59655, Villeneuve d'Ascq, France tony.lefebvre@univ-lille.fr; tel. + 33 (0) 20 43 47 58

Running title: The head-to-head FASN-OGT interaction

Key-words: Fatty acid synthase, O-GlcNAc transferase, O-GlcNAcylation, N-terminal region, liver cancer cells

Abstract

Fatty Acid Synthase (FASN) is part of the *de novo* lipogenesis pathway. By producing fatty acids, the lipogenic enzyme is implicated in numerous crucial cellular processes, but it is also often overexpressed in cancer. *O*-GlcNAc Transferase (OGT) governs the addition of N-acetylglucosamine residues onto cytosolic, nuclear and mitochondrial proteins. As FASN, OGT promotes carcinogenesis. We previously showed that OGT regulates FASN in different *ex vivo* and *in vivo* models. Conversely, FASN promotes OGT expression and activity. The two enzymes physically interact together and contribute to cancer cell survival. It is therefore fundamental to define the respective interaction region of each enzyme to explore new therapeutic solutions for patients with hepatic cancer. By using the hepatocarcinoma cell line Hep3B, we show thanks to two series of deletants that both enzymes preferentially interact *via* their respective *N*-terminal region. Examination of the *O*-GlcNAc status of the different FASN deletants indicates that higher is the interaction with OGT, the higher is the glycosylation, arguing that OGT catalyzes the transfer of GlcNAc without real substrate specificity.

Introduction

FASN is a large enzyme widely expressed in living organisms, active as a homodimer in animals [1,2]. Although a major fraction of the enzyme resides in the cytosol, a small portion interacts with raft lipids [3]. FASN requires no less than 7 catalytic activities to produce fatty acids, which are keto-acyl synthase (KS), malonyl/acetyl transferase (MAT), dehydrogenase (DH), enoyl-reductase (ER), keto-acyl reductase (KR) and thioesterase (TE) (**Figure 1**). The acyl carrier protein (ACP) domain facilitates substrate shuttling between the catalytic domains through its phosphopantetheine group covalently attached to S2156. After esterification, fatty acids constitute a major energy reserve in the form of triglycerides, but are also major components of biological membranes, are the basis of protein acylation, or even allow the synthesis of second messengers [4]. This is why FASN is highly active in cell proliferation and plays a leading role in the processes of carcinogenesis [5]. FASN expression level is increased by *O*- β -N-acetyl-D-glucosaminylation (*O*-GlcNAcylation) [6], a versatile post-translational modification (PTM) whose dynamics is regulated by a pair of enzymes, *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) [7]. From the nucleotide-sugar UDP-GlcNAc, OGT adds N-acetyl-D-glucosamine (GlcNAc) groups onto serine and threonine moieties of a myriad of cytosolic, nuclear and mitochondrial proteins while OGA hydrolyses the residues off the proteins [8,9]. Structurally, at the *N*-terminus part, human OGT possesses 13.5 tetratricopeptide repeats (TPRs) [10]. TPRs are degenerated consensus sequences of 34 amino acids found in a large myriad of proteins that serve as protein-to-protein interaction. At the *C*-terminal part, OGT displays two distinct catalytic domains (CD), CDI and CDII, in addition to a nuclear localization sequence (NLS) [11]. As with FASN, OGT is highly expressed in a large variety of cancers where it participates to the processes of carcinogenesis [12].

Because of their pro-oncogenic activities, defining the respective domain of FASN and OGT that drive their interaction is of special interest so as to propose novel therapeutic avenues to treat cancer, especially hepatocarcinomas. In previous studies, we showed that FASN and OGT physically interact [6]. *In vivo* and *ex vivo*, *O*-GlcNAcylation of FASN leads to a decrease in its ubiquitination by promoting its interaction with the deubiquitinase Ubiquitin Specific Peptidase 2a (USP2a) which reduces its sensitivity to the proteasome. Moreover, inhibiting FASN diminishes OGT content and, in turn, inhibition of OGT reduces level of FASN, which as the consequence to deregulate cell cycle and to decrease cell viability [13]. To promote cancer cell survival, it has been shown that FASN inhibits OGA when oxidative stress was induced [14], whereas *O*-GlcNAcylation increase FASN activity of cancer cells during starvation [15]. Lastly, Li and collaborators [16] recently highlighted OGT interaction at S1483 of FASN in mouse hepatocytes (AML-12 cells). The authors argue that *O*-

GlcNAcylation at S1483 reduces FASN K48-ubiquitination and degradation, increasing its activity and subsequent hepatic lipid accumulation. Altogether, these data shed light to the complex relationship between FASN and OGT.

By combining co-immunoprecipitations, GST pull-down, and bioinformatics, we revealed an *N*-terminal-to-*N*-terminal interaction between FASN and OGT. These findings may support the identification of FASN-OGT interaction inhibitors as potential anti-cancer therapeutics.

Materials and methods

Cell culture and transfection

The hepatocarcinoma Hep3B human cell line came from the ONCOLille Institute (Lille) and is certified by the ATCC. Cells were cultured in Minimal Essential Medium (MEM, Biowest) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% (v/v) fetal calf serum and penicillin/streptomycin at 1:100 dilution (Gibco) and placed at 37°C, in a 5% (v/v) CO₂-enriched humidified atmosphere. Cells were transiently transfected for 24 hours with 10 µg of plasmids in 150 cm² flasks using jetOPTIMUS (Polyplus).

Cell lysis, SDS-PAGE and Western blotting

After washing in ice-cold phosphate-buffered saline (PBS), Hep3B cells were lysed in lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 0.5% NP-40 (v/v), 50 mM sodium fluoride, 1 mM sodium orthovanadate, protease inhibitors, pH 8.0) for 10 min. The supernatants were collected by centrifugation at 20,000×g for 15 min at 4°C. Protein contents were evaluated by using the micro-BCA protein assay kit (Thermo Fisher Scientific). Samples were mixed with Laemmli buffer (250 mM Tris-HCl, 5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 40% (v/v) glycerol, pH 6.8), and heated to 95°C for 7 min. Proteins were separated by 8% SDS-PAGE in electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.8), and electro-transferred onto nitrocellulose (HybondTM-C EXTRA, GE Healthcare) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, pH 8.8). After staining with Ponceau red (5% (v/v) acetic acid and 0.1% (w/v) Ponceau red) to check efficiency of the transfer and equal loading, membranes were destained in TBS (Tris-Buffered Saline)-Tween20 (20 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween20 (Sigma-Aldrich), pH 7.5) (TBS-T), blocked in 5% (w/v) nonfat dry milk in TBS-T, and incubated overnight at 4°C with primary antibodies (**Supp. Table 1**). Membranes were washed in TBS-T three times and then incubated with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (**Supp. Table 1**) for 1 h at room temperature (RT). Membranes were washed again in TBS-T three times and signal was detected using enhanced

chemiluminescence (West Pico Plus or Femto, ThermoScientific). Images were captured using a CCD camera (Fusion Solo, Vilbert Lourmat) and analyzed using Image J software to measure optical densities.

Co-immunoprecipitation

Hep3B cells were incubated in lysis buffer as described above. Lysates containing 1 mg of proteins were first pre-cleared by incubation with 100 μ L of a fifty-fifty mix of A/G protein-coupled agarose beads (Cytiva) for 1 hour at 4°C. The supernatants were collected following gentle centrifugation of the beads, and incubated with 2.5 μ g of an anti-Flag antibody (Merck, mouse monoclonal anti-Flag M2, F1804) or the same amount of mouse IgG-UNLB (Southern Biotech) as a control. Thirteen and 40 μ L agarose-beads coupled to A or G proteins respectively were incubated with the protein samples for 1 hour at 4°C. Beads were washed three times with lysis buffer and heated to 95°C in Laemmli buffer for 7 min.

WGA-beads enrichment

Hep3B cell lysates were diluted into lysis buffer to obtain a final dilution of 1 mg/mL of proteins and incubated with 20 μ L of WGA-labeled beads (Vectolab) overnight at 4°C (specificity of interaction was checked by adding 0.5 M of free GlcNAc). WGA-bound proteins were collected by spin-centrifugation, and beads were washed in the following buffer: 10 mM Tris/HCl, 100 mM NaCl, 0.4% (w/v) sodium deoxycholate, 0.3% (w/v) SDS, and 0.2% (v/v) NP-40, pH 7.5, four times, and heated to 95°C for 7 min in Laemmli buffer

GST pull-down assay

The bacterial expression vectors pGEX-2T for GST and GST-OGT fusion proteins were generously provided by Pr. Xiaoyong Yang from the Yale University School of Medicine, CT. To produce the GST-recombinant proteins, the *E. coli* BL21 DE-3 strain was transformed with the vectors and cultured in a LB medium supplemented with ampicillin at 50 μ g/mL. When exponential growth phase was attained, protein production was induced by addition of 0.1 mM IPTG for 4 h at RT. Bacteria were harvested by centrifugation and resuspended in PBS supplemented with protease inhibitors (Sigma-Aldrich). Bacteria were lysed by high-pressure homogenization (Emulsiflex-C3, Avestin, Mannheim, Germany). A centrifugation was performed at 2,400 $\times$ *g* for 10 min, and GST-fused proteins were bound onto Glutathione Sepharose 4B beads (GE Healthcare) for 2 h at 4°C. Beads were washed for 5 min in a buffer containing 20 mM Tris at pH 7.5 first, then two times with 20 mM Tris pH 7.5 added to 0.1% (v/v) Triton X-100 and 100 mM NaCl, twice, and finally with 50 mM Tris pH 8. Then, 700 μ g of proteins from Hep3B cells overexpressing 3xFlag-FASN were added with the beads, and gentle incubation was done for 2 h at 4°C. Bound proteins were washed first in

PBS, then three times in PBS added to 0.1% (v/v) Triton X-100, in PBS added to 0.1% (v/v) Triton X-100 and 150 mM NaCl, and finally in 50 mM Tris at pH 8. The bound proteins were eluted by incubating beads with 50 μ L of elution buffer (50 mM Tris (pH 8) with 50 mM reduced glutathione (Sigma-Aldrich) for 10 min by gentle vortex.

FASN cloning and generation of the deletion mutants

FASN gene was cloned into the pCMV10-3xFlag expression vector thanks to the Hind III - FASN sense primer and FASN - Xba I antisense primer (Eurogentec) (**Supp. Table 2**) The construction of the various FASN deletion mutants was allowed by introducing STOP codons at the desired position to abort protein synthesis (QuikChange II XL Site-Directed Mutagenesis Kit, Agilent). The primers used are listed in **Supp. Table 2**, and the chemicals were 1 μ L dNTPs, 3 μ L Quick Solution, 2.5 Unit Pfu turbo ultra, completed to 50 μ L distilled water. PCR conditions were the following: 60 s at 95°C, 18 denaturation cycles at 95°C for 50 s, hybridization step at 60°C for 50 s, elongation step at 68°C for 14 min, and 7 min at 68°C. At the end of the PCR procedure, the non mutated parent vector was digested by incubating the PCR mix with Dpn I enzyme for 1 h at 37°C.

