Université des Sciences et Technologies de Lille
Institut de Biologie de Lille, CNRS UMR 8161
Ecole Doctorale Biologie-Santé

Thèse de doctorat

Préparée par

Jonathan LEFEBVRE

En vue de l'obtention du grade de docteur d'Université

ETUDE DES PROPRIETES APOPTOTIQUES DU RECEPTEUR TYROSINE KINASE MET

Soutenue le 16 Décembre 2011

Devant le jury composé de :

Président :	Pr Philippe DELANNOY
Rapporteurs :	Dr Flavio MAINA Dr Catherine BRENNER
Examinateur :	Dr Patrick MEHLEN
Directeur de thèse :	Dr David TULASNE

" Nos vérités sont provisoires : battues en brèche par les vérités de demain, elles s'embroussaillent de tant de faits contradictoires que le dernier mot du savoir est le doute."

Jean-henri Fabre

Souvenirs entomologiques (1900)

Remerciements

Je tiens à remercier l'ensemble des membres de mon jury qui ont accepté de juger mon travail. Merci aux Drs Catherine Brenner et Flavio Maina d'avoir accepté d'être mes rapporteurs, au Pr Philippe Delannoy de présider le jury et au Dr Patrick Mehlen d'être mon examinateur.

Je remercie le Pr Philippe Delannoy pour son soutien lors de mes recherches de post-doctorat. Je remercie également Aurélie Cazet, Marie Bobowski et Agatha Steenackers pour nos collaborations et mon initiation au monde des gangliosides.

Merci au Dr Catherine Brenner de m'avoir accueilli au sein de l'unité INSERM UMR S-769 pour y apprendre les techniques d'isolement de mitochondries. Je remercie également le Dr Christophe Lemaire et son étudiante en thèse Cindy Gallerne pour leur encadrement ainsi que nos échanges et discussions.

Je remercie Dr Flavio Maina pour ses précieux conseils lors de mon comité de suivi de thèse.

Je tiens à remercier mon directeur de thèse, le Dr David Tulasne, pour son soutien inconditionnel. Tu as dirigé l'ensemble de ce travail (et les autres) avec enthousiasme et énergie. Merci de m'avoir fait confiance et d'avoir laissé libre cours à mon imagination. La qualité de ton encadrement sur le plan scientifique et humain m'ont conduit à tirer le meilleur de moi-même. J'espère que l'axe Met/Mitochondries/Cancer aura de beaux jours devant lui.

Enfin, je remercie le Pr Yvan de Launoit de m'avoir accueilli au sein de l'UMR8161.

TABLE DES MATIERES

TABLE DES MATIERES ... 3

RESUME ... 7

ABSTRACT ... 9

TABLE DES FIGURES ... 11

LISTE DES ABREVIATIONS ... 13

INTRODUCTION ... 16

I) L'HGF est le ligand du Récepteur Tyrosine Kinase Met ... 17
- A) Découverte de Met ... 17
- B) L'HGF/SF : deux noms, un même facteur ... 18
- C) Activation de Met par l'HGF/SF ... 19

II) Implications physiologiques du couple HGF/SF-Met ... 22
- A) Au cours de l'embryogenèse ... 22
- B) Chez l'adulte ... 25

III) Signalisation du couple HGF/SF-Met ... 26
- A) Un couple ligand-récepteur, de nombreuses réponses cellulaires ... 26
- B) La signalisation de Met ... 27
  - 1) GAB1 ... 29
  - 2) Grb2 ... 29
  - 3) La PI3-K ... 30
  - 4) STAT3 ... 30
  - 5) Cibles transcriptionnelles ... 31
- C) Les partenaires membranaires de la signalisation de Met ... 31
  - 1) Les intégrines ... 32
  - 2) CD44 ... 33
  - 3) Les plexines/sémaphorines ... 34
  - 4) Les récepteurs tyrosine kinase ... 35
  - 5) Fas R ... 35

IV) Implications du couple HGF/SF-Met dans le cancer ... 36
- A) Réarrangement chromosomique ... 36

B) Mutations de Met ... 37
C) Surexpression de Met et/ou de l'HGF/SF ... 40
D) Autocrinie, paracrinie ... 41
E) Thérapies ... 42

V) Dégradation du récepteur Met ... 44
A) Dégradation dépendante du ligand ... 44
B) PS-RIP ... 73

VI) L'apoptose ... 74
A) La voie dite extrinsèque ... 76
B) La voie dite intrinsèque ... 76
1) La mitochondrie ... 78
2) Perméabilisation de la membrane mitochondriale externe et apoptose ... 80
a) Le pore de transition de perméabilité ... 80
b) Formation de canaux protéiques Bax/Bak ... 82
3) La boîte de Pandore ... 85
a) L'apoptosome ... 85
b) Les autres facteurs mitochondriaux apoptogènes ... 87
C) Les caspases ... 88
1) Classification des caspases ... 88
2) Structure et activation ... 89
3) Conséquences fonctionnelles des clivages par les caspases ... 91
a) Clivage perte de fonction ... 92
b) Clivage gain de fonction ... 92
c) Cas des récepteurs à dépendance ... 93
d) Met, un substrat des caspases ... 96

RESULTATS ... 98

I) p40 Met a rendez-vous avec la mitochondrie ... 99
Abstract ... 101
Introduction ... 102

II) A la recherche de nouveaux partenaires ... 139

III) Implications des gangliosides dans l'activation de Met indépendante du ligand ... 140

CONCLUSION 142

BIBLIOGRAPHIE 146

ANNEXES 164

RESUME

Le récepteur Met et son ligand l'HGF/SF sont essentiels pour le développement embryonnaire tandis que la dérégulation de la signalisation du récepteur Met est associée à la progression tumorale. Activé par son ligand, Met induit un large panel de réponses biologiques telles que la survie cellulaire, la migration ou la prolifération. En revanche, le fragment p40 Met issu du clivage du récepteur par les caspases participe à l'apoptose. Cette dualité fonctionnelle est caractéristique de la famille des récepteurs à dépendance. Bien que la signalisation positive des RTK soit bien décrite, les mécanismes moléculaires leur permettant de participer aux processus de mort restent encore méconnus.

Nous avons montré que le fragment p40 Met est capable d'amplifier l'apoptose indépendamment de l'activité kinase bien qu'il comprenne l'intégralité du domaine catalytique. Au cours de l'apoptose, nous avons montré à partir de lignées cellulaires épithéliales ou dans des foies de souris que p40 Met présente une localisation mitochondriale indispensable à ses fonctions apoptotiques. De manière intéressante, p40 Met possède au sein du site de fixation de l'ATP un domaine BH3 comparable à celui des protéines pro-apoptotiques Bcl-2 de type BH3-only. A l'image des BH3-only, ce domaine est impliqué à la fois dans l'activité apoptotique du fragment et dans son association avec la protéine Bcl-2. Le fragment p40 Met est capable d'induire également la perméabilisation mitochondriale de manière caspase-indépendante, conduisant au relargage du cytochrome *c*. Enfin, au cours de l'apoptose, l'extinction de l'expression de Met entraîne un retard du relargage du cytochrome *c*, démontrant ainsi la participation du récepteur dans la voie intrinsèque de l'apoptose.

ABSTRACT

The receptor tyrosine kinase Met and its ligand, the hepatocyte growth factor/scatter factor, are essential to embryonic development, whereas deregulation of Met signaling pathways is associated with tumorigenesis and metastasis. Ligand-activated Met induces multiple cellular responses including cell survival, migration and proliferation. Nonetheless, Met is cleaved under stress conditions by caspases within its intracellular region, generating a 40 kDa fragment (p40 Met) which is able to induce apoptosis. By triggering both survival and apoptosis responses in presence or absence of ligand respectively, Met belongs to the family of the dependence receptor. While the survival responses induced by membrane full length RTK are well known, the molecular mechanisms initiated by dependence receptors to promote apoptosis are poorly understood.

We demonstrate in Tet-on inducible epithelial cells that p40 Met expression accelerates cell death only when apoptosis is induced beforehand. Consistently, using an antibody directed specifically against the fragment, we observed that it is generated early during apoptosis when caspase activity is not maximal yet. Although p40 Met contains the entire kinase domain, it is not a constitutive active kinase. However, p40 Met is able to translocate both in mitochondria and nucleus and its forced relocalisation at the plasma membrane decreases its apoptotic property. Interestingly, p40 Met contains a BH3 domain required for both its pro-apoptotic property and association to the anti-apoptotic Bcl-2 proteins. In addition, p40 Met is able to induce mitochondrial permeabilization in a caspase-independent manner, leading to cytochrome *c* release and further caspase activation. Extinction of Met expression in epithelial cells delays cytochrome *c* release during apoptosis, showing indeed its involvement in the regulation of mitochondrial cell death.

TABLE DES FIGURES

Figure 1 : Domaines et structures tridimensionnelles de Met 18

Figure 2 : Domaines et structures tridimensionnelles de l'HGF/SF 19

Figure 3 : Modélisation de l'interaction HGF/SF-Met 21

Figure 4 : Phénotypes des souris sauvages et KO HGF/SF ou Met $^{-/-}$ 23

Figure 5 : Effet du KO HGF/SF ou Met $^{-/-}$ dans le développement des muscles 24

Figure 6 : Partenaires recrutés par le récepteur Met activé par l'HGF/SF 28

Figure 7 : Les différents partenaires membranaires du récepteur Met 32

Figure 8 : Caractéristiques permettant la transformation cellulaire 36

Figure 9 : Mutations de Met identifiées chez l'Homme 38

Figure 10 : Stratégies d'inhibition du couple HGF/SF-Met 42

Figure 11 : Illustrations de cellules apoptotiques 75

Figure 12 : Rôle de l'apoptose dans le développement du membre antérieur chez la souris 76

Figure 13 : Voies extrinsèque et intrinsèque de l'apoptose, ... 77

Figure 14 : Morphologie de la mitochondrie 79

Figure 15 : Composition du pore de perméabilité de transition 81

Figure 16 : La famille BCL2 82

Figure 17 : Modèles de l'activation de Bax et Bak par les BH3-only 84

Figure 18 : Structure tridimensionnelle du complexe Bcl-XL/Bad 84

Figure 19 : Modèle d'activation de Bax par une protéine BH3-only 85

Figure 20 : Formation de l'apoptosome 86

Figure 21 : Mécanismes d'action des facteurs mitochondriaux libérés pendant l'apoptose 88

Figure 22 : Activation des caspases initiatrices et effectrices de l'apoptose 89

Figure 23 : Spécificité des substrats protéiques des caspases 91

Figure 24 : Dualité fonctionnelle du récepteur Met 97

Figure 25: Contrôle de l'apoptose mitochondriale par le récepteur à dépendance Met 143

LISTE DES ABREVIATIONS

AIF : Apoptosis Inducing Factor
ADAM : A Disintegrin and metalloproteinase domain-containing protein
APAF-1 : Apoptotic Protease Activating Factor 1
ATP : Adenosine 5'-Triphosphate
Bad : BCL-2 Antagonist of cell Death
Bak : BCL-2-Antagonist/Killer
Bax : BCL-2-Associated X Protein
Bcl-XL : BCL-2 related Protein, Long Isoform
BH : BCL-2 Homology domain
Bid : BH3-Interacting-domain Death agonist
Bim : BCL-2-interacting mediator of cell death
CARD : Caspase Recruitment Domain
Caspase : Cysteinyl aspartic acid-protease
c-FLIP : cellular FLICE (FADD-like interleukin-1b-converting enzyme)-Inhibitory Protein
CBL : Casitas B-lineage Lymphoma
CTF: C-terminal Fragment
DAPK : Death-Associated Protein Kinase
DCC : Deleted in Colorectal Cancer
DED : Death Effector Domain
DISC : Death-Inducing Signalling Complex
EGFR : Epidermal Growth Factor Receptor
ERK : Extracellular signal-Regulated Kinase
FADD : Fas-Associated Death Domain
FasL : Fas Ligand
FGF : Fibroblast Growth Factor
GAB1 : Grb2-associated binder-1
GRB2 : Growth factor receptor-bound protein 2
HGF/SF : Hepatocyte growth factor/Scatter factor
HtrA2/Omi : High-temperature requirement A2/Omi stress-regulated endoprotéase
HIF1 : Hypoxia-inducible factor
IMM : Inner Mitochondrial Membrane
IAP : Inhibitor of Apoptosis Protein
MAPK : Mitogen-Activated Protein Kinase
MDCK: Madin-Darby Canine Kidney
MDM2 : Murine Double Minute 2
MSP : Macrophage Stimulating Protein
NGF : Nerve Growth Factor
NTF : N-Terminal Fragment
OMM : Outer Mitochondrial Membrane
PH : Pleckstrin Homology
PIP3 : Phosphatidylinositol (3,4,5)-trisphosphate
PLCγ : Phospholipase C-gamma
PI3-K : Phosphoinositide 3-kinase
PKC : Protein kinase C
PTB : Phosphotyrosine-Binding
Puma : p53 upregulated modulator of apoptosis
PS-RIP : Presenilin-regulated intramembrane proteolysis
RET : REarranged during Transfection

RON : Récepteur d'Origine Nantais
RTK : Receptor Tyrosine Kinase
SH2 : Src Homology 2
SH3 : Src Homology 3
SHC : Src homology 2 domain containing
SMAC/DIABLO : Second Mitochondria-derived Activator of Caspases/Direct IAP-Binding protein with LOw Pi
SOS : Son-of Sevenless
STAT3 : Signal transducer and activation of transcription
TKB : Tyrosine kinase binding
TNFα : Tumor Necrosis Factor alpha
TPR : Translocated promoter region
TRADD : TNF Receptor-Associated Death Domain
TRAIL : TNF related apoptosis-inducing ligand
VDAC : Voltage-Dependent Anion Channel
VEGF : Vascular Endothelial Growth Factor
XIAP : X-chromosome-linked inhibitor of apoptosis protein

INTRODUCTION

I) L'HGF est le ligand du Récepteur Tyrosine Kinase Met

A) Découverte de Met

Le Récepteur Tyrosine Kinase (RTK) Met a été initialement découvert en 1984 sous la forme oncogénique TPR-Met (Translocated Promoter Region) à partir de cellules HOS (Human osteosarcoma) traitées par un carcinogène chimique (Cooper *et al.*, 1984). Cette protéine chimérique est le résultat d'un réarrangement chromosomique entre un premier fragment de 10 kb du chromosome 1 et un second fragment de 9 kb du chromosome 7 (Park *et al.*, 1987). Le premier correspond à une séquence codant un domaine de dimérisation d'une nucléoporine appelée TPR (Translocated Promoter Region) (Rodrigues and Park, 1993). La séquence du second fragment possède une forte homologie avec celle du domaine intracellulaire des récepteurs de la famille des RTK (Park *et al.*, 1987). Ce nouveau récepteur est baptisé Met en référence à l'agent carcinogène utilisé le N-<u>Met</u>hyl-N'-nitro-N-nitroso-guanidine (MNNG) (Rhim *et al.*, 1975). Le domaine TPR induit une dimérisation permanente des protéines TPR-Met ce qui a pour conséquence d'activer Met de manière constitutive, et, de ce fait, la protéine est transformante (voir pour revue (Peschard and Park, 2007)).

Le gène humain *c-met* est localisé sur la région q21-31 du chromosome 7 et couvre 120 kb, répartis en 21 exons séparés par 20 introns (Lin *et al.*, 1998). La protéine Met fonctionnelle de 190 kDa est le résultat de la glycosylation d'un précurseur de 170kDa puis de son clivage en une sous-unité α de 50 kDa et d'une sous-unité β de 140 kDa qui restent associées par des ponts disulfures (Tempest *et al.*, 1988). La chaîne α, exclusivement extracellulaire, et les 500 premiers acides aminés de la chaîne β forment un domaine caractéristique de la sémaphorine, le domaine Sema (Winberg *et al.*, 1998). La cristallographie de ce domaine révèle une structure de celui-ci en 7 pales d'hélices composées de feuillets β. Il est immédiatement suivi d'un domaine riche en cystéines baptisé PSI car commun aux Plexines, Sémaphorines et Intégrines, et de quatre domaines IPT (domaine homologue aux immunoglobulines) noté 1 à 4 qui forment une structure longitudinale séparant le domaine Sema du domaine transmembranaire (Benvenuti and Comoglio, 2007). La région intracellulaire de la chaîne β porte le domaine tyrosine kinase. Sa structure bilobée avec une partie N-terminale constituée de feuillets β qui s'articulent sur une partie C-terminale composée d'hélices α est classique parmi les récepteurs tyrosine kinase (RTK) (Birchmeier *et al.*, 2003 ; Cristiani *et al.*, 2005; Wang *et al.*, 2006) (Figure 1).

Le récepteur Met

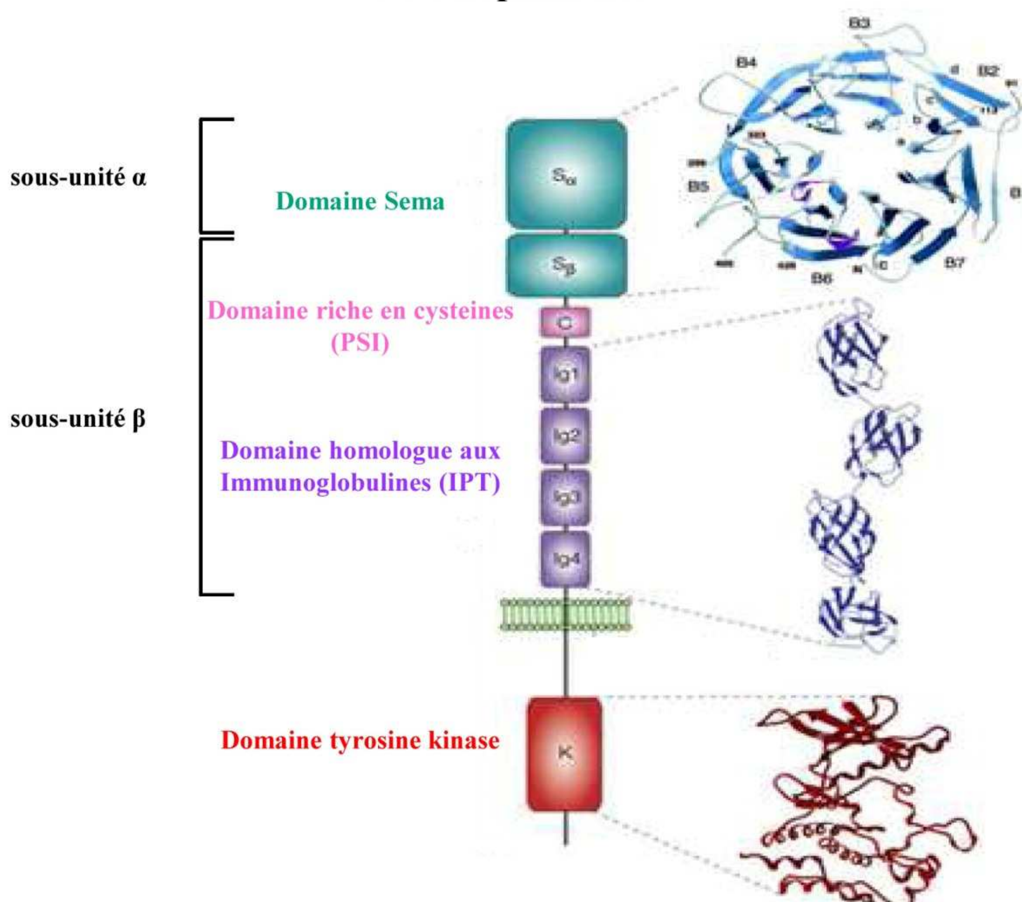


Figure 1 : Domaines et structures tridimensionnelles de Met

Structure du récepteur Met : S=domaine Sema, C=domaine riche en cystéines (PSI), Ig= domaine homologue aux immunoglobulines (=IPT), K=domaine kinase. α et β représentent les sous-unités du récepteur Met après maturation. D'après (Birchmeier et al., 2003).

B) L'HGF/SF : deux noms, un même facteur

La double dénomination du ligand de Met, HGF/SF, vient de la désignation du même facteur de croissance par deux équipes différentes. Il a tout d'abord été identifié comme un facteur capable d'induire la prolifération des hépatocytes de rat (« Hepatocyte Growth Factor ») (Birchmeier 2003, nakamura 1984). Trois ans plus tard, il est identifié comme un facteur sécrété par les fibroblastes embryonnaires capable d'induire la dispersion (« Scatter Factor ») des cellules épithéliales (Stoker *et al.*, 1987 ; Birchmeier *et al.*, 2003).

En 1991, une équipe a montré que ces deux facteurs étaient codés par la même séquence,qu'ils possédaient la même spécificité pour le récepteur Met et que les réponses biologiques qu'ils induisaient étaient interchangeables (Naldini *et al.*, 1991).

Le gène *hgf/sf* est situé sur la bande q21.1 du chromosome 7 et est constitué de 18 exons et de 17 introns (Seki *et al.*, 1991). L'HGF/SF est une glycoprotéine qui est sécrétée sous la forme d'un précurseur (pro-HGF). Il devient biologiquement actif après un clivage protéolytique unique libérant deux chaînes polypeptidiques (α et β) qui s'associent par un pont disulfure. L'activation de l'HGF/SF peut être assuré par différentes protéases comme la matriptase, l'activateur de l'HGF (HGF-A), l'hepsine ou le facteur XIIA (Owen *et al.*).

L'HGF/SF

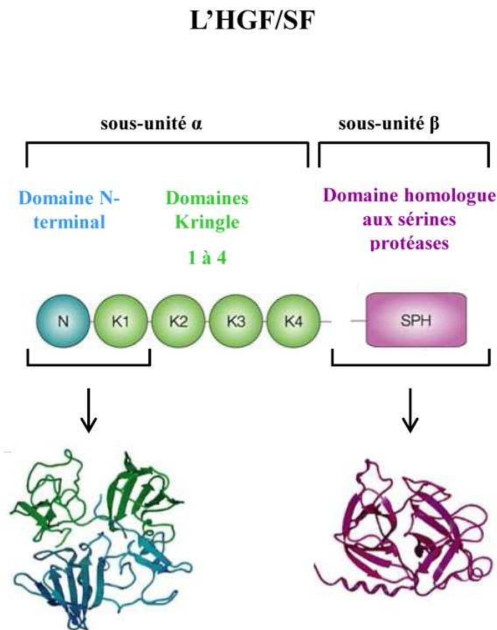


Figure 2 : Domaines et structures tridimensionnelles de l'HGF/SF

Structure de l'HGF/SF : N=domaine N-terminal, K1-4=domaines Kringle, SPH=domaine homologue aux sérines protéase. Structure cristallographique de NK1 et du domaine SPH.D'après (Birchmeier et al., 2003).

L'HGF/SF est composé d'un domaine N-terminal suivi de quatre domaines « kringle » (structure en triple boucles stabilisée par des ponts disulfures) et d'un domaine carboxy-terminal apparenté au domaine catalytique des protéases à sérines (SPH) mais dépourvu d'activité enzymatique (Birchmeier *et al.*, 2003). La sous-unité α couvre la région N-terminale et les 4 domaines « kringle ». Quant au domaine SPH, il constitue à lui seul la chaîne β (Figure 2).

C) Activation de Met par l'HGF/SF

En 1991, Met est identifié comme étant le récepteur à l'HGF/SF. En effet, suite à une stimulation à l'HGF/SF, une protéine phosphorylée de 145 kDa a été détectée puis identifiée

comme étant la sous-unité β du récepteur Met (Bottaro *et al.*, 1991). D'autres résultats ont montré que des cellules COS-7 exprimant le récepteur Met après transfection fixaient l'HGF/SF avec une haute affinité (constante de dissociation ou Kd = 30 pM), contrairement aux cellules non-transfectées (Higuchi *et al.*, 1992).

De nombreuses études ont été réalisées pour déterminer l'interaction entre ces deux partenaires et ont montré que les domaines Sema et IPT du récepteur Met constituent respectivement des sites de basse et de haute affinité pour la fixation du ligand (Basilico *et al.*, 2008). La sous-unité β de l'HGF/SF reconnaît une zone située entre les pales 2 et 3 du domaine Sema avec une faible affinité (*Kd* = 90 nM). Cette interaction permet la phosphorylation de Met malgré cette faible affinité (Kirchhofer *et al.*, 2004). La sous-unité α permet une interaction de forte affinité avec les domaines IPT 3 et 4 (Stamos *et al.*, 2004; Basilico *et al.*, 2008). La forme pro-HGF, tout comme la sous-unité α, possède une forte affinité (*Kd* : 0.2-0.3 nM) pour Met mais n'induit pas la phosphorylation du récepteur (Lokker *et al.*, 1992). De manière intéressante, aucune de ces trois formes (sous-unité α, sous-unité β, pro-HGF) n'induisent de réponses biologiques et seul l'HGF/SF mature, qui interagit à la fois avec le domaine PSI et le domaine Sema, est capable d'activer le récepteur et d'induire des réponses biologiques (Matsumoto *et al.*, 1998).

Met est capable de fixer une molécule d'HGF/SF. Cette fixation permet la dimérisation de 2 molécules d'HGF/SF entraînant la dimérisation des récepteurs (Figure 3) qui permet alors la trans-autophosphorylation des récepteurs sur des résidus tyrosines (Gherardi *et al.*, 2006).