Bacteria transformation

Transformation of *E. coli* (XL10 Gold or DH5 α strains) was carried out by incubating plasmids with bacteria (45 μ L) in 2 μ L of 2-mercaptoethanol for 10 min directly on ice. Two μ L of the PCR product were added prior 30 min incubation at 0°C, then 30 s at 42°C, and finally 2 min at 0°C. *E. coli* were placed in 500 μ L of LB medium for 1 h at 37°C under agitation before plating in Petri dishes containing LB agar supplemented with carbenicillin at a final concentration of 50 μ g/mL.

Plasmids extraction and amplification

Extraction of plasmids was performed with the Plasmid DNA purification-nucleospin Plasmid kit from Macherey Nagel. Colonies were seeded in 5 mL of LB medium containing carbenicillin and maintained overnight at 37°C. Bacteria were then centrifuged at 2,400xg. Plasmids were extracted following the recommendations of the manufacturer, and assay of DNA was done by measurement of optical density at 260 nm with a NanoDrop apparatus. After careful verification of the sequences (Eurofins Genomics), plasmids were amplified (NucleoBond Xtra-Midi kit, Macherey Nagel). At last, amounts of DNA were evaluated by NanoDrop assay.

FASN dimer and OGT structure prediction

The FASN structure (Uniprot: P49327) was predicted by using AlphaFold3 [17] with default parameters. ipTM and pTM scores are respectively 0.68 and 0.7 for the top ranked prediction. The OGT monomer (Uniprot: O15294) was modeled before with AlphaFold2 with a mean pLDDT=92.11. A complex made of a single FASN trimmed to 1-972 and a single OGT trimmed to 1-286 was modeled with AlphaFold3 to predict the interface between both. ipTM and pTM scores are respectively 0.48 and 0.76 for the top ranked prediction. This complex was then used to superimpose the full OGT monomers and full FASN dimer structures. The rendering and superimpositions were done with PyMol 2.3.0 (Ref: The PyMOL Molecular Graphics System, Version 2.3.0 Schrödinger, LLC.).

Statistical analysis

Data (means $\pm$ SEM) were analyzed by one-way ANOVA or Student's *t*-test (two-tailed and unpaired) using Graph-Pad Prism 8.0.2 (GraphPad Software, Inc.).

Results

FASN preferentially interacts with OGT via its N-terminal region

In the attempt to determine which respective domain of FASN and OGT interact, we first engineered a series of 3xFlag-FASN deletants (**Figure 1**, constructions B to G, A being the empty vector only expressing the FLAG peptide). The constructions were transfected into Hep3B cells as described in the materials and methods section. Co-immunoprecipitations were performed with the anti-Flag antibody, and the interaction with OGT was evaluated by Western blot (**Figure 2A**). Although OGT interacts with full-length FASN (construction G), the stronger complexes were formed by the association of OGT with constructions B and C. In parallel of the co-IP experiments, we evaluated the ability of the various FASN deletants to be O-GlcNAcylated. The glycosylation level of the different constructions was determined by Wheat Germ Agglutinin (WGA)-beads enrichment (**Figure 2B**). Interestingly, the most pull-down constructions (B and C) correspond to FASN N-terminal region comprising the KS, MAT and DH domains. These observations prove that more OGT interact with a FASN deletant form, more the glycosylation occurs.

OGT preferentially interacts with FASN via its N-terminal region

Most of OGT substrates interact with its TPRs, corresponding to OGT N-terminal domain [10]. Thus, we conversely used GST-OGT deletants [18] to determine OGT interaction region with FASN. After production in *E. coli* each construction (1 to 4) (**Figure 2C**) was incubated with lysates prepared from Hep3B cells overexpressing 3xFlag-FASN, and then GST pull-

down assays were performed (**Figure 2C**). We detected a strong interaction between FASN and full-length OGT (lane 1) as previously reported by us and others [6, 13, 15, 16]. Interestingly, OGT lacking only the sixth first TPRs (construction 2) no more interact with FASN (lane 2). Of particular interest, the use of only the sixth first TPRs (construction 3) showed an interaction with FASN (lane 3). Although the latter construction less interacts with FASN than full-length OGT, these data highlight the requirement of OGT TPRs 1-6 for interaction with FASN.

FASN and OGT N-terminal-to-N-terminal interaction modeling

Finally, we aimed to model the *N*-terminal-to-*N*-terminal interaction between FASN and OGT. Using the AlphaFold 3 software (**Figure 3**), we were able to visualize the FASN homodimer with each monomer interacting with one monomer of OGT. A modeling of FASN 1-972 and OGT 1-286 interaction was also predicted. These predictions are consistent with our at-the-bench observation, strengthening the head-to-head interaction of the two enzymes in Hep3B cells. The list of the interface residues of each partner is given in **Supp. Table 3**.

Discussion

The lipogenic enzyme FASN is considered a master hepatic pro-oncogenic enzyme since its expression level can be decisive in the initiating tumorigenesis [18]. *O*-GlcNAcylation, and the enzyme responsible for this modification, OGT, are both usually increased in tumors and promote many hallmarks of cancer including hepatocellular carcinoma (HCC) [19-21]. Therefore, investigating FASN and OGT interaction in HCC is of special interest. In the present report, we show that the *N*-terminal part of FASN strongly interacts with the *N*-terminal part of OGT which contains TPR domains pivotal in the recruitment of OGT's substrates [10]. Although little is known about FASN interactions with protein partners, our data regarding OGT is consistent with previous reports [22-25]. To go further, the mutation of specific amino acids at the FASN-OGT interface could help in identifying those holding a key-position for the interaction. Furthermore, the observation that the more OGT interacts with the different FASN deletants, the more these deletants are glycosylated (**Figure 2B**), is an argument for the lack of specificity of the glycosyltransferase, whose substrate discrimination mechanism remains very poorly understood. Most likely, a third partner playing a role as a regulatory subunit of OGT is necessary, each client protein potentially requiring its own regulatory subunit.

To date, no FASN inhibitor has been approved for cancer treatment. Toxicity and metabolic side effects remain potential concerns. However, inhibitors of Protein-Protein Interactions

(PPIs) have gained insights in oncology. For instance, the approval of venetoclax, an inhibitor of BCL2-BAX interaction, to treat leukemia [26], provides tangible evidence of the therapeutic potential of targeting PPIs. Hence, targeting OGT-FASN interaction using this kind of therapeutic approach is of particular interest. Several studies report that *O*-GlcNAcylation of FASN increases its stability and activity [6, 13, 15, 16]. Thus, disrupting the interaction between the two pro-oncogenic enzymes FASN and OGT should reduce FASN *O*-GlcNAcylation and, consequently, decrease its subsequent activity and pro-tumoral effects. This hypothesis requires intensive further investigation.

At last, due to the tight relationship between *O*-GlcNAcylation and *de novo* lipogenesis, investigating OGT-FASN interaction in pathologies associated with nutritional disorders would be of great interest. As a whole, these results could open exploration of targeting OGT-FASN interaction, and may offer potential novel therapeutic solutions for patients with liver cancer.

Acknowledgements

The authors thank the University of Lille, and the “Centre National de la Recherche Scientifique (CNRS)” for funding this study. DV is a recipient of a fellow from the “Ministère de l’Enseignement Supérieur et de la Recherche”.

Authors’ contributions

conceptualization, DV and TL; methodology and investigation, DV, CS, GB, NR, PGM, ML and ASVE; data curation, DV and TL; draft writing, DV and TL; editing, all authors; funding acquisition, TL; supervision, TL

Data statement

All data generated or analyzed during this study are included in this published article. Any additional information is available upon reasonable request to the corresponding author.

Declaration of competing interests

The authors declare that they have no competing interest.

Figure captions

Figure 1: FASN constructions used in this study.

The delimitation of each domain is indicated by numbering the amino-acids. Each construction encodes a Flag-peptide located at the *N*-terminus.

ACP, Acyl Carrier Protein; DH, DeHydrogenase; ER, Enoyl-Reductase; KR, Keto-acyl Reductase; KS, Keto-acyl Synthase; MAT, Malonyl/Acetyl Transferase; TE, ThioEsterase.

Figure 2: FASN and OGT interact *via* their respective *N*-terminal part.

A and B. Hep3B cells were transfected with an empty vector (A) or with the different 3xFlag-FASN deletant constructs (B to G) in combination with OGT-HA overexpression. Protein samples were incubated with an anti-Flag antibody for co-immunoprecipitation with OGT

("IP" lanes for "Immuno-Precipitated") (**panel A**) or with WGA-agarose beads ("P" lanes for "Purified") (**panel B**).

A. Co-immunoprecipitation of the deletants with OGT analyzed by Western blotting. Optical densities of co-IP OGT were measured and normalized with the corresponding IP deletant (red arrows; n=3).

B. *O*-GlcNAcylation levels of the deletants analyzed by Western blotting. Specificity of WGA was checked by adding 0.5 M GlcNAc ("P+Gn" lane). Optical densities of the "purified" *O*-GlcNAcylated constructs were measured and normalized with inputs (red arrows; n=3). * $p < 0.05$; ** $p < 0.01$.

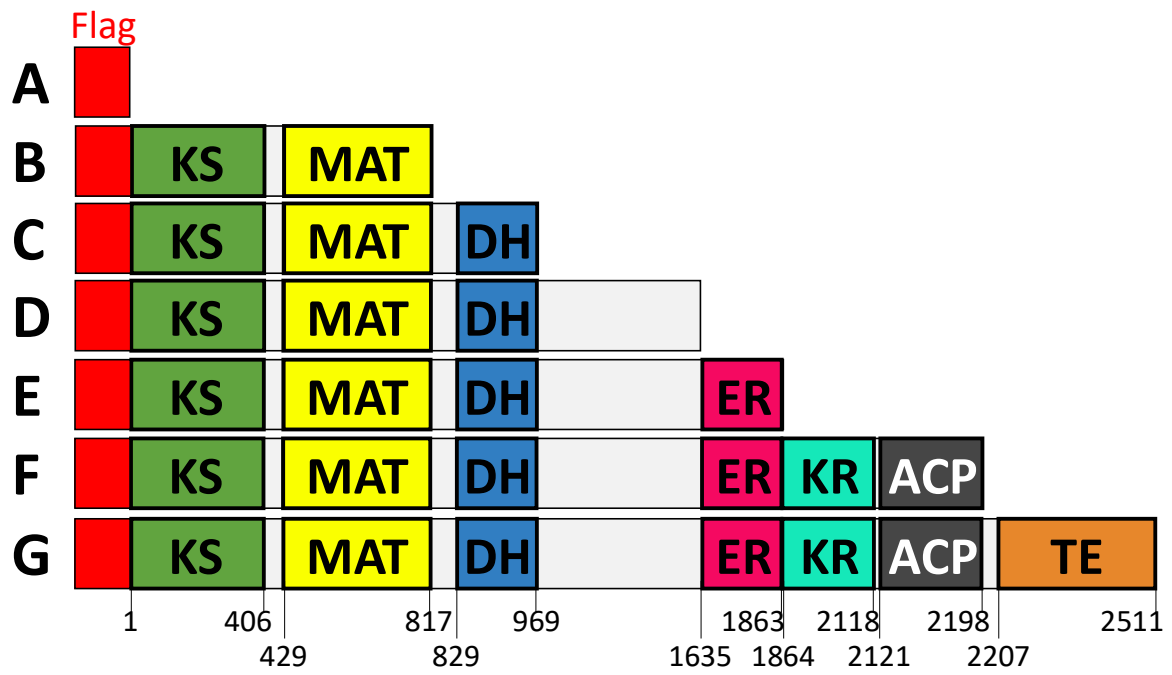
C. Hep3B cells were transfected with 3xFlag-FASN. Protein samples were incubated with a GST-OGT construct (1 to 3) or GST only (4). After GST pull-down, interaction of the GST-OGT constructs (red arrows) with 3xFlag-FASN was analyzed by Western blotting. Optical densities of pull-down 3xFlag-FASN were measured and normalized with the corresponding construct. Interaction with GST only was subtracted from the other bands (n=4). CD, catalytic Domain; GST, Glutathione-S Transferase; NLS, Nuclear Localization Signal; TPR, Tetratricopeptide Repeats.

Molecular mass markers are indicated on the left (kDa).

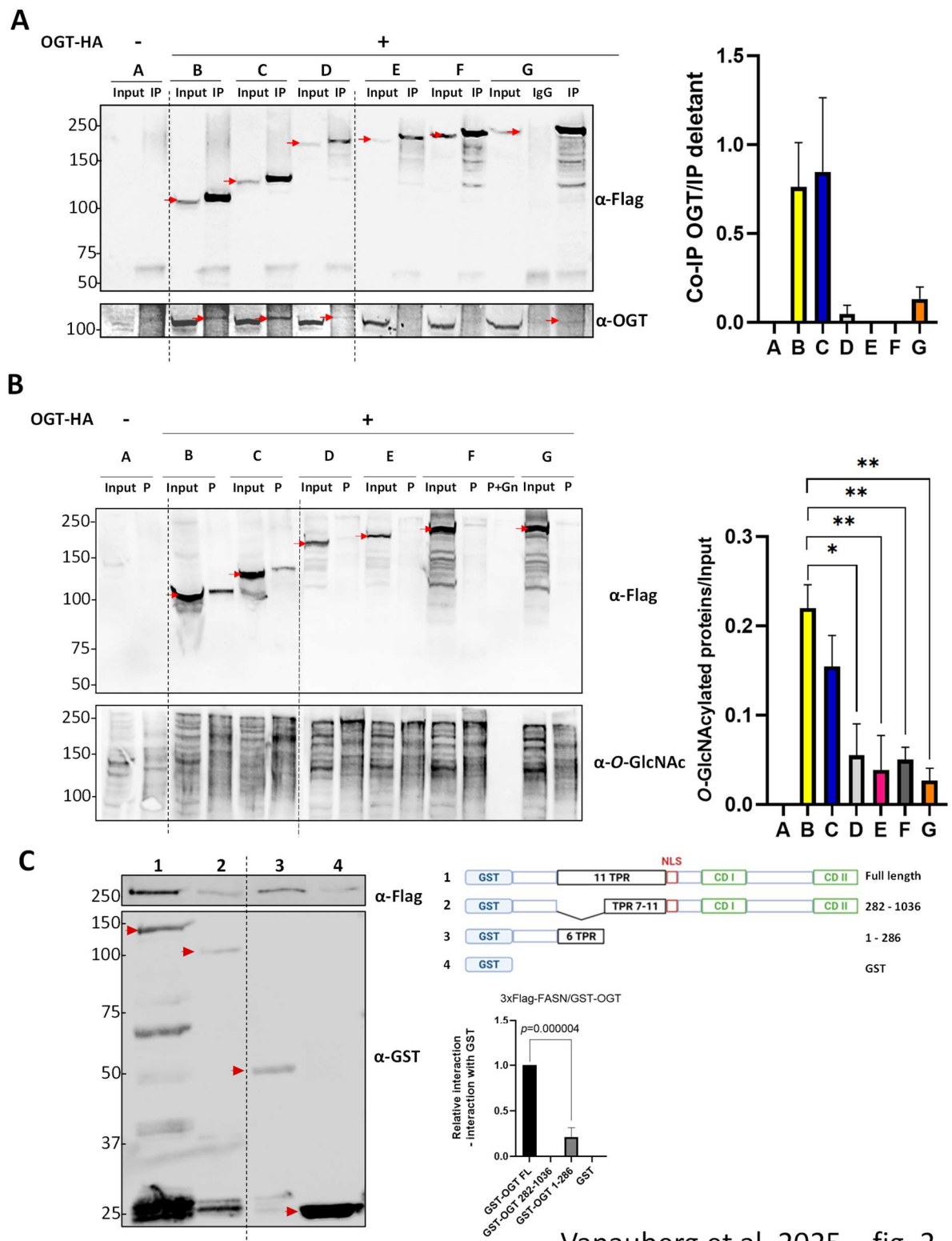
Figure 3: FASN and OGT interaction modeling.

A. Modeling of FASN dimer and two OGT monomers; both full sequences were modeled with AlphaFold (see Materials and methods) and rendered in cartoon representation, FASN dimer in cyan and magenta (2511 residues each) and OGT monomers in green and yellow (1046 residues each) respectively.

B. Modeling of FASN *N*-ter (cyan) and OGT *N*-ter (green); the FASN sequence was trimmed to 1-972 and the OGT sequence was trimmed to 1-286; the structure was predicted with AlphaFold3 with default parameters and rendered in cartoon representation.

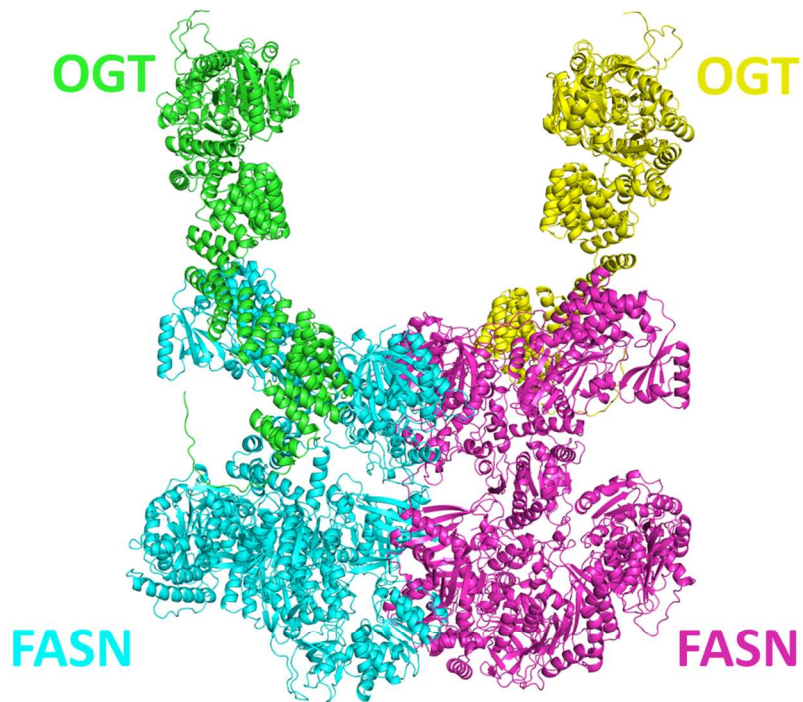


Vanauberg et al. 2025 – fig. 1

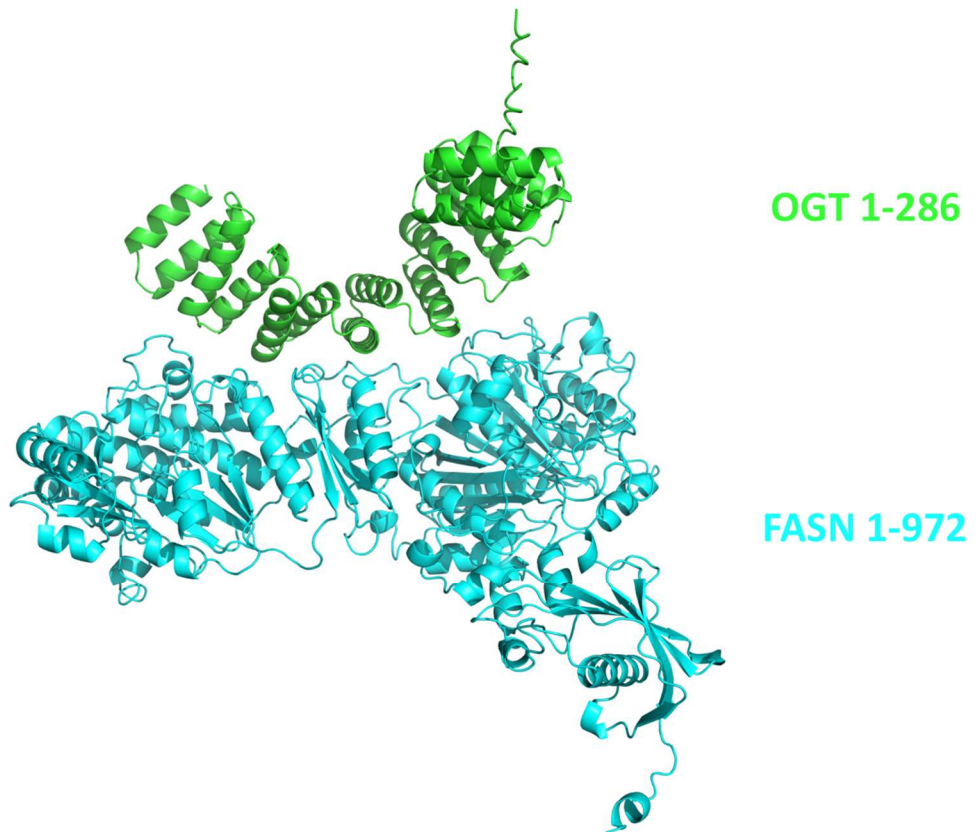


Vanauberg et al. 2025 – fig. 2

A



B



Vanauberg et al. 2025 – fig. 3

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Declaration of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary table 1: Interface residues within 5Å between OGT and FASN.