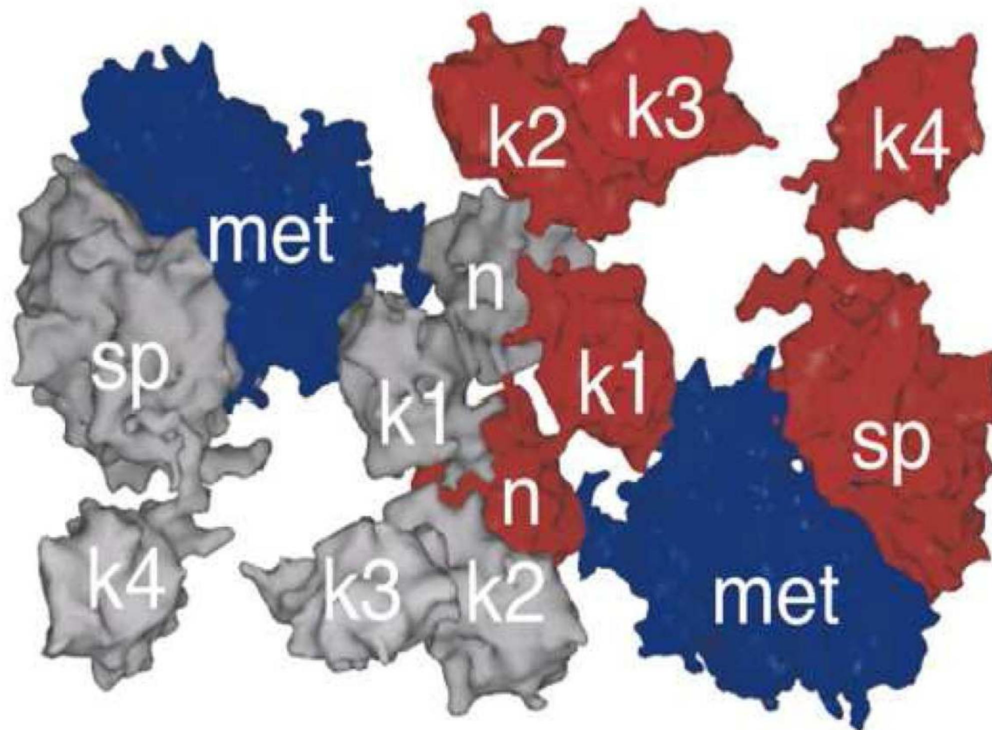


Figure 3 : Modélisation de l'interaction HGF/SF-Met

Le récepteur Met est capable de fixer une molécule d'HGF/SF formant ainsi des dimères. Ces dimères s'associent ensuite tête-bêche à travers une interaction entre chaque molécule d'HGF/SF via les domaines N-terminaux et Kringle 1.
sp : domaine d'homologie à la sérine protéase, k : domaine kringle, n : domaine N-terminal. D'après (Gherardi et al., 2006)

Met se phosphoryle sur les tyrosines 1234 et 1235 situées au niveau du domaine kinase. La phosphorylation de ces résidus permet une meilleure accessibilité de l'ATP à ce domaine et par conséquent participe à l'amplification de l'activité enzymatique (Ferracini *et al.*, 1991) (Naldini *et al.*, 1991). Ces résidus sont indispensables à l'activité catalytique du récepteur puisque leur mutation abolit toute activité du récepteur Met (Longati *et al.*, 1994). L'activation de Met permet à son tour la phosphorylation de la tyrosine 1003 située dans la dans la région juxtamembranaire mais aussi dans la région C-terminale avec la phosphorylation des tyrosines 1349 et 1356 (Ponzetto *et al.*, 1994). Les tyrosines C-terminales phosphorylées et les acides aminés environnants constituent alors un site d'interactions protéines-protéines qui intervient dans le recrutement de la majeure partie des protéines de signalisation activées par Met. Le recrutement de ces protéines est à la base de la transduction du signal intracellulaire qui mènera à l'élaboration d'une réponse biologique. Depuis, il a été montré que d'autres tyrosines (Y1313, Y1365) pouvaient être phosphorylées et impliquées

dans les réponses biologiques induites par Met (Maulik *et al.*, 2002 ; Cristiani *et al.*, 2005). Dans le même temps, la liaison de l'HGF/SF par Met induit sa phosphorylation sur le résidu tyrosine 1003 situé au niveau du domaine juxtamembranaire. Ce résidu phosphorylé recrute alors la protéine c-CBL, une E3 ubiquitine ligase connue pour être impliquée dans la dégradation de nombreux RTK (Weidner *et al.*, 1995). La protéine c-CBL est à l'origine de l'ubiquitination de Met et permet son internalisation ainsi que son adressage vers les voies de dégradation (Schmidt and Dikic, 2005). Le domaine juxtamembranaire intervient donc directement dans la régulation négative du récepteur et dans le contrôle des réponses biologiques induites par le ligand tandis que le domaine C-terminal de Met intervient dans l'initiation des réponses biologiques.

II) Implications physiologiques du couple HGF/SF-Met

A) Au cours de l'embryogenèse

Au cours de l'embryogenèse, l'HGF/SF et Met sont exprimés de manière précoce et conjointement dans l'endoderme ainsi que dans une partie de la ligne primitive du mésoderme. Après la gastrulation, processus qui fait intervenir plusieurs mouvements cellulaires permettant la mise en place des différents feuillets embryonnaires, leur co-expression perdure dans la corde et la notocorde. Il a été proposé que l'action autocrine de l'HGF/SF sur Met intervient dans ces mouvements cellulaires. Au cours de l'organogenèse, leur expression concomitante persiste dans le cœur, les somites et les cellules des crêtes neurales avant de se dichotomiser (Andermarcher *et al.*, 1996). L'HGF/SF est alors exprimé dans les tissus mésenchymateux tandis que Met est retrouvé dans les tissus épithéliaux. Cette particularité suggère un mode d'action paracrine et un rôle important de ce couple dans les interactions épithélium-mésenchyme au cours de l'organogenèse (Andermarcher *et al.*, 1996; Wilhelmsen *et al.*, 2006).

Afin de déterminer le niveau d'implication de ce couple dans le développement, des invalidations de *met* ou de l'*hgf/sf* par knock out (KO) ont été réalisées chez des souris. L'extinction de l'un ou l'autre de ces gènes entraîne la mort *in utero* des embryons dès le 15ème jour de développement (E15), démontrant leur rôle essentiel au cours du développement 4). Ces embryons présentent alors des phénotypes tout à fait similaires, ce qui confirme le lien fonctionnel entre le récepteur et son ligand. Les embryons présentent un défaut d'organisation

du trophoblaste labyrinthique du placenta, réduisant ainsi les échanges materno-fœtaux qui serait la cause de leur mort *in utero* (Uehara *et al.*, 1995; Birchmeier and Gherardi, 1998). Ces embryons présentent également une réduction de la taille du foie, liée à un défaut de prolifération des hépatocytes et à une apoptose accrue dans cet organe (Bladt *et al.*, 1995 ; Schmidt *et al.*, 1995).

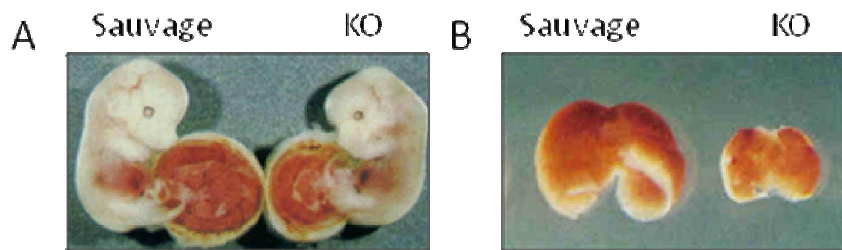


Figure 4 : Phénotypes des souris sauvages et KO HGF/SF ou Met $^{-/-}$
A) Apparence des embryons de souris sauvages (à gauche) et HGF/SF $^{-/-}$ (à droite) à E13.5 montrant les défauts de formations du plancenta (Uehara et al., 1995).
B) Apparence des foies d'embryons de souris sauvages (à gauche) et HGF/SF $^{-/-}$ (à droite) à E14.5, (Schmidt et al., 1995).

Les études phénotypiques ont également révélé une absence de muscles squelettiques dans les bourgeons des membres chez les individus KO *met* ou *hgf*. Ces défauts sont liés à un défaut de migration des précurseurs myoblastiques. Ces précurseurs expriment habituellement Met sous l'action du facteur de transcription Pax3 et migrent vers les bourgeons de membre où l'HGF/SF est exprimé soulignant ainsi l'action chémo-attractive du ligand (Figure 5) (Bladt *et al.*, 1995 ; Epstein *et al.*, 1996 ; Birchmeier *et al.*, 2003).

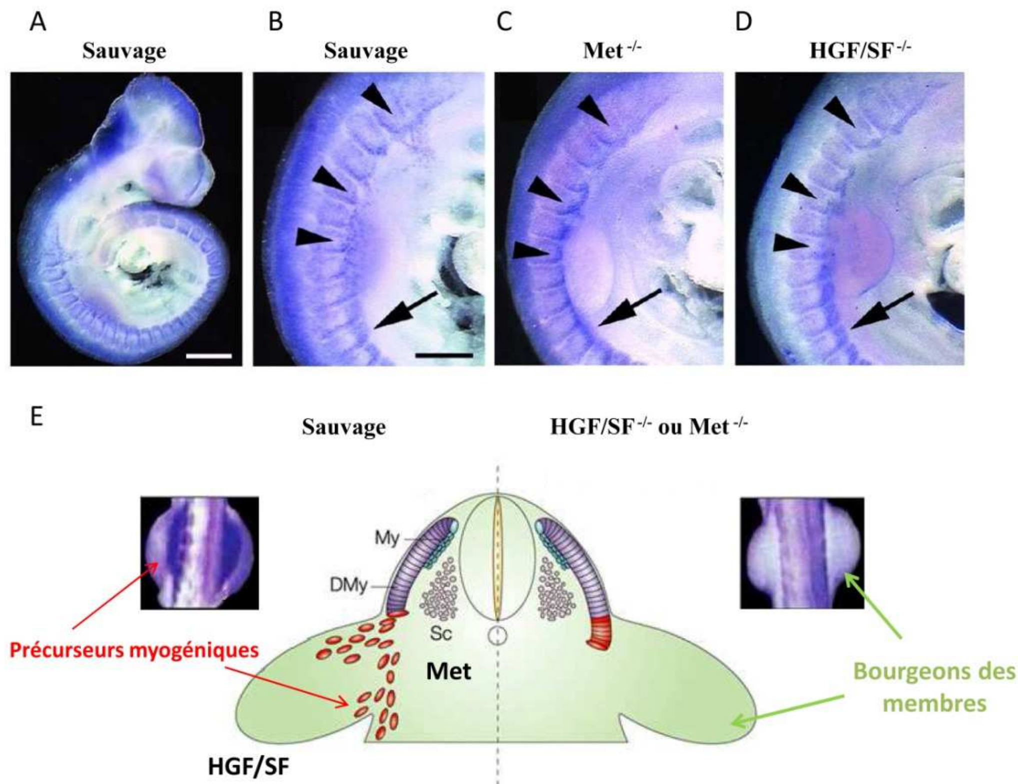


Figure 5 : Effet du KO HGF/SF ou Met $^{-/-}$ dans le développement des muscles
(A, B, C, D) Hybridation in situ de Pax3 sur des embryons in toto de souris à E10, Barre d'échelle (A) 500 µm, (B) 300 µm.
(E) Schéma des bourgeons des membres et détection des précurseurs myogéniques chez des embryons E9,5. My :Myotome, Dmy : Dermomyotme, Sc : Sclérotome
D'après (Epstein et al., 1996; Birchmeier et al., 2003).

Le couple HGF/SF-Met agit également au niveau du système nerveux. L'expression de Met et de l'HGF/SF est détectée au cours du développement du cerveau et persiste à l'âge l'adulte. Met est également exprimé dans de nombreuses structures du système nerveux à différents stades du développement. Ainsi, on observe son expression dans les cellules des crêtes neurales, les motoneurones de la moelle épinière et les ganglions de la racine dorsale (Sonnenberg *et al.*, 1993; Jung *et al.*, 1994; Andermarcher *et al.*, 1996; Yamamoto *et al.*, 1997).

In vitro, l'HGF/SF est capable d'induire la croissance des neurites à partir de neurones issus d'explants du néocortex. En plus de son rôle de facteur de croissance, il est également capable d'orienter la trajectoire des axones issus des motoneurones et agit donc sur les cellules comme facteur chimioattractant (Ebens *et al.*, 1996; Hamanoue *et al.*, 1996). De plus, *in vitro,* l'HGF/SF induit la survie et la prolifération des neuroblastes mais aussi leur différenciation. Ainsi, l'utilisation d'anticorps dirigés contre l'HGF/SF réduit le nombre de

neurones sympathiques issus de la différentiation des neuroblastes tandis que l'invalidation de *met* dans ces cellules provoque une apoptose massive (Maina *et al.*, 1998).

De nombreuses études ont été réalisées *in vivo* afin de mieux comprendre le rôle de ce couple dans la neurogenèse. Ainsi, à la suite d'un knock in (KI) de *met,* où le gène sauvage est remplacé par une version codant un récepteur dépourvu d'activité kinase, les embryons présentent des défauts dans l'innervation sensorielle des muscles des membres (Maina *et al.*, 1997). D'autres études ont utilisé la stratégie du KO conditionnel afin d'étudier le rôle de Met. Le gène d'intérêt est alors flanqué par des séquences de recombinaison de l'ADN (Lox) tandis que l'expression de l'enzyme capable de recombiner ces sites, la recombinase Cre, est sous le contrôle d'un promoteur spécifique. Ainsi, en utilisant le promoteur de la nestine, une protéine exprimée spécifiquement dans les cellules nerveuse, une équipe a pu réaliser le KO conditionnel de Met dans les cellules neuroépithéliales. Ce KO conditionnel entraîne des défauts dans la survie d'une sous-catégorie de motoneurones qui interviennent dans l'innervation du petit pectoral ou le maintien de l'expression du facteur de transcription *runx1* et souligne l'importance de la signalisation HGF/SF-Met dans ces processus (Lamballe *et al.*). Enfin au niveau du cerveau, le KO inductible du récepteur Met empêche la migration des neurones dépendants de GABA (Gamma-AminoButyric Acid) dans l'hippocampe (Martins *et al.*, 2007).