OGT

Chain A, Residue LEU 116
Chain A, Residue PRO 120
Chain A, Residue ASP 121
Chain A, Residue TYR 126
Chain A, Residue VAL 147
Chain A, Residue SER 148
Chain A, Residue LEU 150
Chain A, Residue GLN 151
Chain A, Residue TYR 152
Chain A, Residue ASN 153
Chain A, Residue PRO 154
Chain A, Residue ASP 155
Chain A, Residue ARG 160
Chain A, Residue LEU 163
Chain A, Residue GLY 164
Chain A, Residue LEU 167
Chain A, Residue LYS 168
Chain A, Residue ARG 172
Chain A, Residue LEU 173
Chain A, Residue GLU 174
Chain A, Residue GLU 175
Chain A, Residue ALA 176
Chain A, Residue LYS 177
Chain A, Residue ALA 178
Chain A, Residue CYS 179
Chain A, Residue TYR 180
Chain A, Residue LEU 181
Chain A, Residue LYS 182
Chain A, Residue ALA 183
Chain A, Residue ILE 184
Chain A, Residue GLU 185

Chain A, Residue THR 186
Chain A, Residue GLN 187
Chain A, Residue PRO 188
Chain A, Residue PHE 190
Chain A, Residue ALA 193
Chain A, Residue TRP 194
Chain A, Residue ASN 196
Chain A, Residue LEU 197
Chain A, Residue GLY 198
Chain A, Residue VAL 200
Chain A, Residue PHE 201
Chain A, Residue ASN 202
Chain A, Residue GLN 204
Chain A, Residue GLY 205
Chain A, Residue GLU 206
Chain A, Residue ILE 207
Chain A, Residue TRP 208
Chain A, Residue LEU 209
Chain A, Residue ALA 210
Chain A, Residue ILE 211
Chain A, Residue HIS 212
Chain A, Residue HIS 213
Chain A, Residue PHE 214
Chain A, Residue GLU 215
Chain A, Residue LYS 216
Chain A, Residue THR 219
Chain A, Residue ILE 240
Chain A, Residue ASP 242
Chain A, Residue ARG 243
Chain A, Residue PRO 426

FASN

Chain B, Residue GLU 349
Chain B, Residue HIS 350
Chain B, Residue GLY 351
Chain B, Residue LEU 352
Chain B, Residue GLN 379
Chain B, Residue PRO 380
Chain B, Residue LEU 381
Chain B, Residue PRO 382
Chain B, Residue VAL 383
Chain B, Residue ARG 384
Chain B, Residue PRO 410
Chain B, Residue PRO 411
Chain B, Residue PRO 412
Chain B, Residue ALA 413
Chain B, Residue PRO 414
Chain B, Residue ALA 415
Chain B, Residue PRO 416
Chain B, Residue HIS 417
Chain B, Residue ALA 418
Chain B, Residue THR 419
Chain B, Residue LEU 420
Chain B, Residue PRO 421
Chain B, Residue ARG 422
Chain B, Residue GLN 440
Chain B, Residue GLY 441

Chain B, Residue LEU 442
Chain B, Residue ARG 443
Chain B, Residue HIS 444
Chain B, Residue SER 445
Chain B, Residue GLN 446
Chain B, Residue ASP 447
Chain B, Residue LEU 448
Chain B, Residue PHE 450
Chain B, Residue LEU 451
Chain B, Residue GLY 474
Chain B, Residue GLY 475
Chain B, Residue GLU 476
Chain B, Residue LEU 508
Chain B, Residue MET 511
Chain B, Residue ARG 512
Chain B, Residue ASP 514
Chain B, Residue ARG 517
Chain B, Residue LYS 528
Chain B, Residue LEU 539
Chain B, Residue THR 541
Chain B, Residue ASP 791
Chain B, Residue LEU 793
Chain B, Residue GLU 794
Chain B, Residue PHE 816
Chain B, Residue PRO 817

Chain B, Residue GLU 820
Chain B, Residue PRO 824
Chain B, Residue ARG 825

Chain B, Residue GLY 826
Chain B, Residue THR 827
Chain B, Residue PRO 828

Supplementary table 2: Antibodies used in this study.

Antibodies	Dilution for WB
Primary antibodies (supplier, reference)	
Mouse monoclonal anti-O-GlcNAc (Thermo Scientific, RL2)	1 : 1,000
Rabbit polyclonal anti-OGT (Sigma-Aldrich, DM-17)	1 : 1,000
Mouse monoclonal anti-Flag M2 (Sigma-Aldrich, F1804)	1 : 1,000
Mouse monoclonal anti-GST (Thermo Scientific, 740007M)	1 : 5,000
Secondary antibodies (supplier)	
Sheep anti-mouse IgG/HRP conjugated (GE Healthcare)	1 : 10,000
Donkey anti-rabbit IgG/HRP conjugated (GE Healthcare)	1 : 10,000

Supplementary table 3: Primers used in this study.

Primer type	Name	Sequence (5' → 3')
Cloning	Hind III_FASN_Fw	CCCAAGCTTATGGAGGAGGTGGTGAATGCCGGC
	FASN_Xba I_Rv	GCTCTAGATTACGTAGAATCGAGTCCGAGGAGAGGGTTAGGGATAG
Mutagenesis	FASN G826stop_Fw	AGTTCCCAGCTCCCCGATGAACTCCCCTCATCTC
	FASN G826stop_Rv	GAGATGAGGGGAGTTCATCGGGGAGCTGGGAACT
	FASN E973stop_Fw	CTCTTCGACCACCCGTAAAGCCCCACCCCCAACCCC
	FASN E973stop_Rv	GGGGTTGGGGGTGGGGCTTTACGGGTGGTCAAGAG
	FASN W1634stop_Fw	CTGTCACCGGACTTCCTCTGAGATGTGCCTTCCAAGTGG
	FASN W1634stop_Rv	CCAGTTGGAAGGCACATCTCAGAGGAAGTCCGGTGACAG
	FASN E1862stop_Fw	TTGCGGAGGAGCCGTAGGCAGTGTGAAGGGGGCC
	FASN E1862stop_Rv	GGCCCCCTTCAACACTGCCTACGGCTCCTCCGCAA
	FASN C2202stop_Fw	GAGCTGGCATGACCCACGCCCAAGGAG
	FASN C2202stop_Rv	CTCCTTGGGCGTGGGTGATGCCAGCTC
Sequencing	FASN_Fw1	CGCCACCATCCTGAACG
	FASN_Fw2	TGATGACATCGTCCATTC
	FASN_Fw3	CTTCCCCAACGGTTCAG
	FASN_Fw4	GCTGAAGATGGTGGTGC
	FASN_Fw5	CTTGGTGAAGTGTCTCC
	FASN_Fw6	GTCGCTTCCTGGAAATTG
	FASN_Fw7	GTGGAGACGATGAGCAC
	hGH_PA_Rv	CAGCTTGGTTCCCAATAG

DISCUSSION

Cancers are a worldwide public health issue. By 2050, the Global Cancer Observatory estimates an approximately 90% increase in cancer-related deaths. As for many subtypes of cancers, hepatocellular carcinoma (HCC) treatment is challenging. The multikinase inhibitor sorafenib is capable of reducing tumor progression by targeting kinases like VEGFR or Raf (Rapidly accelerated fibrosarcoma). However, only around 30% of patients can benefit sorafenib, and the development of drug resistance is common (Tang et al., 2020). Thus, many studies have identified other target candidates to improve patient outcome like FASN. This pro-oncogenic enzyme, basically involved in *de novo* FA synthesis, gained insights in many hallmarks of cancer (Vanauberg et al., 2023). Hence, understand how this enzyme is regulated in HCC could shed light to new therapeutic avenues. Following this aim, for several years my team studied FASN regulation by the nutrient-dependent modification *O*-GlcNAcylation in HCC. Likewise FASN, this glycosylation mediated by OGT is involved in all hallmarks of cancer (Ferrer et al., 2016) (**Figure 20**).

My team highlighted FASN *O*-GlcNAcylation as a modification preventing its proteasomal degradation (Baldini et al., 2016). More recently, my colleagues discovered a positive cross-regulation between FASN and OGT (Raab et al., 2021). Both enzymes are overexpressed in HCC tissues from patients (Raab et al., 2022) and interact together in different phases of cell cycle (Raab et al., 2021). During my thesis, we showed that *O*-GlcNAcylation positively regulates fatty acids synthesis of HepG2 cells (**Publication I - Figures 1 and 2**), in accordance with previous reports (Baldini et al., 2016; Raab et al., 2021; Wong et al., 2022). But, to our knowledge, this is the first study that shows that modulating *O*-GlcNAcylation levels selectively impacts the synthesis of individual fatty acids. While the previous studies have reported a global role for *O*-GlcNAc in lipogenesis, our data provide a more detailed view of how this modification may influence the production of specific fatty acid species.

Then, we characterized FASN *O*-GlcNAcylation at the site-specific level and particularly identified and investigated two sites of *O*-GlcNAcylation: S595 and T980.

Given the considerable length of FASN (2,511 amino acids), it may seem surprising to focus on only two *O*-GlcNAcylation sites. Nevertheless, my thesis proves that such a focused approach can yield substantial insights, highlighting the value of in-depth investigations of PTMs.

Furthermore, targeting a limited number of sites enables a deeper and faster exploration of molecular and cellular mechanisms.

Although FASN is made up of a high number of amino acids, little is known about its PTMs. In addition to ubiquitination and *O*-GlcNAcylation (Baldini et al., 2016; X. Li et al., 2025; Malard et al., 2025; Wong et al., 2022), FASN is also phosphorylated. According to the PhosphoSitePlus® Database (Hornbeck et al., 2015), different sites of phosphorylation were reported. Interestingly, T980 phosphorylation was already reported in a lung cancer cell line (Tsai et al., 2015) but its function remains unknown. However, phosphorylation at Y66 has been characterized by Jin and collaborators (Jin et al., 2010). These authors showed that this phosphorylation, mediated by HER2, activates FASN and subsequently promotes invasion of breast cancer cells.