B) Chez l'adulte

Chez l'adulte, le couple HGF/SF-Met joue un rôle majeur dans la régénération de nombreux organes. Ainsi, lors de la régénération du foie de souris après hépatectomie, l'extinction de Met provoque un délai de régénération plus long ce qui corrèle avec une diminution des capacités de prolifération des hépatocytes (Borowiak *et al.*, 2004; Huh *et al.*, 2004 ; Phaneuf *et al.*, 2004). D'autres organes bénéficient également de l'effet régénérateur de la signalisation HGF/SF-Met. Ainsi, chez la souris, l'ablation partielle du rein ou l'induction de lésions rénales aigües par l'administration de chlorure de mercure en intraveineuse, corrèle avec une augmentation du taux d'HGF/SF, suggérant une implication du couple HGF/SF-Met dans la régénération de ces organes. *In vivo*, l'injection en intraveineuse d'HGF/SF accélère la régénération rénale et la réparation des lésions tissulaires (Kawaida *et al.*, 1994). De la même façon, on observe une augmentation du taux d'HGF/SF après un infarctus du myocarde, des blessures pulmonaires ou une section de la moelle

épinière (Ohmichi *et al.*, 1996 ; Jin *et al.*, 2003 ; Shimamura *et al.*, 2007), tandis que l'administration d'HGF/SF accélère leur régéneration. Le couple est également impliqué dans le processus de cicatrisation. En effet, après une blessure cutanée, le taux d'HGF/SF augmente et permet aux cellules exprimant Met de migrer et de proliférer sur le site de la blessure. Ainsi, à la suite d'un KO conditionnel de *met* obtenu au niveau kératinocytes (l'expression de la recombinase est alors sous le contrôle du promoteur de la kératine 14), on observe une inhibition du processus de cicatrisation au niveau de l'épiderme (Chmielowiec *et al.*, 2007). Enfin, en période de gestation, le niveau d'expression de l'HGF et de Met augmente au niveau de la glande mammaire pendant la période de tubulogenèse, suggérant que ce couple régule également la morphogénèse de branchement. En revanche, leurs expressions diminuent après la grossesse, ce qui coïncide avec la période de lactation lorsque démarre la phase de différenciation cellulaire mammaire (Pepper *et al.*, 1995; Soriano *et al.*, 1998). Plus récemment, il a été montré que les cellules à caractère souche situées dans la zone sous ventriculaire du cerveau (une des zones connues où persiste la neurogenèse chez l'adulte) exprime le récepteur Met. *In vivo,* l'administration d'HGF par injection intracérébroventriculaire entraine une prolifération des cellules souches mesurables à l'aide d'un marquage à la BrdU. Ces mêmes cellules, isolées et cultivées après l'injection d'HGF/SF, prolifèrent et sont capables de former des amas appelés neurosphères. De plus, par l'utilisation d'anticorps capables de neutraliser l'HGF/SF, les auteurs montrent que l'HGF agit de manière endogène puisqu'ils observent une diminution d'incorporation de la BrdU par les cellules de la SVZ (zone sous-ventriculaire) par rapport à la condition contrôle (injection d'anticorps aspécifiques) ce qui est synonyme d'une baisse de la prolifération de ces cellules (Nicoleau *et al.*, 2009). Ces travaux ouvrent ainsi des perspectives sur des stratégies thérapeutiques basées sur l'utilisation de ces cellules.

III) Signalisation du couple HGF/SF-Met

A) Un couple ligand-récepteur, de nombreuses réponses cellulaires

Activé par son ligand, le récepteur Met est capable d'induire de nombreuses réponses biologiques dans des cultures cellulaires. Ainsi, l'HGF/SF est un puissant mitogène pour les cellules hépatiques (Nakamura *et al.*, 1984) mais également un facteur capable d'induire la dispersion de nombreux types cellulaires (Maulik *et al.*, 2002). Ces différents mécanismes ont d'ailleurs été à l'origine de la découverte de l'HGF/SF. Le ligand permet aussi la mise en

place d'une réponse biologique plus complexe comme la morphogenèse lorsque les cellules épithéliales sont cultivées en présence de composants de la matrice extracellulaire. Ainsi, des cellules épithéliales cultivées dans un gel de collagène s'organisent sous forme de kystes et forment des branchements au sein du gel en réponse à l'addition de l'HGF/SF (Montesano *et al.*, 1991). L'HGF/SF est alors le premier facteur de croissance identifié capable d'induire cette croissance en trois dimensions. De la même façon, de nombreuses lignées cellulaires sous l'effet de l'HGF/SF sont capables de reproduire *in vitro* des structures proches de leur tissu d'origine (Brinkmann *et al.*, 1995).

L'HGF/SF protège également les cellules de l'apoptose induite par une déplétion en sérum et/ou des inducteurs d'apoptose (TNF-α, cis-platine…) (Kosai *et al.*, 1998 ; Yo *et al.*, 1998). Cependant, malgré le rôle de survie largement illustré dans la littérature, des fonctions pro-apoptotiques et cytotoxiques du couple HGF/SF-Met ont été décrites dans plusieurs lignées cellulaires transformées (Tajima *et al.*, 1991), notamment la lignée de sarcome-180 où le couple HGF/SF-Met est capable d'activer la caspase-3 (Arakaki *et al.*, 1998). Les travaux portant sur ces fonctions ont d'ailleurs été à l'origine de l'identification d'un facteur baptisé F-TCF (pour Fibroblast-derived Tumor Cytotoxic Factor) qui n'était autre que l'HGF/SF (Higashio *et al.*, 1990; Shima *et al.*, 1991a ; Shima *et al.*, 1991b).

Pour conclure, les réponses cellulaires observées *in vitro* sont cohérentes avec les rôles du couple HGF/SF-Met observés *in vivo.*

B) La signalisation de Met

Nous l'avons vu précédemment, la mise en place d'une réponse biologique contrôlée par Met nécessite sa trans-autophosphorylation sur des résidus tyrosines. Ces résidus et les acides aminés environnants vont alors constituer des sites de recrutement reconnus par des protéines portant des domaines spécifiques SH2 (Src Homology 2), SH3 (Src Homology 3) ou PTB (PhosphoTyrosine Binding) (Figure 6). En effet, des souris exprimant par knock-in une séquence codant Met dont les tyrosines C-terminales ont été remplacées par des résidus non phosphorylables présentent des défauts similaires aux souris n'exprimant pas le récepteur, preuve du rôle majeur de ce site de recrutement (Maina *et al.*, 1997). Pour autant, toutes les réponses ne sont pas abolies puisqu'il a été montré que des récepteurs mutés sur ces tyrosines étaient capables d'induire des réponses de dispersion, suggérant que des signaux pouvaient être transmis indépendamment de la présence d'un site de recrutement multisubstrat fonctionnel (Tulasne *et al.*, 1999).

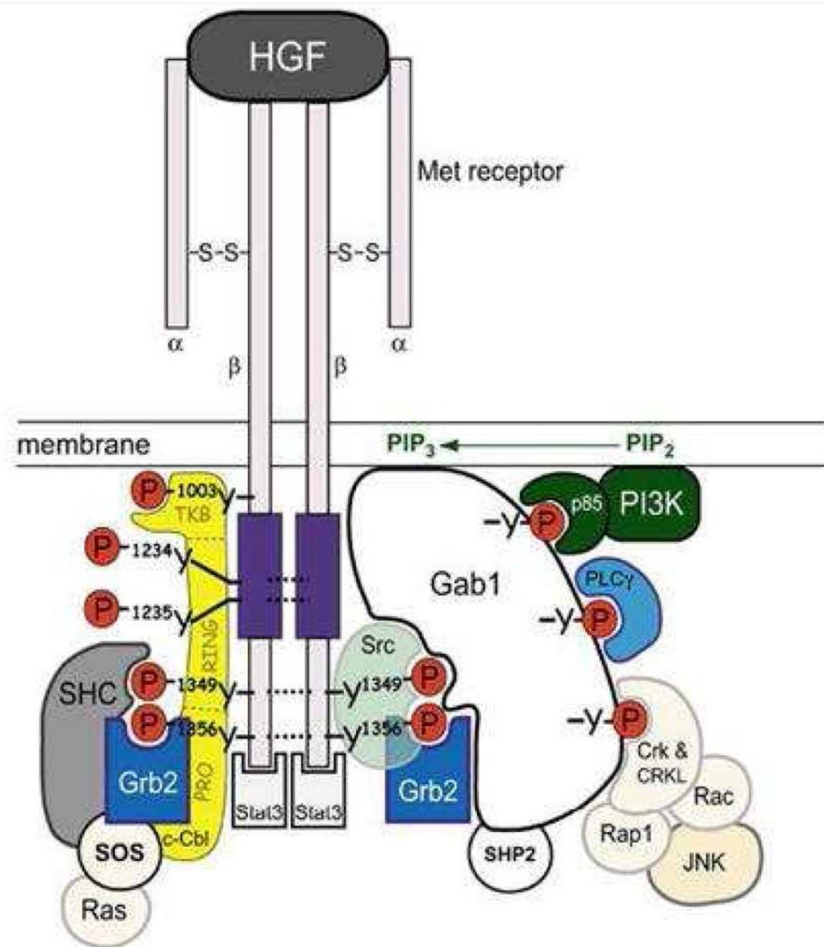


Figure 6 : Partenaires recrutés par le récepteur Met activé par l'HGF/SF
D'après (Hammond et al., 2004)

Met recrute un nombre important de protéines qui initient les voies de signalisation permettant l'intégration du signal perçu par le récepteur. Parmi ces recrutements, on distingue les recrutements directs de protéines effectrices comme celui de la protéine PI3-K (Phosphatidyl Inositol 3 Kinase) ou le recrutement indirect par l'intermédiaire de protéines adaptatrices comme GAB1 (Grb2 Associated Binding 1) ou Grb2 (Growth factor Receptor-Bound 2). Ces adapatateurs, contrairement à la PI3-K, ne possèdent pas d'activité enzymatique et sont capables de participer au recrutement d'autres partenaires protéiques impliqués dans la signalisation de Met. Enfin, Met peut également recruter le facteur de transcription STAT 3 (Signal Transducers and Activator of Transcription 3) et réguler ainsi de manière directe l'expression de gènes cibles. Ces partenaires sont retrouvés de manière récurrente et vont servir d'exemple pour détailler l'importance des différentes voies de signalisation participant à la mise en place des réponses biologiques induites par le couple HGF/SF-Met.

1) GAB1

GAB1 est une protéine adaptatrice impliquée dans la signalisation de Met. Elle interagit directement avec la phospho-tyrosine 1349 de Met par son domaine MBD (Met Binding Domain). GAB1 est également recrutée par la tyrosine 1356 *via* une interaction avec Grb2. Ce recrutement indirect semble prépondérant puisque la mutation dans Gbr2 du site de liaison à Met réduit fortement le recrutement de GAB1 (Bardelli *et al.*, 1997; Fixman *et al.*, 1997). GAB1 possède également un domaine PH (Pleckstrin Homology) lui permettant d'interagir à la membrane avec les PIP3 (phosphatidylinositol 3, 4, 5 triphosphate) qui sont produits par la PI3-K, recrutée elle aussi par Met (Rodrigues *et al.*, 2000). L'inhibition du recrutement de GAB1 à la membrane réduit fortement les réponses de morphogenèse. La localisation membranaire intervient donc directement dans la signalisation du récepteur Met (Maroun *et al.*, 1999a; Maroun *et al.*, 1999b). Le phénotype des souris GAB1$^{-/-}$, qui est très proche de celui des souris Met$^{-/-}$, témoigne du rôle majeur de cette protéine dans la signalisation de Met. Arrimée à Met, GAB1 est phoshorylée et peut alors servir de plateforme de recrutement aux protéines de signalisation telles que PLCγ (PhosphoLipase Cγ), SHP2 (Src Homology 2-containing tyrosine Phosphatase) ou encore PI3-K (Lock *et al.*, 2000 ; Schaeper *et al.*, 2000; Lock *et al.*, 2003) induisant certaines réponses biologiques. Ainsi, Src est plutôt associée à la croissance sans ancrage (Rahimi *et al.*, 1998), SHC (Src Homology 2 domain-Containing) à l'angiogenèse (Saucier *et al.*, 2004), PLCγ (PhosphoLipase Cγ) à la morphogenèse (Gual *et al.*, 2000).

2) Grb2

Grb2 est recrutée par Met et possède également un rôle d'adaptateur. La tyrosine 1356 phosphorylée de Met constitue un site de recrutement direct pour la protéine Grb2 qui peut également être recrutée indirectement *via* la protéine SHC (Pelicci *et al.*, 1995). Le recrutement de Grb2 associée à l'échangeur de guanidine SOS (Sons Of the Sevenless), va permettre l'activation de la GTPase membranaire Ras en catalysant le remplacement du GDP en GTP (Li *et al.*, 1993 ; Ponzetto *et al.*, 1994). La forme active Ras-GTP active alors Raf, une sérine/thréonine kinase qui active MEK1 (MAP/ERK kinase 1) qui active à son tour ERK (Extracellular signal Regulated Kinase). Cette activation en cascade constitue la voie des MAPK (Mitogen-Activated Protein Kinase). ERK activée constitue alors un centre à partir duquel rayonnent différentes voies, impliquées entre autres dans le réarrangement du

cytosquelette et l'activation des facteurs de transcription ETS-1 (E Twenty-Six 1) et c-Fos (Furge *et al.*, 2000 ; Tulasne *et al.*, 2002).

L'importance de cette voie de signalisation a été démontrée par l'utilisation de souris KI exprimant un récepteur Met incapable de recruter Grb2. Ces souris sont viables jusqu'à la naissance. Elles ne présentent pas de malformation au niveau du placenta contrairement aux souris KO pour Met ou pour l'HGF/SF. Cependant, ces souris meurent après leur naissance, probablement par un défaut respiratoire (aspect cyanosé des embyrons) et présentent notamment une insuffisance musculaire au niveau des membres. Contrairement au KO de *met*, ce KI n'affecte pas la migration des précurseurs myoblastiques mais plutôt leur prolifération (Maina *et al.*, 1996). Ces résultats rejoignent d'autres résultats obtenus *in vitro* puisqu'une version de Met mutée, toujours capable de lier les protéines comme SHC, ou PLCγ mais incapable de recruter Grb2, ne modifie pas la migration induite par l'HGF/SF (Ponzetto *et al.*, 1996).