Other reported PTMs of FASN include *S*-nitrosylation, acetylation and SUMO (small ubiquitin-like modifier)-ylation. Like *O*-GlcNAcylation, SUMOylation (Floris et al., 2020) and *S*-nitrosylation (M. S. Choi et al., 2016) seem to promote FASN activity. On the contrary, FASN acetylation, a PTM frequently reduced in human HCC samples, trigger its proteasomal degradation (H.-P. Lin et al., 2016). Despite these interesting findings, a deeper site-specific investigation is needed to decipher the complex regulation of this metabolic enzyme.

On our side, FASN T980 was shown to be a major *O*-GlcNAcylation site regulating the enzyme's properties. FASN *O*-GlcNAcylation at T980 was already reported by Woo and collaborators (Woo et al., 2018). In Hep3B cells, we demonstrated the key role of FASN T980 *O*-GlcNAcylation by sustaining the ability of the enzyme to dimerize and to subsequently produce FA. This modification also supports FASN stability and membrane association (**Publication I - Figure 6**). The latter observation correlates with another study in prostate cancer cells showing FASN localization to membranes, particularly to lipid rafts (Di Vizio et al., 2008). It would be interesting to check whether FASN T980A lipid rafts localization is altered in our model. We have previously tried to raise this point, but without success.

Because of its pro-oncogenic features, we also analyzed the impact of T980 *O*-GlcNAcylation on FASN ability to promote cell survival, cell proliferation and cell cycle progression (**Publication I - Figure 5**). Consistent with the experiments described before, this modification is necessary for FASN pro-oncogenicity. It would be of particular interest to study this aspect

at the *in vivo* level. However, *in vivo* experiments require a stable expression of the different constructs. Unfortunately, we were unable to establish stable cell lines because of technical issues using lipofection and electroporation: high cell mortality, low transfection rate (partly due to the high size of FASN complementary DNA (cDNA)), loss of plasmid expression overtime... This suggests that other techniques, like gene editing such as CRISPR-Cas9 technology, should be tested. Additionally, the potential phosphorylation of FASN at T980 (**Figure 23**) warrants further molecular and cellular investigation in HCC.

We wondered whether the reduced stability observed for FASN T980A could be rescued by proteasome inhibition. Hence, transfected Hep3B cells were treated with the proteasome inhibitor MG132 (**Publication I - Supp Figure 4**): only a partial rescue of FASN protein level was obtained. This suggests that FASN T980 *O*-GlcNAcylation may protect the enzyme from another degradation pathway. Although my team demonstrated that *O*-GlcNAcylation protects FASN from proteasomal degradation (Baldini et al., 2016), we can speculate that modification at T980 could protect FASN from autophagic degradation. Indeed, Shpilka and collaborators demonstrated FASN degradation by autophagy upon nitrogen starvation in yeast (Shpilka et al., 2015). It would be of great interest to test this hypothesis.

Deng and collaborators recently identified Heat shock protein 90 α (Hsp90 α) as a crucial partner of FASN, enhancing its stability in HCC (Deng et al., 2025). The FASN interactome represents a promising area of investigation for uncovering the functional relevance of FASN T980 *O*-GlcNAcylation. While the role of FASN *O*-GlcNAcylation in promoting enzyme expression and activity is well established (Baldini et al., 2016; X. Li et al., 2025; Wong et al., 2022), our data further identify the T980 residue as a potentially critical determinant in HCC pathogenesis. We propose that OGT-mediated modification at T980 may enhance the binding affinity between FASN and Hsp90 α , thereby stabilizing the enzyme and augmenting its activity.

To go further on these data, we then aimed to determine how FASN and OGT interact. By combining co-immunoprecipitations, GST-pull down assays and bioinformatics, we highlighted a *N*-terminal-to-*N*-terminal interaction (**Publication II - Figures 2 and 3**). OGT *N*-terminal domain being commonly recognized as the major interaction surface with substrates, our data are consequently in accordance with the literature (Potter et al., 2024).

Our data also support the idea of the lack of specificity of OGT, its substrate recognition mechanism being poorly understood (Mauri et al., 2021). Indeed, we observed that the more OGT interacts with a FASN deletant, the more this deletant is glycosylated.

Remarkably, in addition to post-translationally modified proteins, *O*-GlcNAcylation is a co-translational modification of nascent proteins that prevents their premature degradation, as shown for Specificity protein 1 (Sp1) and Nuclear pore protein p62 (Nup62) (Y. Zhu et al., 2015). FASN is part of these co-translationally *O*-GlcNAcylated proteins (Y. Zhu et al., 2020) which could be consistent with our data. Indeed, it seems that FASN *N*-terminal is the most *O*-GlcNAcylated region of the enzyme (**Publication II - Figure 2B**). Protein synthesis proceeding from the *N*-terminus to the *C*-terminus, we hypothesize that OGT mainly *O*-GlcNAcylates FASN in the early stages of its translation, whereas in the later stages, FASN may have reduced exposure to OGT. As a result, FASN *N*-terminus would be the most *O*-GlcNAcylated part of the enzyme. Sustaining this hypothesis, Zhu and collaborators demonstrated that nascent proteins are more heavily *O*-GlcNAcylated than mature proteins (Y. Zhu et al., 2015) to potentially prevent premature degradation: if this is the case for FASN, this deserves further investigation.

We next reported sites exposed at the FASN-OGT interaction surface (**Publication II - Supp Table 1**). Determine those actually required for FASN - OGT interaction remains an open question. Identifying hot spots reinforcing this interaction could subsequently facilitate the discovery of small molecules inhibitors capable of disrupting FASN and OGT respective binding. This blockade should reduce FASN *O*-GlcNAcylation and, consequently, lower its pro-oncogenicity. Thus, preventing FASN - OGT interaction could impact HCC progression and constitute a novel therapeutic approach. In that way, inhibition of the interaction between two partner proteins has gained insights in oncology. For instance, interaction of p53 with its negative regulator Murine double minute 2 (MDM2) can be targeted by Idasanutlin (RG7388) (Mezzanotte-Sharpe et al., 2025). Well studied in clinics, this inhibitor prevents p53 ubiquitination and subsequent degradation. Thanks to its tumor suppressing activity, p53 is therefore able to counteract tumor progression.

Thus, novel generation protein interaction inhibitors are a promising avenue in cancer therapy due to their targeting specificity and limited off-target effects.

CONCLUSION AND PERSPECTIVES

My thesis works deciphered the regulation of lipogenesis by *O*-GlcNAcylation and the interaction between the two enzymes mediating these processes, namely FASN and OGT.

Modulation of HepG2 cells *O*-GlcNAcylation levels, by siRNA or inhibitors, significantly impacted FA synthesis that we measured by GC-FID. Reduced or elevated global *O*-GlcNAcylation decreased or increased FA synthesis, respectively. Precisely, it impacted several types of FA including FASN main product, palmitic acid (C16:0). Thus, we showed that *O*-GlcNAcylation positively regulates FA synthesis.

By LC-MS/MS, we identified two *O*-GlcNAc modifications on FASN at S595 and T980. Using site-directed mutagenesis, mutation of these sites into alanine allowed to determine the function of their respective glycosylation. While S595A mutation of FASN moderately affected its behavior, the T980A mutation significantly impeded the enzyme's properties.

We showed by cell fractionation that FASN T980A mutation reduced its membrane localization in Hep3B cells. By cycloheximide chase assay, we observed a significant reduction of FASN stability with this mutation. Native-PAGE experiments revealed a major decrease in FASN homodimers amount. This resulted in a reduced enzymatic activity, as shown by our BODIPY assays.

Altogether, the FASN T980A mutation suppressed its pro-oncogenic activity. Indeed, using different cellular assays we demonstrated that this mutation deeply reduced FASN ability to promote cell survival, as well as cell proliferation and cell cycle progression.

Our LC-MS/MS analyses showed that the T980 residue of FASN can also be phosphorylated (**Figure 23**). Hence, we potentially highlighted a “yin-yang” mechanism at T980 by engineering a phosphomimetic substitution (FASN T980D): while *O*-GlcNAcylation at T980 promotes FASN expression, phosphorylation dramatically induces its degradation.

Finally, our findings using co-immunoprecipitations and FASN deletants revealed that FASN preferentially interacted with OGT *via* its *N*-terminal domain. Likewise, we showed by GST-pull down with GST-OGT deletants that OGT preferentially interacted with FASN *via* its *N*-terminal domain. These observations were strengthened by bioinformatics modelling.

In perspectives, our data shed the light to a potent new therapeutic avenue for patients suffering from HCC. By preventing this *N*-terminal-to-*N*-terminal interaction between FASN and OGT, it is tempting to suppose that this could decrease FASN *O*-GlcNAcylation. As a result, the enzyme's pro-oncogenic properties would be abrogated, leading to a reduced tumor load (**Figure 24**).

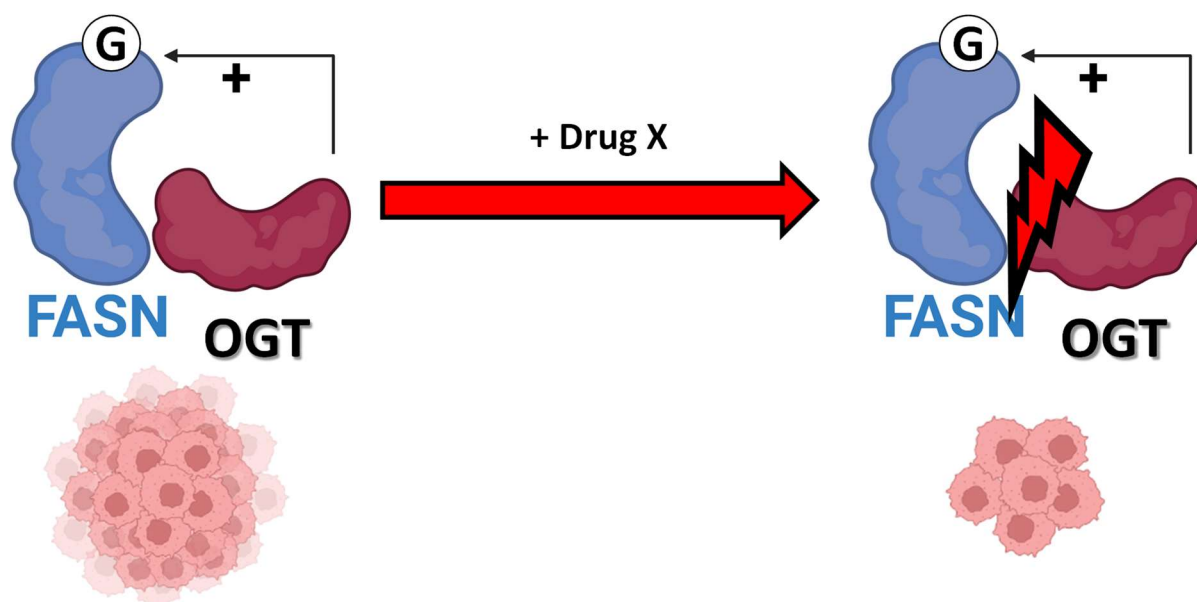


Figure 24: Target FASN-OGT interaction as a therapeutic avenue in HCC. The interaction between FASN and OGT promotes tumor progression. Hence, inhibiting this interaction with a “Drug X”, could reduce tumor load.

Such a drug would consist of an inhibitor of protein-protein interactions, *i.e.*, FASN - OGT interaction. To identify candidate compounds, structure-based drug design combined with virtual screening of a chemical library could be employed. Subsequent *in vitro* and cell-based assays would help assess the efficiency of the most promising hits. Finally, validation in an *in vivo* model of HCC could pave the way for clinical trials.

Due to the pivotal status of FASN T980 *O*-GlcNAcylated site, targeting this residue may represent a promising therapy. Blocking *O*-GlcNAcylation at T980 may suppress FASN pro-oncogenic character in HCC. Consequently, it would be of great interest to check this hypothesis *in vitro* and *in vivo*.

REVIEW ARTICLE OPEN



Involvement of the pro-oncogenic enzyme fatty acid synthase in the hallmarks of cancer: a promising target in anti-cancer therapies

Dimitri Vanauberg¹, Céline Schulz¹ and Tony Lefebvre¹✉

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An accelerated de novo lipogenesis (DNL) flux is a common characteristic of cancer cells required to sustain a high proliferation rate. The DNL enzyme fatty acid synthase (FASN) is overexpressed in many cancers and is pivotal for the increased production of fatty acids. There is increasing evidence of the involvement of FASN in several hallmarks of cancer linked to its ability to promote cell proliferation via membrane biosynthesis. In this review we discuss about the implication of FASN in the resistance to cell death and in the deregulation of cellular energetics by increasing nucleic acids, protein and lipid synthesis. FASN also promotes cell proliferation, cell invasion, metastasis and angiogenesis by enabling the building of lipid rafts and consequently to the localization of oncogenic receptors such as HER2 and c-Met in membrane microdomains. Finally, FASN is involved in immune escape by repressing the activation of pro-inflammatory cells and promoting the recruitment of M2 macrophages and T regulatory cells in the tumor microenvironment. Here, we provide an overview of the involvement of the pro-oncogenic enzyme in the hallmarks of cancer making FASN a promising target in anti-cancer therapy to circumvent resistance to chemotherapies.

Oncogenesis (2023)12:16; <https://doi.org/10.1038/s41389-023-00460-8>

A LONG ENZYME IN A FEW WORDS

Fatty acid synthase (FASN), previously characterized as OA-519 for Oncogenic Antigen-519 [1], is a ubiquitous and cytosolic enzyme. FASN is the second enzyme of de novo lipogenesis (DNL) catalyzing the synthesis of palmitic acid (C16:0) by using malonyl-CoA and acetyl-CoA as substrates, and NADPH, H⁺, as co-substrate. FASN is functional as a head-to-tail [2] or head-to-head [3] homodimer. Each monomer exhibits seven distinct enzymatic activities working in a sequential and coordinated manner plus an acyl carrier protein (ACP) that carry the acyl group during its elongation [4]. The final product is released by hydrolysis catalyzed by the thioesterase (TE) domain of FASN. The complete structure of mammalian FASN has been solved by X-ray crystallography except for the flexible ACP and TE domains [5]. FASN is not only involved in energy storage in the form of triglycerides but it also actively participates in the synthesis of membrane components and second messengers, and, indirectly, in protein modification by acylation [4]. At the organism level FASN is essential for gestation [6], lactation [7], respiration [8] and ageing [9].

From a pathological point of view, the implication of FASN in tumorigenesis was first identified in human breast carcinoma cells in 1994 [10]. Thus, FASN is overexpressed in breast cancer [11] but also in many other types of cancers: colorectal [12], prostate [13], stomach [14], esophageal [15], lung [16], pancreatic [17], ovarian [18], hepatic [19, 20], melanoma [21], glioma [22] and in primary effusion lymphoma (PEL) [23]. FASN is often a poor prognostic marker, its overexpression being correlated to a decrease in the

survival of patients. However, it is not considered as an oncogene as its overexpression in healthy cells does not induce a malignant transformation as shown for hepatocytes [24]. Through the years, many studies have highlighted the involvement of FASN in different hallmarks of cancer including cell metabolism, proliferation, migration, invasion, resistance to cell death, immune escape, and angiogenesis (Fig. 1): this makes the lipogenic enzyme an interesting and promising target in anti-cancer therapies.

FASN AND THE DEREGLATION OF CELLULAR ENERGETICS

One of the hallmarks of cancer cells is metabolic reprogramming [25]. This includes an increased DNL unlike healthy cells which have a reduced lipogenesis, fatty acid needs being satisfied usually through dietary (exogenous) intake. The acceleration of lipogenesis observed in tumors promotes cell proliferation, survival, invasion and resistance to chemotherapy [26]. The increase in the lipogenic flux is partly related to the Warburg effect and to the overexpression of several metabolic enzymes including FASN. This increase in DNL flux has been not only described in hepatocellular carcinoma (HCC) [26] but also in breast cancer [27], glioblastoma [28], colorectal cancer [29] and in hematologic malignancies like acute myelogenous leukemia and endemic Burkitt lymphoma [30].

The highest levels of the lipogenic enzymes ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACC), stearyl-coenzyme A desaturase 1 (SCD1) and FASN have been detected in HCC characterized by an aggressive phenotype [31, 32]. These enzymes

¹Univ. Lille, CNRS, UMR 8576 - UGSF - Unité de Glycobiologie Structurale et Fonctionnelle, F-59000 Lille, France. ✉email: tony.lefebvre@univ-lille.fr

Received: 9 December 2022 Revised: 1 March 2023 Accepted: 3 March 2023
Published online: 18 March 2023

are generally overexpressed in HCC explants in association with an increase of triglycerides, fatty acids and cholesterol compared to the adjacent non-tumoral tissue [31]. Recently, it has been shown in non-Hodgkin lymphoma that FASN promoted nucleotide biosynthesis required for supporting malignant proliferation [33]. By using NADPH, H^+ to produce palmitate, FASN limits the level of the co-substrate and thus promotes 6-phosphogluconate dehydrogenase (PGDH) activity of which NADPH, H^+ is an allosteric inhibitor. PGDH is the second enzyme of the pentose-phosphate pathway (oxidative branch) that generates ribulose-5-phosphate then converted into ribose-5-phosphate by the keto-isomerase (non-oxidative branch). As a result, FASN indirectly increases DNA/RNA synthesis required for cell proliferation. FASN also promotes protein synthesis through the activation of mammalian/mechanistic target of rapamycin (mTOR) activity in HepG2 [34] and in HCT116 cells [35] (Fig. 2).

FASN AND PROLIFERATIVE SIGNALING

Overexpression of FASN in breast cancer cells promotes their survival and proliferation via the hyperactivation of HER1/2 receptors [11]. In turn, HER1/2 induce expression of FASN via activation of the MAPK and PI3K/Akt pathways (Fig. 3) highlighting a positive feedback to maintain high levels of FASN in cancer cells [11]. Jin et al. have demonstrated that inhibition of FASN by C75 (Table 1) in the breast cancer cell lines SKBR3 and BT474 was responsible for an increase in the internalization and degradation of HER2 associated with a decrease in the level of HER2 mRNA [36]. FASN promotes the expression and activation of HER2 probably by allowing its localization in lipid rafts and, conversely, HER2 favors the expression and activity of FASN (Fig. 3). The overexpression of FASN in prostate cancer cells (LNCaP) increases cell proliferation and soft agar growth [13]. In addition, mice bearing xenografts of esophageal squamous cell carcinoma cells (Colo680N) treated with the FASN inhibitor C93 (Table 1) showed a significant inhibition of tumor growth [15]. Together, these observations strongly support a role for FASN in cell proliferation and tumor growth.

FASN is overexpressed in breast cancer stem cells (BCSC), promotes stemness, and is hyperactivated in stemness-enriched samples [37]. Its inhibition with the antioxidant epigallocatechin-3-gallate (EGCG) or with the EGCG synthetic derivative G28 (Table 1) diminishes stemness and cell proliferation. It has been recently demonstrated that mTOR promotes FASN expression in HCC cells

[34] and in breast cancer cells [38] (Fig. 3). In non-small cell lung cancer (NSCLC) cells with a mutant EGFR, FASN induces indirectly EGFR palmitoylation which prevents its inhibition by tyrosine kinase inhibitors (TKI). This results in a persistent signaling driven by mutated EGFR and consequently to cell proliferation [39] (Fig. 3). DNL is more active in glioblastoma CSCs compared to non-stem cancer cells [40]. FASN is necessary for the expression of markers of glioma stem cells, and for the maintenance of stemness [40]. In this sense, the inhibition of FASN with the natural antibiotic cerulenin (Table 1) blocks cell proliferation and decreases the expression of the glioblastoma stemness markers Nestin, Sox2 and Fatty Acid Binding Protein 7 (FABP7) [40]. On the contrary, cerulenin increases the expression of the differentiation marker glial fibrillary acidic protein (GFAP). At last, FASN is overexpressed in induced pluripotent stem cells, neural stem and progenitor cells and glioma stem-like cells [41]; it is therefore likely that FASN is involved in the maintenance of stemness of CSCs (Fig. 3).

FASN AND RESISTANCE TO CELL DEATH

Migita et al. reported that the expression of FASN in human prostate tumor tissues was statistically significantly inverse to the apoptotic rate [13]. The activation of the intrinsic pathway of apoptosis with camptothecin in the prostate cancer LNCaP cells stably overexpressing FASN did not induce cell death contrary to control LNCaP cells [13] suggesting that FASN blocks the intrinsic pathway of apoptosis. In the same report, the authors showed that transgenic mice overexpressing FASN in the prostate harbored a prostatic hyperplasia associated with a weaker apoptotic rate compared to wild-type mice [13]. A study conducted on LNCaP cells demonstrated that knock-down of FASN favored apoptosis as evidenced by the increased-signal of Annexin V-EGFP and staining with propidium iodide. On the contrary, no effect was observed on fibroblasts viability [42].