3) La PI3-K

La protéine PI3-K est composée d'une sous-unité qui porte l'activité catalytique et d'une seconde spécialisée dans l'interaction avec les deux tyrosines du domaine de recrutement multisubstrat de Met (Vanhaesebroeck *et al.*, 2005). Une fois complexée à Met, la PI3-K produit le PIP3, un second messager lipidique localisé à la membrane. Le PIP3 permet alors le recrutement de la protéine Akt à la membrane pour y être activée par la kinase membranaire PDPK1 (3-PhosphoInositide Dependent Protein Kinase-1). Akt est alors capable de phosphoryler de nombreux effecteurs sur des résidus sérines et thréonines, comme la protéine BAD (Bcl-2-Associated Death promoter) ou la pro-caspase-9 qui perdent alors leurs capacités pro-apoptotiques (Fresno Vara *et al.*, 2004).

4) STAT3

Suite à son activation par l'HGF/SF, Met est ubiquitinylé et internalisé. L'internalisation de Met est un mécanisme connu pour initier la voie de dégradation. Ce même mécanisme intervient également dans la signalisation contrôlée par Met. Ainsi, l'internalisation de Met activé entraîne sa colocalisation avec le facteur de transcription STAT3, induit sa phosphorylation puis la translocation de la forme active de STAT3 dans le noyau (Boccaccio *et al.*, 1998; Kermorgant and Parker, 2008). STAT3 est alors capable

d'induire la transcription de différents gènes cibles dans le compartiment nucléaire (Levy and Darnell, 2002). Bien que les gènes cibles contrôlé par STAT3 en réponse à une stimulation à l'HGF/SF soient méconnus, les études ont déterminé qu'il intervenait dans les réponses de morphogenèse (Zhang *et al.*, 2002).

5) Cibles transcriptionnelles

Il a été montré que les réponses induites par Met nécessitent la néo-synthèse de protéines puisque l'inhibition de la traduction protéique par l'utilisation de cycloheximide sur des MDCK (Madin-Darby Canine Kidney) bloque la dispersion induite par l'HGF/SF (Ridley *et al.*, 1995). En effet, Met contrôle ses réponses biologiques en régulant au niveau transcriptionnel l'expression de nombreux acteurs de signalisation directement impliqués dans le mécanisme de dispersion et de migration. Ainsi, l'HGF/SF peut activer l'expression de diverses protéases comme MMP 1 et 9, ainsi que l'UPA (Urokinase-type Plasminogen Activator) qui sont capables de dégrader les composants de la matrice extracellulaire (Fafeur *et al.*, 1997; Hanzawa *et al.*, 2000). Des études montrent également que la dispersion induite par l'HGF/SF est dépendante de l'expression des intégrines $\alpha 2$ et $\alpha 3$ qui corrèle avec l'activation de la protéine ERK et sa translocation dans le noyau (Liang and Chen, 2001).

Enfin, l'HGF/SF protège également de l'apoptose par différents mécanismes. Il a été observé que l'HGF/SF active l'expression des IAP (X-chromosome-linked inhibitor of apoptosis protein), des inhibiteurs des caspases et de la protéine Mdm 2, une ubiquitine ligase impliquée dans la dégradation de la protéine p53, facteur de transcription aux propriétés apoptotiques (Yamamoto *et al.*, 2001; Moumen *et al.*, 2007).

C) Les partenaires membranaires de la signalisation de Met

L'induction des réponses biologiques nécessite le recrutement de nombreux partenaires intracellulaires. D'autres études montrent également que les réponses biologiques induites par Met dépendent également de co-récepteurs situés à la membrane plasmique (Figure 7).

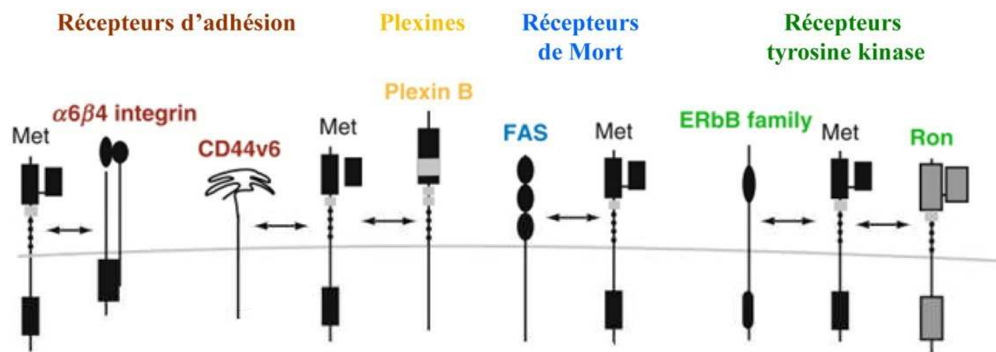


Figure 7 : Les différents partenaires membranaires du récepteur Met
D'après (Hanna et al., 2009)

1) Les intégrines

Les intégrines constituent une superfamille qui participe à l'adhésion des cellules aux composants de la matrice extracellulaire, favorisant ainsi leur survie et leur prolifération. Elles sont également à l'interface entre le cytosquelette et la matrice extracellulaire (Wilhelmsen *et al.*, 2006). Ces protéines sont composées d'une sous-unité α et une sous-unité β. A ce jour, les 18 sous-unités α et β connues peuvent s'associer et former 24 dimères distincts. La complémentarité de leurs domaines extracellulaires permet la spécificité au ligand. De par cette spécificité, les intégrines ont été classées en différents groupes : les intégrines à laminine, les intégrines à collagène, les intégrines leucocytaires et les intégrines reconnaissant des motifs protéiques RGD (Arginine-Glycine-Aspartate) (Takada *et al.*, 2007). Ces hétérodimères interviennent également dans les liaisons cellule-cellule puisque l'intégrine α6β4 participe majoritairement à l'architecture des hémidesmosomes (van der Neut *et al.*, 1996). En accord avec ses fonctions motogènes, l'HGF/SF induit la phosphorylation de la sous-unité β, déstabilisant ainsi les hémidesmosomes et prévenant leur formation. Cette phosphorylation convertit l'intégrine α6β4 en une plateforme de signalisation capable de recruter les protéines de signalisation PI3-K et SHC. Ces recrutements initient alors des voies de signalisation qui aboutissent à des réponses de migration (Trusolino *et al.*, 2001).

De la même façon, il a été montré que l'integrine α3β1 et Met s'associent physiquement. En réponse à l'HGF, ce complexe permet de recruter les protéines de signalisation GAB1 et PI3-K. Cette association permet alors le contrôle de la voie Wnt qui intervient dans la tubulogenèse rénale et la survie cellulaire. Les explants de papilles rénales

issus de souris KO pour l'integrine $\alpha3\beta1$ présentent alors un défaut de croissance qui corrèle avec une chute d'expression des gènes contrôlés par la voie Wnt. Ainsi, Met peut coopérer avec les intégrines pour médier des fonctions biologiques (Liu *et al.*, 2009).

Met peut également être activé par les intégrines. En effet, après stimulation de l'intégrine $\alpha5\beta1$ par son ligand, la fibronectine (un composant de la matrice extracellulaire), Met est activé en absence d'HGF/SF par la sous-unité $\alpha5$. Ce mécanisme d'activation est étroitement lié au processus de métastatisation dans un modèle cancereux de l'ovaire puisque l'utilisation d'anticorps dirigés contre la sous-unité α de l'intégrine $\alpha5\beta1$ contrecarre l'activation de Met en absence d'HGF/SF dans ces cancers ainsi que le développement de métastases (Mitra *et al.*, 2003).

2) CD44

CD44 regroupe les nombreuses isoformes d'une protéine transmembranaire qui peuvent résulter de l'épissage alternatif de 20 exons. CD44 a été localisé pour la première fois à surface des lymphocytes T et sa première fonction décrite est celle de récepteur à l'acide hyaluronique (Toole, 1990). Ensuite, il a été montré que CD44 est capable de fixer des facteurs de croissance tels que le FGF-2 (Fibroblast Growth Factor-2), HB-EGF (Human Heparin Binding *Epidermal* Growth Factor) et l'HGF/SF (Bennett *et al.*, 1995 ; Jackson *et al.*, 1995 ; van der Voort *et al.*, 1999).

Cette capacité de fixation de l'HGF intervient dans la signalisation HGF/SF-Met puisqu'elle favorise la phosphorylation de Met et par conséquent l'activation de la voie de signalisation MAPK (van der Voort *et al.*, 1999). Il a été proposé que le CD44 permettait de faciliter la présentation de l'HGF/SF au récepteur Met. L'isoforme CD44v6, qui possède spécifiquement l'exon 11, favorise quant à lui la signalisation de Met en deux étapes. Par son domaine extracellulaire, il favorise l'activation entre l'HGF/SF et Met au sein d'un complexe ternaire et l'utilisation d'anticorps dirigés contre CD44v6 bloque alors cette activation. Enfin, par son domaine intracellulaire, CD44v6 recrute les protéines de la famille ERM (Ezrine, Radixine et Moesine) qui s'associent à l'actine du cytosquelette et favorisent l'activation de la protéine Ras et de la voie des MAPK (Orian-Rousseau *et al.*, 2002; Orian-Rousseau *et al.*, 2007).

Des souris KO pour CD44 ont été développées afin de démontrer ce rôle de co-récepteur de Met dans un contexte *in vivo.* Malgré l'importance du CD44 dans les réponses

induites *in vitro* par l'HGF/SF, les souris CD44$^{-/-}$ sont viables et ne présentent aucun des défauts observés dans les souris Met$^{-/-}$. Ce phénotype a permis aux auteurs de suggérer l'existence d'un mécanisme de compensation pour pallier l'absence de CD44. Dans les cellules issues des souris CD44$^{-/-}$, la protéine ICAM-1 est capable d'interagir avec le récepteur Met, de plus cette interaction intervient dans l'activation de Met en réponse à l'HGF/SF. Il a donc été proposé qu'ICAM-1 pouvait compenser le déficit de la protéine CD44 en jouant le rôle de co-récepteur de Met et ce exclusivement dans les souris CD44$^{-/-}$ (Olaku *et al.*).

3) Les plexines/sémaphorines

Nous l'avons vu précédemment, le récepteur Met possède dans sa région extracellulaire un domaine Sema qui partage une forte homologie de séquence avec celui des sémaphorines et des plexines. Les sémaphorines constituent une famille de protéines secrétées ou associées à la membrane. Les plexines, une famille de protéines transmembranaires, sont les récepteurs aux sémaphorines. Elles s'associent par leur domaine Sema et sont impliquées dans la motilité, la guidance des axones ou l'angiogenèse (Kruger *et al.*, 2005). De par leurs homologies pour ce domaine, trois membres des plexines type B sont ainsi capables d'interagir avec Met (Conrotto *et al.*, 2004). En absence de leur ligand respectif, il a été montré que Met s'associe avec la plexine B1. La fixation de la sémaphorine 4D par la plexine B1 entraîne l'activation de Met et leur phosphorylation respective. Cette activation en relais de Met par la plexine B1 est même nécessaire pour la réponse biologique en réponse à la sémaphorine 4D (Giordano *et al.*, 2002 ; Artigiani *et al.*, 2004). De même, il a été montré que l'effet angiogénique de la sémaphorine 5A, qui comprend la migration des cellules endothéliales, est sous le contrôle de Met. (Sadanandam *et al.*). Pourtant, plus récemment, des études dans des mélanomes qui surexpriment Met ont montré que la surexpression de la plexine B1 abolit les réponses biologiques induites par l'HGF/SF. Cette activité de suppresseur de tumeurs portée par la plexine B1 serait liée à sa capacité à interagir avec Met, ce qui entraînerait une diminution de l'affinité de Met pour l'HGF/SF (Stevens *et al.*).

4) Les récepteurs tyrosine kinase

Met dialogue également avec plusieurs récepteurs membranaires à activité tyrosine kinase comme la famille des récepteurs à l'EGF 1et 3 (Epidermal Growth Factor), le récepteur VEGFR (Vascular Endothelial Growth Factor) et Ron, un homologue de Met (Récepteur d'Origine Nantais). Ainsi, il a été observé qu'une stimulation à l'HGF/SF pouvait activer les récepteur RON et EGFR. Inversement, on observe une activation de Met suite à stimulation au MSP (Macrophage Stimulating Protein), le ligand du récepteur RON, ou par le GDNF (glial cell derived neurotrophic factor), le ligand du récepteur RET (REarranged during Transfection) (Follenzi *et al.*, 2000 ; Popsueva *et al.*, 2003; Spix *et al.*, 2007 ; Ishibe *et al.*, 2009). Ces interactions sont importantes dans des processus physiologiques comme le développement du rein puisque l'inhibition de Met ou l'EGFR entraîne un défaut de formation de cet organe. Ces mêmes coopérations ont été retrouvées dans plusieurs cancers. Ainsi, dans les cancers gastriques où Met est surexprimé suite à une amplification génique, on observe une phoshorylation basale de EGFR3 et de l'EGFR qui peut être inhibée par un inhibiteur spécificique de l'activité de Met (Bachleitner-Hofmann *et al.*, 2008). Certaines études ont montré que Met et l'EGFR pouvait former des complexes hétéro-dimériques, suggérant une activation directe des deux entités (Jo *et al.*, 2000 ; Guo *et al.*, 2008). Cependant, d'autres données suggèrent plutôt un mécanisme d'activation indirecte par l'intermédiaire de Src. Activée par Met, Src pourrait alors phosphoryler l'EGFR ce qui conduirait à son activation (Mueller *et al.*, 2008).

5) Fas R

Met est également capable de s'associer avec le récepteur Fas (Fas-R) par son domaine extracellulaire. En présence de son ligand, le Fas-Ligand (Fas-L), Fas-R se trimérise et permet l'activation de caspases initiatrices qui mèneront à la mort cellulaire par apoptose. Cette association FasR-Met permet de séquestrer le récepteur de mort et l'empêche d'interagir avec son ligand, entraînant ainsi une résistance des cellules à la mort médiée par Fas-L. A l'inverse, une forte dose d'HGF/SF déstabilise l'association de Met avec Fas-R, ce qui permet à ce dernier d'interagir avec Fas-L, sensibilisant ainsi les cellules à l'apoptose (Wang *et al.*, 2002). Les données *in vivo* montrent que des souris surexprimant le domaine extracellulaire de Met sont plus résistantes à l'apoptose hépatique dépendante de Fas. Cette interaction a

également été démontrée dans des cellules endothéliales (HUVEC) ce qui leur permet dans de résister à l'apoptose induite par Fas (Smyth and Brady, 2005).

IV) Implications du couple HGF/SF-Met dans le cancer

Selon Weinberg et Hanahan la transformation cellulaire qui permet le passage d'une cellule normale à une cellule tumorale nécessite l'acquisition d'une dizaine de propriétés (Hanahan and Weinberg) (Figure 8). Parmi ces caractéristiques, on retrouve, entre autres, l'indépendance vis-à-vis des signaux stimulant la prolifération, la protection contre l'apoptose, une angiogenèse importante ou l'invasion métastatique. Plusieurs travaux ont montré que Met pouvait être directement impliqué dans les mécanismes de transformation cellulaire qui aboutissent au développement de cancers.

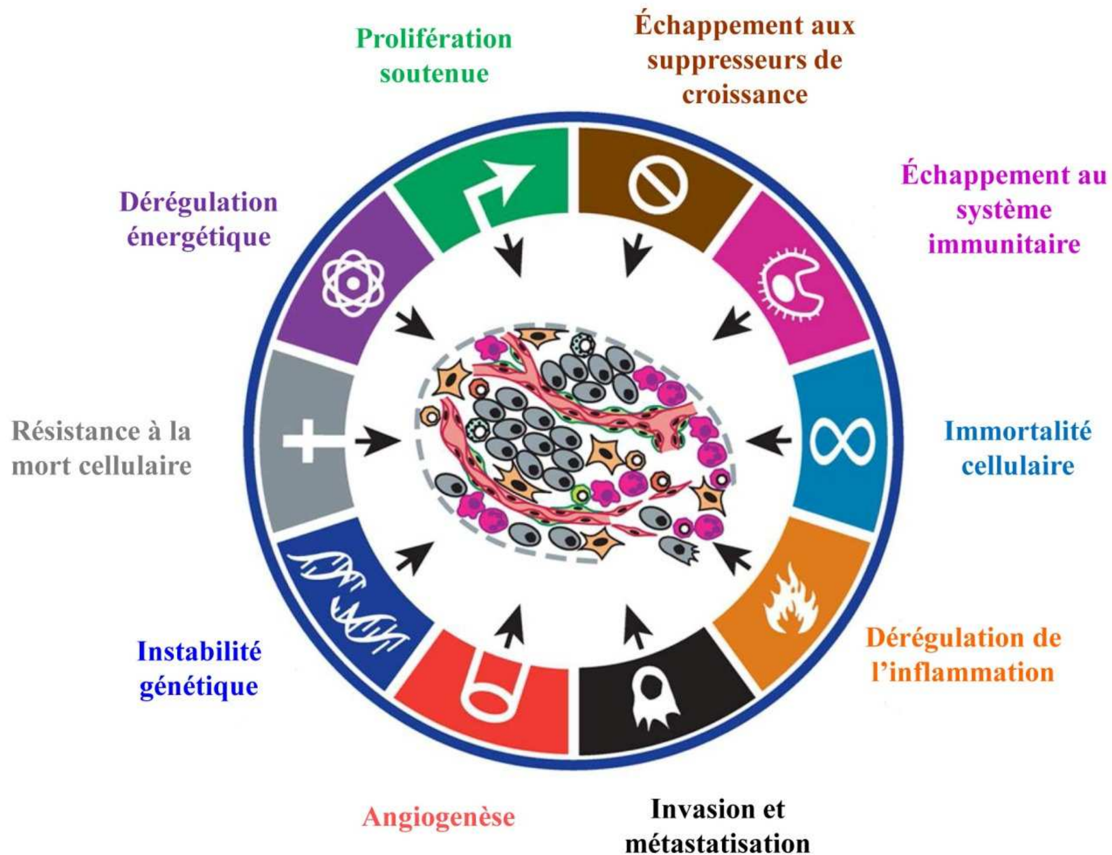


Figure 8 : Caractéristiques permettant la transformation cellulaire
D'après (Hanahan and Weinberg)

A) Réarrangement chromosomique

Met a été découvert sous la forme d'une protéine de fusion oncogénique TPR-Met. Les domaines TPR situés en région N-terminale dimérisent constitutivement la région

intracellulaire de Met qui est dépourvue de la région juxtamembranaire. Cette protéine présente une phosphorylation continue synonyme d'une activité catalytique soutenue (Rodrigues and Park, 1993). Il en résulte une activation constitutive de Met dont le potentiel oncogénique a pu être évalué par la formation de foyers de fibroblastes transformés et par la formation de tumeurs dans des souris transgéniques (Liang *et al.*, 1996). L'existence de cette protéine de fusion dans des cas de cancers est pourtant controversée. En 1991, une équipe a détecté l'existence de la protéine TPR-Met dans des biopsies et des lignées cellulaires issues de cancers gastriques. Cependant, des travaux similaires réalisés par une autre équipe n'ont pas permis de confirmer ces résultats (Soman *et al.*, 1991 ; Heideman *et al.*, 2001).

B) Mutations de Met

Plus tard, un lien direct entre Met et le cancer a pu être mis à jour par la découverte de mutations activatrices situées au niveau du domaine kinase dans des carcinomes papillaires rénaux héréditaires (Jeffers *et al.*, 1997a; Schmidt *et al.*, 1997). Depuis, plus d'une centaine de mutations de la séquence nucléotidique de Met a été répertoriée. Elles sont à l'origine d'une quarantaine de substitutions d'acides aminés dans la séquence protéique de Met. De manière intéressante, ces mutations sont majoritairement concentrées dans trois régions impliquées dans la régulation de l'activité de Met : la région extracellulaire (impliqué dans la fixation du ligand), le domaine juxtamembranaire (une zone de régulation négative) et le domaine kinase (Figure 9).

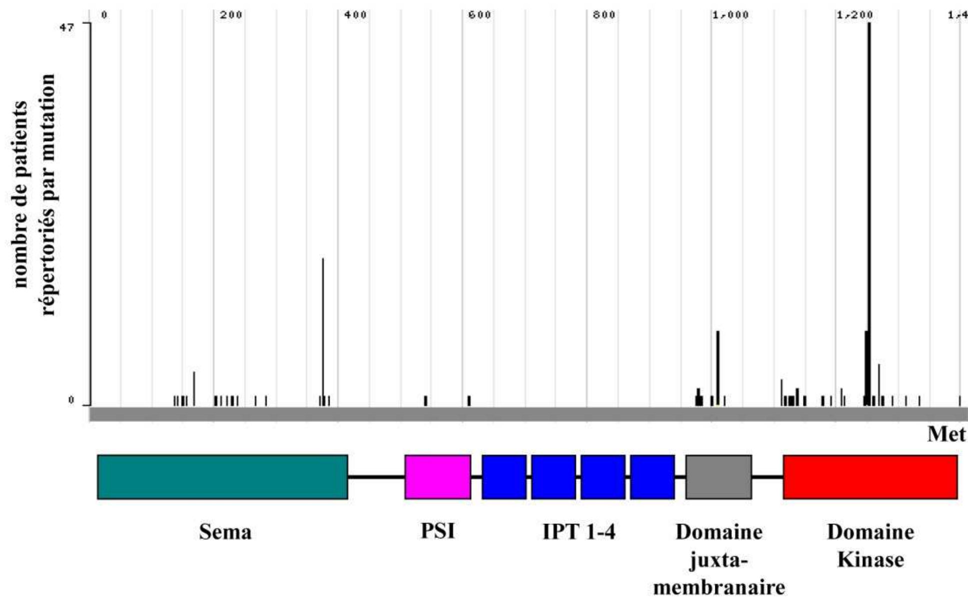


Figure 9 : Mutations de Met identifiées chez l'Homme
Répartition des mutations le long de la séquence protéique de Met
D'après la banque de données du site http://www.sanger.ac.uk/genetics/CGP/cosmic/

Comme l'indique le site Sanger, plus d'une vingtaine de mutations affecte le domaine kinase du récepteur. Ces mutations sont préférentiellement observées dans les tumeurs des voies aérodigestives supérieures (VADS) et des reins. Pour quelques unes de ces mutations, le mode d'action a pu être élucidé. Ainsi, il a été montré que certaines de ces mutations augmentent l'activité kinase de Met ou activent le récepteur sans phosphoryler les tyrosines 1234 et 1235(Jeffers *et al.*, 1997a; Jeffers, 1999; Cristiani *et al.*, 2005). Le résidu Y1235, une des deux tyrosines située dans la poche catalytique et phosphorylée en réponse au ligand, est mutée en acide aspartique dans 10% des cancers des VADS. En mimant la charge négative d'un groupement phosphate, la mutation de la tyrosine 1235 en acide aspartique active constitutivement le récepteur, indépendamment de son ligand (Aebersold *et al.*, 2003; Cristiani *et al.*, 2005). Pour d'autres mutations du domaine kinase, on observe une diminution du seuil d'activation du récepteur permettant une phosphorylation de Met avec de faible concentration d'HGF/SF (Chiara *et al.*, 2003). De manière globale, les récepteurs portant ces mutations sont constitutivement phosphorylés, induisent la transformation des NIH-3T3 et leur expression favorise la formation de tumeurs dans des souris immunodéprimées (Jeffers *et al.*, 1997a). Enfin, des souris exprimant par KI une version de Met portant une mutation activatrice développent de nombreux cancers, principalement des lymphomes et des carcinomes mais aussi des sarcomes (Graveel *et al.*, 2004). Ces études, en plus de confirmer les capacités tumorigènes de ces mutations, ont permis d'identifier une duplication du gène

met dans ces cellules tumorales. Il a ainsi été proposé qu'en plus de la mutation du récepteur, l'augmentation du nombre de copies de Met serait une étape nécessaire à la tumorigenèse.

Les mutations de la partie extracellulaire concernent majoritairement le domaine Sema, un domaine non-catalytique de Met qui partage des homologies de séquences avec les plexines, les sémaphorines (Jagadeeswaran *et al.*, 2006; Finegold *et al.*, 2008; Ma *et al.*, 2008). Ces mutations ont été répertoriées dans des cancers du poumon. Contrairement aux mutations du domaine kinase, ces mutations n'activent pas le récepteur et, pour certaines d'entre elles, diminueraient même l'interaction du récepteur avec le ligand. Le mécanisme d'action de ces mutations reste donc à élucider et pourrait redéfinir l'implication du domaine Sema dans le contrôle de l'activité de Met (Ma *et al.*, 2008).

Comme nous l'avons décrit précédemment, le domaine juxtamembranaire de Met intervient dans la dégradation du récepteur après son activation par le ligand. Trois mutations du domaine juxtamembranaire, (R970C, P991S et T992I) ont été retrouvées dans des cancers des VADS, du poumon et de la thyroïde mais ne semblent pas induire l'activité kinase de Met (Tyner *et al.*, ; Ma *et al.*, 2003). Le caractère oncogénique de la mutation T992I a pu être illustré par l'injection de cellules NIH-3T3 exprimant cette forme mutée de Met à des souris immunodéprimées. Celle-ci produisent alors davantage de tumeurs que l'injection de NIH3T3 exprimant une version sauvage de Met (Lee *et al.*, 2000). De plus, il a été montré que la susceptibilité des souris SWR/J au cancer du poumon était liée à la mutation R970C de Met (Zaffaroni *et al.*, 2005). Toutefois, en l'absence de mécanisme d'action clairement identifié, une équipe a suggéré que ces deux mutations puissent être de simples polymorphismes (Tyner *et al.*). Enfin, il a été montré que plusieurs mutations situées dans les régions introniques de Met induisent la délétion de l'exon 14 qui code pour le domaine juxtamebranaire de Met. Ces récepteurs délétés de l'exon 14 ne possèdent plus le résidu Y1003 et ne peuvent plus recruter la protéine c-CBL. Le récepteur présente alors un défaut de dégradation : la signalisation induite par l'HGF/SF est alors prolongée et aboutit à la transformation des fibroblastes (Asaoka *et al.*, ; Ma *et al.*, 2005; Kong-Beltran *et al.*, 2006; Onozato *et al.*, 2009). Des résultats similaires ont été obtenus suite à la mutation ponctuelle de la tyrosine 1003 (Peschard *et al.*, 2001; Abella *et al.*, 2005).

C) Surexpression de Met et/ou de l'HGF/SF

Malgré l'existence de ces mutations, il a été montré que dans la plupart des cancers liés à Met, on observe plutôt une surexpression de Met et/ou de l'HGS/SF. De façon cohérente, la surexpression de Met ou de son ligand par transgenèse chez la souris induit le développement de tumeurs et de lésions métastatiques (Takayama *et al.*, 1997). A l'inverse, la diminution de l'expression de Met ou de l'HGF/SF dans des cellules tumorales humaines atténue leur potentiel tumorigène (Abounader *et al.*, 2002).