Inhibition of FASN with C75 in HepG2 cells induces a G2-phase arrest associated with an increase of p53 expression [43]. Similarly, the treatment of PEL cells with C75 triggers apoptosis contrary to primary B cells [23]. The treatment of MDA-MB-231 cells with the natural FASN inhibitor curcumin (Table 1) or silencing FASN decreases the level of the anti-apoptotic protein Bcl-2 and concomitantly increases the pro-apoptotic protein Bax [44]. These observations strengthen the potent ability of FASN to oppose cancer cell apoptosis likely through their dependence upon DNL compared to healthy cells. Nevertheless, future investigations are necessary to decipher and understand the precise mechanism

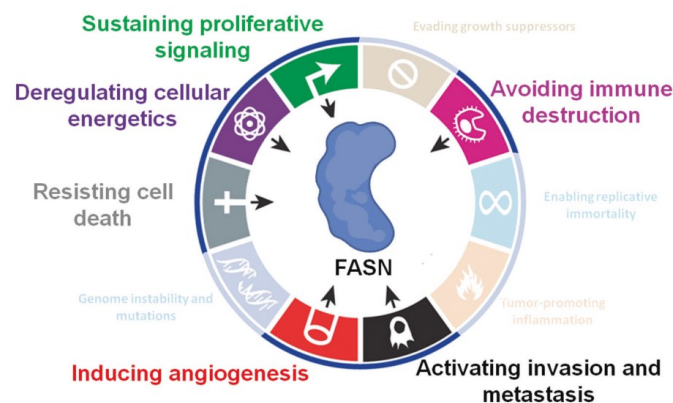


Fig. 1 Upregulation of FASN correlates with several hallmarks of cancer. The pro-oncogenic enzyme FASN is involved in sustained proliferation, dysregulation of cell energetics, resistance to cell death, induction of angiogenesis, activation of invasion and metastasis, and prevention of immune destruction. Adapted from Hanahan and Weinberg, 2011 [25].

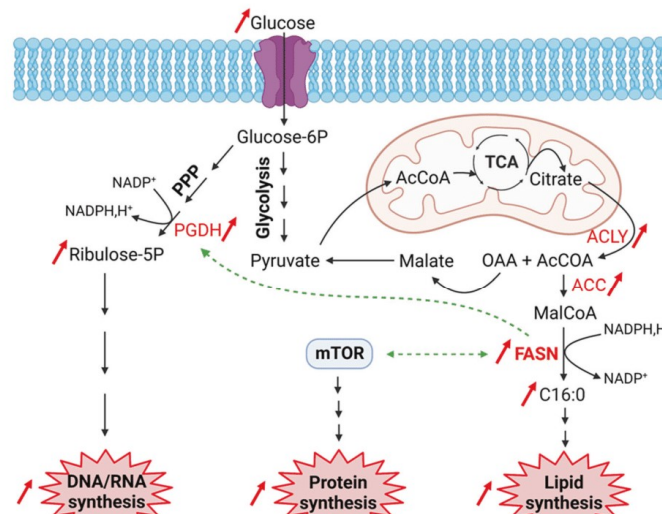


Fig. 2 FASN deregulates cell metabolism. Many enzymes are upregulated in cancer cells including the lipogenic enzymes ACLY, ACC, and FASN. Their overexpression leads to an increased lipid synthesis sustained by the Warburg effect. In addition FASN promotes mTOR activation resulting in an increased protein synthesis. FASN also favors the activity of the PPP (pentose-phosphate pathway) enzyme PGDH by increasing the pool of NADP^+ , a co-substrate of the latter. PPP upregulation concomitantly increases DNA/RNA synthesis. The dotted arrows represent an indirect effect. PGDH, 6-phosphogluconate dehydrogenase; AcCoA, acetyl-CoA; MalCoA, malonyl-CoA; TCA, tricarboxylic acid cycle; OAA, oxaloacetic acid; ACLY, ATP citrate lyase; ACC, acetyl-CoA carboxylase; PPP, pentose-phosphate pathway; mTOR, mammalian/mechanistic target of rapamycin; FASN, fatty acid synthase.

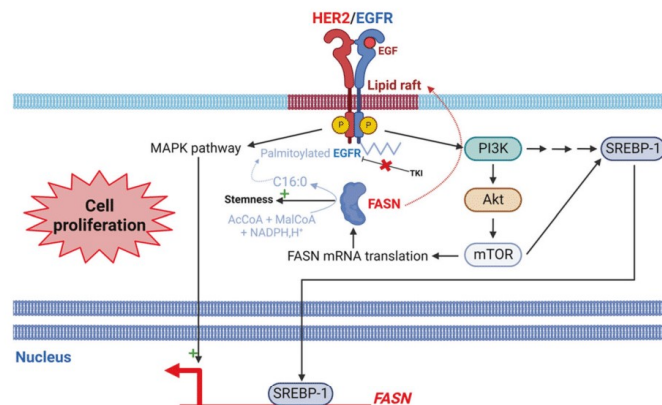


Fig. 3 FASN promotes cell proliferation. FASN participates in the elaboration of lipid rafts required for signaling-associated receptors including the oncogenic receptors HER2 and EGFR. Thus, a positive feedback is ON as these receptors drive FASN expression via the activation of the MAPK and PI3K/Akt/mTOR pathways. PI3K and mTOR govern the expression of the transcription factor SREBP-1 which in turn induces FASN expression. mTOR also regulates FASN mRNA translation. In addition, FASN favors indirectly EGFR palmitoylation which prevents its inhibition by TKI. Finally, FASN promotes stemness and therefore cell proliferation.

linking FASN to the extrinsic and intrinsic pathways of apoptosis at the molecular level.

FASN AND CELL INVASION/METASTASIS

Jin et al. demonstrated that FASN was phosphorylated on Tyr66 by HER2 [36]. This phosphorylation activates FASN leading to cell

invasion via an increase in the activity of the metalloproteinase MMP-9 (Fig. 4). Furthermore, FASN mediates the epithelial-mesenchymal transition (EMT) of breast cancer cells as its inhibition with cerulenin increases and decreases respectively the expression of E-cadherin and vimentin, and inhibits cell migration [45]. Hung et al. showed that the inhibition of FASN with the natural compound osthole (Table 1) or with C75 induced a loss

Table 1. Summary of the different FASN inhibitors discussed in this review.

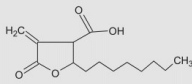
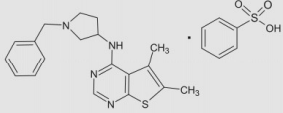
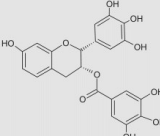
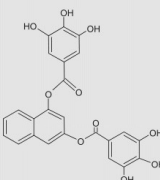
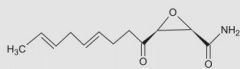
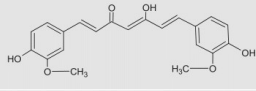
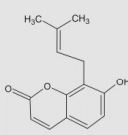
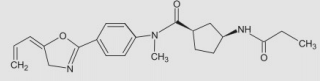
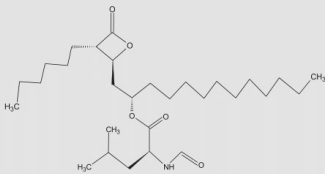
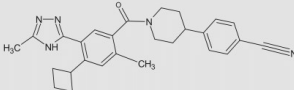
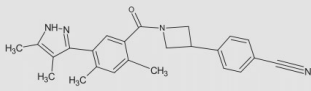
Compound	Structure	Origin	Targeted domain	Development stage
C75		Cerulenin synthetic derivate	β -ketoacyl synthase, thioesterase, enoyl reductase	Pre-clinic
C93		Synthetic	β -ketoacyl synthase	Pre-clinic
EGCG		Plant-derived polyphenol (natural component of green tea)	β -ketoacyl reductase	Pre-clinic
G28		EGCG synthetic derivative	Same as EGCG?	Pre-clinic
Cerulenin		Antibiotic produced by <i>Cephalosporium caerulens</i>	β -ketoacyl synthase	Pre-clinic
Curcumin		Antioxidant produced by <i>Curcuma longa</i>	Malonyl/acetyl transferase	Pre-clinic
Osthole		Botanical anti-fungal agent produced by <i>Cnidium monnieri</i>	?	Pre-clinic
BI99179		Synthetic	β -ketoacyl reductase	Pre-clinic

Table 1. continued

Compound	Structure	Origin	Targeted domain	Development stage
Orlistat		Derivative of lipstatin isolated from <i>Streptomyces toxytricini</i>	Thioesterase	FDA-approved anti-obesity drug
TVB-2640		Imidazopyridine-based inhibitor	β -ketoacyl reductase	In phase II trial
TVB-3166		Pre-clinical version of TVB-2640	Same as TVB-2640	Pre-clinic

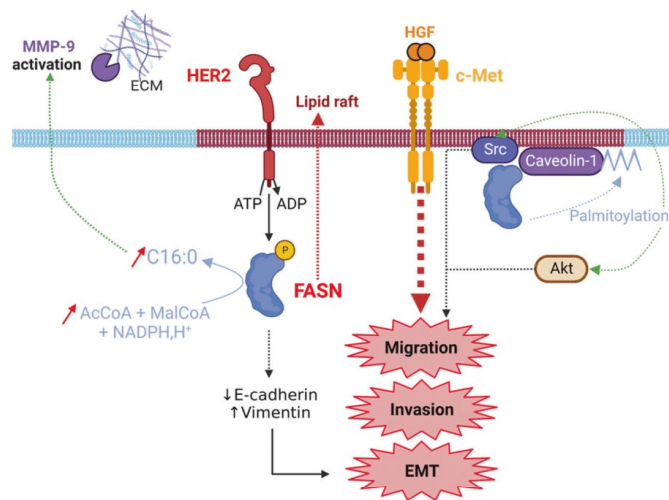


Fig. 4 FASN promotes cell migration and invasion. FASN is phosphorylated by HER2 increasing its activity and being responsible for an activation of MMP-9 that degrades the extracellular matrix (ECM). FASN exerts a positive feedback loop by allowing HER2 localization into lipid rafts. Through a respective reduction and increase of E-cadherin and vimentin expression, FASN promotes EMT reinforced by the proper localization of the c-Met receptor into lipid rafts. FASN interacts with Src and caveolin-1. FASN indirectly participates in caveolin-1 palmitoylation which in turn promotes Src and Akt activity leading to cell migration. The dotted arrows represent an indirect effect.

of the oncogenic receptor c-Met inhibiting consequently EMT, migration and invasion of MCF-7 cells [46]. The deficit of c-Met was prevented by the addition of palmitate in the culture cell medium. Since c-Met is resident in lipid rafts [47] and that in epithelial cancer cells FASN activity is essential for structuring lipid rafts implicated in cell signaling and migration [48], this study suggests that the loss of FASN prevents c-Met localization in lipid

microdomains (Fig. 4). It would be interesting to challenge the correct localization mechanism of c-Met at the level of lipid rafts in near future studies. Also, at the level of these membrane microdomains, FASN has been shown to interact with Src and Caveolin-1. Palmitoylation of caveolin-1, through the indirect activity of FASN, promotes the activation of Src and Akt which increases the migration of prostate cancer cells [49] (Fig. 4).

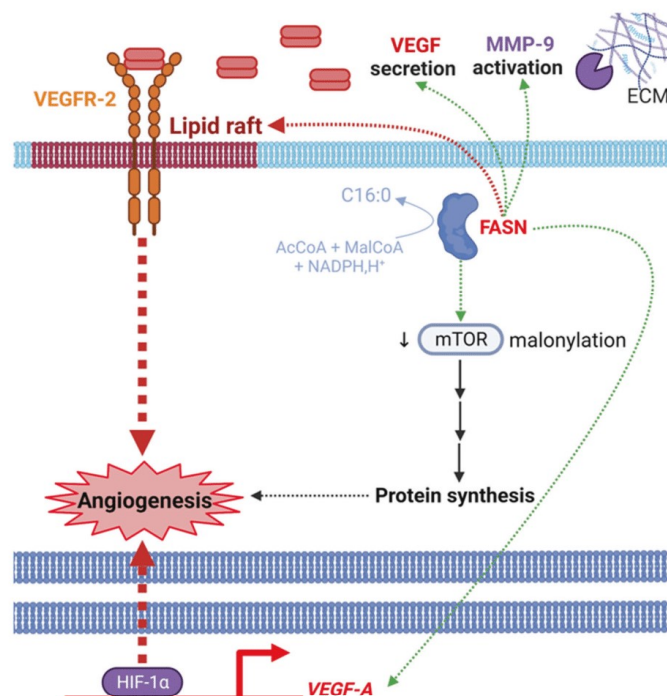


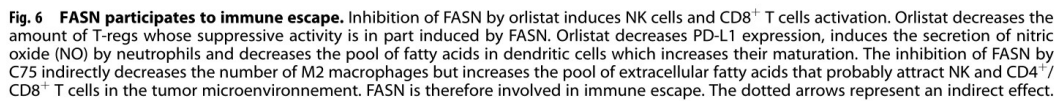
Fig. 5 FASN favors angiogenesis. The catalytic activity of FASN probably allows the localization of the oncogenic receptor VEGFR-2 in lipid rafts, a prerequisite to the regulation of angiogenesis. By producing fatty acids, FASN reduces the pool of malonyl-CoA leading to a decrease of mTOR malonylation which promotes its activity and therefore angiogenesis. FASN also controls VEGF secretion and MMP-9 activation to respectively activate VEGFR-2 and degrade the extracellular matrix (ECM), and consequently to sustain angiogenesis. The expression and activity of the transcription factor HIF-1 α are positively regulated by FASN; this induces VEGF-A expression. The dotted arrows represent an indirect effect.

Expression of FASN in the high metastatic MHCC97H and SK-Hep-1 cell lines is increased compared with low metastatic HCC cell lines [19]. Transfection of HCC SK-Hep-1 cells with shFASN inhibited proliferation, migration and invasion [19]. Supplementation with palmitic acid improves HCC cell migration and activates the TGF- β and Wnt signaling pathways, both responsible for EMT program [50]. The DNL pathway promotes metastasis in mice; inhibiting FASN with the brain-permeable inhibitor BI99179 (Table 1) decreases the growth of brain metastases but does not impact the breast primary tumor [51]. In vivo, inhibiting the transcription factors sterol regulatory element binding proteins (SREBPs), that drive FASN expression, by fatostatin blocks both prostate tumor growth and distant metastasis [52]. Finally, cerulenin blocks cell migration and invasion of glioblastoma CSCs [40]. Consequently, FASN promotes metastasis, making the enzyme a prime target for preventing cancer progression and cancer cells spreading.

FASN AND ANGIOGENESIS

FASN interferes with angiogenesis. The incubation of breast cancer cells with cerulenin results in a decrease expression of vascular endothelial growth factor (VEGF) and VEGFR-2 [45]. Moreover, FASN promotes the localization of VEGFR-2 on the endothelial cell surface [53]. It can be assumed that FASN promotes the localization of VEGFR-2 at the level of lipid rafts, and thus its activation by VEGF. but, to our knowledge this has

never been clearly demonstrated (Fig. 5). In addition, Bruning et al. showed that knockdown of FASN in endothelial cells increased the malonylation of the kinase mTOR due to the excess of malonyl-CoA not used by FASN [54]. Malonylation inhibits mTOR resulting in a decrease in protein synthesis and to a loss of endothelial cell proliferation and angiogenesis (Fig. 5). Interestingly, the inhibition of FASN with orlistat (Table 1), a FDA-approved anti-obesity drug, or with cerulenin, significantly reduces metastases and angiogenesis in a mouse syngeneic model of melanoma [55]. In this model, FASN inhibition induced an increased secretion of an anti-angiogenic isoform of VEGF-A by melanoma cancer cells. Later, Zhou and collaborators showed that the expression level of FASN correlated with microvessels density in human gliomas [22]. They observed in mice orthotopically xenografted with GSCs that silencing FASN using shRNAs or inhibition with C75 decreased microvessels density and tumor growth. Mechanistically, the inhibition of FASN blocked hypoxia-inducible factor-1 α (HIF-1 α)/VEGF-A signaling and upregulated the anti-angiogenic isoform-VEGF-165b. But, the precise link between FASN and HIF-1 α /VEGF-A signaling pathway deserves to be pushed a little further. Zaytseva et al. also demonstrated that the stable knockdown of FASN in colorectal cancer cells induced a decrease of angiogenesis [56]. The culture of human lung microvascular endothelial cells in a medium conditioned from FASN knockdown colorectal cancer cells decreased VEGFR-2 activation, cell proliferation, migration and tubulogenesis. These cancer cells secreted less MMP-9 and



produce more nitric oxide which has antineoplastic effects (Fig. 6). Conversely, lipids produced by ovarian cancer cells overexpressing FASN accumulate in dendritic cells [60]. Dendritic cells are then unable to present the antigens to T lymphocytes and to induce an anti-tumor immune response. Finally, Cioccoloni et al. demonstrated that orlistat impairs the expression of PD-L1 implicated in immune suppression in a T-cell leukemia line [61]. This observation suggests that FASN promotes PD-L1 expression and, as a result, immune escape (Fig. 6).

GENERAL CONCLUSION AND PERSPECTIVES

Due to its pivotal involvement in many features of cancer, FASN is potentially a good anti-cancer therapeutic target. To date, TVB-2640 (Table 1) is the most advanced FASN inhibitor currently used in phase 2 trial. Combined to taxane, TVB-2640 is promising in the treatment of advanced cancers [62]. This first-in-class inhibitor has been tested with patients carrying astrocytoma, NSCLC, breast cancer, peritoneal carcinoma and ovarian cancer [63]. TVB-3166 (Table 1), a smaller analogue of TVB-2640 used *in vitro*, leads to an endoplasmic *reticulum* stress which prevents the translation of ERα mRNAs in tamoxifen-resistant MCF-7 cells and results in a decrease of tumor growth of xenografted tumors in mice [64].

Of particular interest, PROTeolysis Targeting Chimeric molecules, or PROTACs, are promising tools that could be used to develop next-generation therapeutics to target FASN. PROTACs consist of chimeric molecules that bridge any protein to an E3 ligase so as to induce its proteasomal degradation. There are currently several PROTACs in clinical trials for the treatment of cancer such as ARV-471 (breast cancer) expected to enter phase 3 soon or ARV-110 (prostate cancer) which is in phase 2 [65]. These PROTACs are also combined to other drugs like Everolimus for ARV-471 (NCT05501769) or Abiraterone for ARV-110 (NCT05177042) in some clinical trials in an attempt to improve the treatment of patients. Thus, multi-compound therapies represent promising tools for the management of hard-to-cure cancer patients and could apply to FASN in conjunction with inhibitors currently in clinical phase.

FASN may also be responsible for the resistance to trastuzumab (HerceptinTM) in HER2 overexpressing breast cancers. As discussed

previously, there is a cross-talk between FASN and HER2 in which each promotes the expression of the other [36] (Figs. 3 and 4). The combination of trastuzumab with FASN blockade presents a synergistic effect on SK-BR-3 breast cancer cells growth inhibition and apoptosis [66]. Moreover, the inhibition of FASN-driven lipid rafts building also negatively affects EGFR-HER2 cross-talk which is of particular importance for trastuzumab resistance [48]. It has been also shown that tamoxifen promotes FASN expression in ER⁺/HER2⁺ breast cancer cells and increases cell proliferation [67]. Thus, FASN could also induce tamoxifen resistance by promoting cell growth. In NSCLC cells with mutated EGFR and resistant to TKI, the inhibition of FASN with orlistat induces EGFR K48-ubiquitination resulting in a reduction of tumor growth associated with an increase in apoptosis in mice xenografted tumors [39]. It would be particularly interesting to check whether this K48-ubiquitination of EGFR actually induces its proteasomal degradation.

FASN inhibition could be used as an adjuvant anti-cancer therapy to those already existing. For example, a synergistic effect of FASN and Thymidylate Synthase respectively inhibited by cerulenin and 5-fluorouracil is observed on cell viability in breast cancer cells [68]. In the same way, FASN blockade induces a synergistic chemo-sensitization of breast cancer cells to microtubule interfering agents such as docetaxel [69], paclitaxel [70] and vinorelbine [71]. As discussed previously the combined inhibition of FASN and PI3Ka synergistically inhibits tumor growth in a murine allograft model by enhancing anti-tumoral immunity [57]. As well, the inhibition of FASN in patients harboring a resistance to c-Met TKI is a promising therapy [46]. Finally, the FASN inhibitor G28 allows to overcome EGFR TKIs resistance in NSCLC [72]. The potential final approval of TVB-2640 or other anti-FASN drugs could therefore offer novel therapies for patients with cancer in an era of widespread resistance to existing drugs.

DATA AVAILABILITY

All data presented in the current review are publicly available, and all findings summarized here come from articles cited in the reference list and available in the MEDLINE database.

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AUTHOR CONTRIBUTIONS

DV drafted the manuscript, CS and TL edited the manuscript. All authors approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Correspondence and requests for materials should be addressed to Tony Lefebvre.

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