La surexpression de Met peut être due à une amplification génique. De multiples copies de *met* ont d'ailleurs été observées dans 10 à 20 % des cancers gastriques (Smolen *et al.*, 2006), dans des cancers colorectaux et des métastases hépatiques (Zeng *et al.*, 2008) ainsi que dans des cancers du poumon (Toschi and Cappuzzo). Cette amplification génique entraine alors une augmentation de l'expression de Met. Cette surexpression peut mener à une activation du récepteur en absence de ligand (Ponzetto *et al.*, 1991). Ainsi, les cellules tumorales gastriques GTL-16 qui possèdent 11 copies du gène *met* surexprime le récepteur Met et présentent une phosphorylation constitutive (Herrick *et al.*, 2000).

D'autres mécanismes permettent la surexpression du récepteur. Ainsi, la transcription du gène *met* peut être activé par la voie MAPK sous le contrôle de l'oncogène Ras ou par le facteur de transcription Ets1. Dans les cellules tumorales, ces deux oncogènes permettent donc la surexpression de Met qui est retrouvé activé indépendamment du ligand. Une boucle d'auto-activation permet alors à Met d'amplifier l'action de Ras et d'Ets1 (Gambarotta *et al.*, 1996; Ivan *et al.*, 1997).

L'hypoxie est également un facteur capable d'induire la surexpression de Met. Les anomalies structurelles de la vascularisation tumorale qui entraîne une mauvaise oxygénation, associées au métabolisme intense des cellules tumorales, favorisent la présence de régions hypoxiques dans la tumeur. Cet environnement favorise la production d'espèces réactives de l'oxygène (ROS) par la mitochondrie (Guzy *et al.*, 2005). La production de ces ROS permet la stabilisation du facteur HIF-1, un facteur de transcription très instable en conditions de normoxie. Stabilisé, ce facteur est alors capable de réguler l'expression de plus d'une centaine de gènes impliqués dans la survie, l'angiogenèse, l'érythropoïèse ou encore la résistance à l'anoïkie. L'expression de ces gènes favorise la croissance tumorale et la dissémination métastatique en dépit des conditions hypoxiques (Scarpino *et al.*, 2004). Ainsi, on observe que

l'inhibition de l'expression de Met à l'aide de shRNA bloque la dissémination métastatiques induite par HIF-1 dans les conditions hypoxiques (Comito *et al.*).

D) Autocrinie, paracrinie

En conditions physiologiques, l'expression de l'HGF/SF par les cellules mésenchymateuses et de Met par les tissus épithéliaux entraine une activation paracrine de Met. Cette activation paracrine peut être impliquée dans des situations pathologiques suite à une production de manière anormale d'HGF/SF par les tissus mésenchymateux. Cette augmentation d'expression d'HGF/SF, souvent corrélée à un mauvais pronostic, a été observée dans les tumeurs ou dans le sérum de patients atteints de cancers du sein, du poumon, de l'estomac, du colon et des cellules gliales (Yamashita *et al.*, 1994; Siegfried *et al.*, 1997; Toi *et al.*, 1998; Niki *et al.*, 1999; Arrieta *et al.*, 2002; Toiyama *et al.*, 2009). L'activation autocrine a lieu quant à elle lorsque Met et l'HGF/SF sont exprimés conjointement et de manière aberrante par les cellules tumorales. Cette caractéristique a été observée dans des cancers du sein, du poumon, plusieurs myélomes, des leucémies et certains cas de sarcomes (Christensen *et al.*, 2005). Des modèles cellulaires exprimant de manière ectopique l'HGF/SF et/ou Met forment alors des tumeurs en souris nude. De plus, l'expression conjointe de l'HGF/SF et de Met favorise l'angiogenèse et le développement métastatique (Rong *et al.*, 1994; Navab *et al.*, 2009). Cependant, peu de cellules cancéreuses expriment à la fois Met et son ligand, suggérant plutôt une activation paracrine de Met. Des études ont d'ailleurs montré que les cellules tumorales exprimaient tout un panel de facteurs capables d'induire l'expression de l'HGF/SF par les fibroblastes du stroma. Ainsi le caractère invasif de cellules issues de carcinomes dépend directement de la présence de fibroblastes et des facteurs qu'ils secrétent (Matsumoto *et al.*, 1996). Ces mêmes facteurs favoriseraient la tumorigenèse en augmentant l'angiogenèse, la réponse inflammatoire et la prolifération des fibroblastes (Jiang *et al.*, 1999; Matsumoto and Nakamura, 2006).

E) Thérapies

Depuis quelques années, toute une batterie d'outils thérapeutiques a été développée pour inhiber l'activité de Met dans le but de thérapies ciblées anti-cancéreuses (Figure 10).

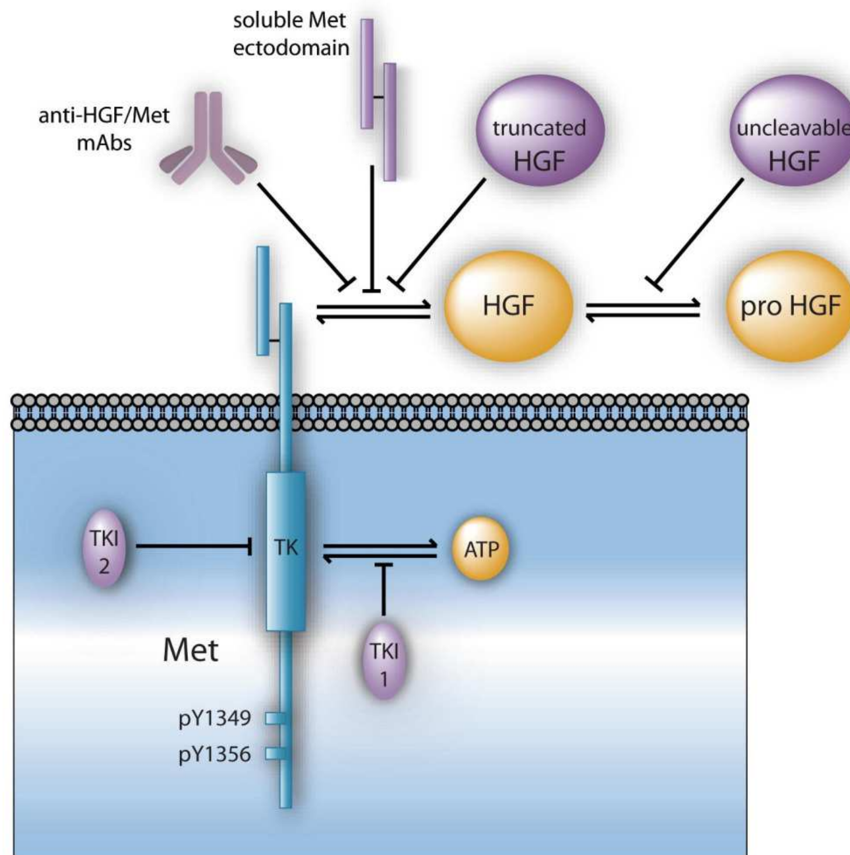


Figure 10 : Stratégies d'inhibition du couple HGF/SF-Met
D'après (Cecchi et al.)

Ces outils peuvent cibler le domaine extracellulaire et sa capacité de liaison au ligand Des molécules antagonistes inspirées de la molécule NK2, un variant naturel de l'HGF/SF qui fixe Met sans l'activer, sont développées pour cibler le domaine extracellulaire du récepteur et sa capacité de liaison au ligand (Montesano *et al.*, 1998). La molécule NK2 comprend le domaine N-terminal et les deux premiers domaines kringles de la sous-unité α de l'HGF/SF (Chan *et al.*, 1993). En se basant sur les caractéristiques de NK2, deux autres molécules compétitrices de l'HGF/SF mature ont été mises au point. Ainsi, NK4, qui correspond à la sous-unité α de l'HGF/SF, a pu être testée dans différents modèles de tumeurs murines où il a montré des activités anti-angiogéniques. De manière similaire, une forme non-activable de l'HGF/SF capable de se fixer sur Met diminue lui aussi la vascularisation des tumeurs et

limite la dissémination métastatique (Matsumoto and Nakamura, 2003; Mazzone *et al.*, 2004; Matsumoto and Nakamura, 2008).

D'autres molécules antagonistes, decoyMet et rSema, correspondant à la partie extracellulaire de Met et jouant le rôle de leurre, ont été développées. Ces molécules correspondant au domaine extracellulaire de Met sont capables d'inhiber les réponses biologiques en réponse au ligand mais aussi les réponses biologiques dans le cadre d'une activation de Met indépendante du ligand. En effet, elles peuvent former des hétérodimères non fonctionnels avec Met, déstabilisant ainsi les homodimères actifs et les réponses qu'ils contrôlent (Kong-Beltran *et al.*, 2004; Michieli *et al.*, 2004).

D'autres stratégies d'inhibition ont été développées sur la base de l'ingénierie des anticorps monoclonaux. Certains des outils crées ciblent directement la liaison entre Met et l'HGF/SF. Cependant, comme c'est le cas pour l'anticorps 5D5, ils sont également capables d'induire la dimérisation et l'activation de Met puisque chaque bras est capable de fixer un récepteur. De ce fait, une version améliorée de cet anticorps, le 5D5 O.A (One Armed), baptisée MetMab, a été développée et empêche efficacement l'activation par l'HGF/SF sans induire la dimérisation du récepteur Met (Martens *et al.*, 2006; Jin *et al.*, 2008). MetMAB/5D5 est aujourd'hui au stade final de la phase II d'expérimentation clinique.

Enfin, d'autres inhibiteurs ciblent quant à eux le domaine kinase de Met. Ce sont des molécules qui, en mimant la structure de l'ATP, sont capables de se loger dans la poche ATP, empêchant ainsi l'activation du récepteur. Suite au développement des premiers inhibiteurs de Met comme le SU1127 (IC_{50}= 20 nM) et le PHA 665752 (IC_{50}= 9 nM), largement utilisés en recherche fondamentale, plusieurs autres inhibiteurs de l'activité kinase de Met ont été développés (Underiner *et al.*, ; Smolen *et al.*, 2006). Ils permettent une meilleure tolérance, possède une IC_{50} inférieure au nM et font l'objet de différents essais cliniques sur des cancers gastriques, des cancers du poumon à non petites cellules et des cancers (Bagai *et al.*, ; Munshi *et al.*).

Nous l'avons vu précédemment, les RTK peuvent coopérer dans leur signalisation au profit de la croissance tumorale. Ces notions de « cross-talk » ont été particulièrement analysées entre Met et l'EGFR et ont conduit à des combinaisons thérapeutiques. Ainsi, *in vitro,* dans des cellules issues de carcinome bronchique, l'inhibition de Met permet notamment de lever la résistance observée avec les inhibiteurs de l'EGFR (Engelman *et al.*, 2007). De plus, l'homologie de structure partagée par les RTK a permis de développer des

molécules à large spectre capables d'inhiber plusieurs RTK dont Met. Les résultats obtenus sur un inhibiteur ciblant 6 RTK, le XL184/BMS90735, semblent confirmer l'efficacité d'un traitement ciblant plusieurs oncogènes. L'utilisation de cette molécule s'inscrit également dans le cadre des résistances aux inhibiteurs à l'EGFR comme l'Erlotinib (Zhang *et al.*).

V) Dégradation du récepteur Met

De nombreux groupes étudient les réponses biologiques induites par l'activation du récepteur Met. Ces travaux ont permis de décrypter le réseau des voies de signalisation induit par la fixation de l'HGF/SF à Met et décrit un peu plus haut. Ces travaux ont aussi montré que Met était également endocyté puis dégradé afin de contrôler dans le temps l'induction de ces réponses. Certains de ces travaux ont permis d'identifier de nombreux fragments du récepteur mais leurs rôles n'ont jamais été élucidés (Prat *et al.*, 1991; Galvani *et al.*, 1995; Jeffers *et al.*, 1997b; Wajih *et al.*, 2002). Avant mon arrivée au laboratoire, l'équipe a montré que des fragments de Met peuvent provenir d'aux moins deux processus de clivages protéolytiques de Met (Tulasne *et al.*, 2004; Foveau *et al.*, 2007; Deheuninck *et al.*, 2008; Foveau *et al.*, 2009). Ces mécanismes de dégradation ne sont pas associés au mécanisme classique de dégradation dépendante du ligand et ont permis d'identifier des fonctions biologiques pour ces fragments, levant ainsi le voile sur la signalisation de Met en absence de d'HGF/SF. L'ensemble de ces clivages apporte ainsi une nouvelle vision de la signalisation et la dégradation du récepteur Met. Les mécanismes de dégradation du récepteur Met, induite par liaison de l'HGF/SF par Met ou induit indépendamment de cette fixation sont décrits dans la revue qui suit. Les caractéristiques du clivage de Met par les caspases seront reprises dans la partie résultats.

A) Dégradation dépendante du ligand

Voir la revue ci-après

<u>Publication 1:</u> Met degradation: more than one stone to shoot a receptor down

<u>**Lefebvre J.**</u>, Ancot F., Leroy C., Muharram G., Lemière A., and Tulasne D.

Soumis à FASEB

Met degradation: more than one stone to shoot a receptor down

Jonathan Lefebvre, Frédéric Ancot, Catherine Leroy, Ghaffar Muharram, Arnaud Lemière, and David Tulasne

CNRS UMR 8161, Institut de Biologie de Lille-Institut Pasteur de Lille-Université de Lille Nord de France, IFR142, B.P.447, 59021 Lille cedex, FRANCE.

Correspondence should be addressed to:
David TULASNE
CNRS UMR 8161
Institut de Biologie de Lille, Institut Pasteur de Lille
B.P.447, 59021 Lille, FRANCE.
Tel: 0 33 3 20 87 12 57
Fax: 0 33 3 20 87 11 11
email: david.tulasne@ibl.fr

Running title: Met degradation

Keywords: receptor tyrosine kinase, cleavages, caspase, metalloprotease, γ-secretase, signaling, leucine-rich repeat proteins

Abbreviations:

HGF/SF: Hepatocyte growth factor/Scatter factor
RTK: Receptor tyrosine kinase
GAB1: Grb2-associated binder-1
SHC: Src homology 2 domain containing
GRB2: Growth factor receptor-bound protein 2
PLCγ1: Phospholipase C-gamma 1
PI3-K: Phosphoinositide 3-kinase
STAT3: Signal transducer and activation of transcription
TPR: Translocated promoter region
HIF1: Hypoxia-inducible factor 1
HSP90: Heat shock protein 90
PIP2: Phosphatidylinositol 4,5-bisphosphate
CBL: Casitas B-lineage lymphoma
TKB: Tyrosine kinase binding
HRS: HGF-regulated tyrosine kinase substrate
CIN85: CBL INteracting protein of 85 kDa
CCV: Clathrin-coated pit vesicles
EPS15: Epidermal growth factor receptor substrate 15
UIM: Ubiquitin-interacting motif
EGFR/ ErbB: Epidermal growth factor receptor
MVB: Multivesicular bodies
PI3P: Phosphatidylinositol 3-phosphate
STAM: Signal transducing adaptor molecule
ESCRT: Endosomal sorting complex for transport
ERK: Extracellular signal-regulated kinases
MAPK: Mitogen-activated protein kinases
GGA3: The Golgi-localized gamma-ear-containing Arf-binding protein 3
Rab4: Ras-related protein-4
PKC: Protein kinase C
Caspase: Cysteine-dependent aspartate-directed proteases
PS-RIP: Presenilin-regulated intramembrane proteolysis
ADAM10: A Disintegrin and metalloproteinase domain-containing protein 10
NTF: N-terminal Fragment
CTF: C-terminal Fragment
ICD: Intracellular domain
LRR: Leucine-rich repeat
LRIG1: Leucine-rich repeat and immunoglobulin-like domain containing protein 1
TIMP: Tissue inhibitors of metalloproteinases
CDK6: Cyclin-dependent kinase 6
InlA/B: Internalin A/B
Rac1: Ras-related C3 botulinum toxin substrate 1
ADPKD: Autosomal dominant polycystic kidney disease
PKD1: Polycystin 1 and 2
MDCK: Madin-Darby canine kidney, epithelial cell line

Abstract

The receptor tyrosine kinase Met and its high-affinity ligand, the hepatocyte growth factor/scatter factor, are essential to embryonic development. Deregulation of their signaling is associated with tumorigenesis and metastasis, notably through receptor overexpression. It is thus important to understand the mechanisms controlling Met expression. The ligand-dependent internalization of Met and its subsequent degradation in the lysosomal compartment are well described. This process is known to attenuate downstream Met signaling pathways. Yet internalized Met takes part directly in intracellular signaling by chaperoning signaling factors in the course of its trafficking. Furthermore, recent studies describe various new degradation mechanisms of membrane-anchored Met, involving proteolytic cleavages or association with novel partners. Although all these degradations are ligand independent, they share, to different extents, some common features with canonical HGF/SF-dependent degradation. Interestingly, activated Met variants display resistance to degradation, suggesting defective degradation is involved in tumorigenesis. Conversely, forced degradation of Met through re-induction of one or more degradation pathways is a promising therapeutic strategy.

Multiple biological responses induced by the Met receptor

Met is a receptor tyrosine kinase (RTK) expressed predominantly in cells of epithelial origin. It consists of an exclusively extracellular α-chain and a β-chain shared between the extra- and intracellular compartments. The intracellular region of the β-chain contains a juxtamembrane domain, a catalytic tyrosine kinase domain, and a C-terminal domain (1). Met is activated by its high-affinity stromal ligand, the hepatocyte growth factor/scatter factor (HGF/SF) (2). HGF/SF and Met are essential to embryonic development, since knockout of either one affects placenta, liver, muscle, and neuron formation (3-6). In adults, the HGF/SF-Met pair is involved in physiological processes such as mammary gland development (7), wound healing, and kidney or liver regeneration (8, 9). *In vitro*, ligand-activated Met stimulates proliferation, scattering, invasion, and morphogenesis of epithelial cells, acts as an angiogenic factor, and has chemoattractant and neurotrophic activities (2).

In response to HGF/SF stimulation and receptor dimerization, the intrinsic tyrosine kinase activity of Met elicits phosphorylation of specific tyrosine residues within the intracellular domain (10). Autophosphorylation of residues Y1234 and Y1235, located within the activation loop of the tyrosine kinase domain, triggers upregulation of its kinase activity (11, 12). This leads to phosphorylation of two neighboring carboxy-terminal tyrosine residues of Met (Y1349 and Y1356), generating a multi-substrate docking site conserved among Met family members. This multi-substrate docking site allows recruitment of many signaling proteins, including adaptors such as GAB1, SHC, and GRB2, enzymes such as PLCγ1 and PI3-K, and signaling proteins with transcriptional activity such as STAT3 (Fig. 1). These recruitments are required for activation of downstream signaling pathways and induction of biological responses (13-15).

Aberrant Met and HGF/SF signaling is involved in promoting tumorigenesis and metastasis. In fact, the Met receptor was originally identified as an oncogene resulting from a chromosomal rearrangement giving rise to a product where the dimerization domain of the translocated promoter region (TPR) is fused with the intracellular region of the Met receptor (16). A direct link between Met and cancer was later evidenced by characterization of receptor-activating mutations in hereditary papillary renal carcinoma (17). About forty different mutations of Met have since been identified in tumor samples of various origins (www.sanger.ac.uk/genetics/CGP/cosmic/). Three mutation hot spots, namely the sema domain, the juxtamembrane region, and the kinase domain, have been mapped. In most cases, mutations in the kinase domain increase the kinase activity. The functional consequences of mutations in the other two regions are unknown. Although many different mutations have

been identified, Met activation in cancer occurs most often through ligand-dependent stimulation, induced by uncontrolled expression of HGF/SF and/or Met, leading to autocrine or paracrine activation (2). In addition, aberrant Met activation is induced by overexpression of the receptor without HGF/SF engagement: ligand-independent activation of receptor tyrosine kinases is typically observed in cells expressing high levels of receptor, leading to spontaneous dimerization and subsequent activation (18).

The Met level is regulated at multiple stages, and an abnormally high Met level can result from many defects. First, misregulation can occur at transcript level through gene amplification as in colorectal cancer (19), by oncogene-enhanced transcription (20, 21), or during hypoxia by activation of the HIF-1 transcription factor (22). Secondly, it has been shown that several miRNAs targeting Met mRNA are downregulated in cancer cells, causing Met overexpression (23, 24). Lastly, treatment with the HSP90 inhibitor geldanamycine induces efficient downregulation of Met in tumor cells, suggesting that HSP90 is a chaperone of Met promoting its stabilization during maturation.

When the mature Met receptor is present at the plasma membrane, it is functional and can be activated by its high-affinity ligand HGF/SF. Like other receptors possessing intrinsic tyrosine kinase activity, Met is downregulated following ligand stimulation. This process is important for desensitization of the signaling cascade, avoiding overstimulation that might lead to cell transformation. This ligand-dependent degradation of Met is a well-known mechanism, but recent reports describe various novel mechanisms involved in Met degradation, most occurring independently of ligand stimulation. In this review we describe these novel degradation mechanisms having a direct impact on Met receptor functions and likely to open the way to unexpected strategies for inhibiting Met expression and activity in pathological situations such as cancer.

Canonical ligand-dependent Met degradation

Stimulation by the ligand induces receptor degradation, observable from about 1 hour after stimulation and peaking at about 8 hours, before a return to the initial situation after 24 hours (25). This ligand-dependent degradation of Met and of RTKs in general requires a complex machinery that has been extensively studied and that we briefly describe below (for a review see (26) (Fig. 1).

Upon HGF/SF stimulation, Met can recruit PI3-K through its C-terminal multisubstrate binding site. Activation of PI3-K leads to formation of PIP2 at the plasma membrane, favoring recruitment of the AP2 complex (27). This complex associates with

clathrin, which leads to formation of clathrin-coated pits containing the activated Met receptor.

Ligand-activated Met also recruits the E3 ubiquitin ligase CBL, promoting receptor ubiquitination. CBL recruitment can occur directly through association between the TKB domain of CBL and the phosphorylated Y1003 of Met and indirectly, through association of the C-terminal proline-rich domain of CBL with GRB2, itself associated with the phosphorylated Y1356 of Met (28). Like EGFR, the activated Met receptor is multi-mono-ubiquitinated (28, 29). Ubiquitination of the receptor is essential to its efficient degradation, but not to its internalization, since the non-ubiquitinated Y1003F Met mutant is still internalized but displays enhanced stability. In a mirror experiment, fusion of mono-ubiquitin to the Met Y1003F variant was found to decrease its stability and to restore phosphorylation of HRS (HGF-regulated tyrosine kinase substrate), a component of the endosomal sorting complex (30).

Met internalization is also controlled by the scaffold function of CBL. Activated CBL can interact with CIN85 (CBL INteracting protein of 85 kDa) (31), which associates with endophilins, regulatory components of clathrin-coated pit vesicles (CCVs) involved in the negative curvature, invagination, and scission of vesicles from the plasma membrane (32, 33). Met receptor endocytosis is also inhibited by expression of a dominant-negative form of dynamin, a GTPase that deforms lipid bilayers and possibly involved in vesicle scission from the membrane (34).

Pinching-off of the clathrin-coated domain requires the action of additional proteins including EPS15, dynamin, and endophilins. EPS15 is recruited to the plasma membrane through its interaction with the AP2 protein (35). Previous reports highlight the importance of the EPS15 ubiquitin-interacting motif (UIM), which recognizes the mono-ubiquitinated residue involved in EGFR endocytosis (36). Yet recruitment of EPS15 to Met is more complex. It involves two distinct domains, a coiled-coil domain and a proline-rich motif that can bind the signaling adaptor molecule Grb2 recruited by activated Met. Although the UIM domain of EPS15 is not essential to recruitment to the Met receptor, an EPS15 mutant lacking this domain fails to promote receptor degradation. This suggests an involvement of this domain in Met trafficking and degradation (37).

Once formed, the CCVs fuse with sorting endosomes to form multivesicular bodies (MVBs) involved in either directing proteins towards lysosomal degradation or recycling them to the cell membrane. Mono-ubiquitinated receptors are retained and endocytosed upon recognition by proteins of the endocytic pathway containing a UIM. One such protein, HRS,

localizes to the surface of the endosome by binding to the PI3P found in microdomains of early endosomes, and can associate with clathrin (38) (39). HRS also associates with EPS15 and STAM, thereby increasing the stability of the complex (40, 41). Met activation and ubiquitination are required for recruitment of the HRS/STAM complex, and when this recruitment does not occur, receptor degradation is inhibited (30). The HRS/STAM complex forms the endosomal sorting complex for transport (ESCRT-0), which initiates recruitment of other ESCRT complexes to the sorting endosome and thereby regulates formation of the MVB (42). The late MVB is then targeted to the lysosome, and the endocytosed Met receptor is degraded. Upon inhibition of the vacuolar ATPase generating the acidic environment necessary for the activity of lysosomal enzymes, Met degradation is also inhibited. Met is thus indeed degraded in this compartment (43).

Although the role of the lysosome in Met degradation is well established, there is evidence suggesting that the proteasome pathway can also participate in this process (43). Met degradation is decreased by proteasomal inhibitors, to which other RTKs such as EGFR are insensitive. Additionally, when Met endocytosis is blocked in a dynamin mutant, the receptor is still degraded, albeit at slower rate.

Not only the ligand-activated Met receptor itself, but also its direct downstream signaling partners are targeted to degradation. In response to HGF/SF, the adaptor protein GAB1 is degraded via a mechanism involving Met tyrosine kinase activity and the proteasome machinery. Accordingly, ligand stimulation induces GAB1 multi- and poly-ubiquitination in a CBL-dependent manner. GAB1 downregulation is associated with loss of activation of the ERK MAPK in response to acute ligand treatment, suggesting the involvement of GAB1 degradation in attenuation of the Met signaling pathways (44).

In addition to its involvement in degradation, the endocytic machinery can sort Met receptors for recycling. Upon HGF/SF stimulation, Met associates with GGA3 (the Golgi-localized gamma-ear-containing Arf-binding protein 3) via the adaptor CRK. Silencing of GGA3 inhibits Met recycling from the Rab4-positive endosome, causing increased Met degradation (Fig.1). Consequently, downstream signaling (including ERK activation and biological responses such as migration) is attenuated. This demonstrates that active recycling of Met from the endosome to the plasma membrane participates in the spatial and temporal control of Met signaling (45).

Internalized Met still participates in signaling

Upon HGF/SF treatment, the Met receptor is internalized into the early endosome and directed to specialized compartments, including the lysosome involved in its degradation. Yet about 2 hours following stimulation of HeLa cells, Met localizes to perinuclear vesicles. This perinuclear localization, appearing independent of receptor degradation and synthesis (46), depends on active trafficking along the microtubules, and PKCα activity is required for this motion (47). The significance of this unexpected localization was unknown. However, during the early steps of HGF/SF-induced endocytosis, Met colocalizes with the transcription factor STAT3. After 2 hours of stimulation, STAT3 translocates to the nucleus to regulate gene expression (48). Strikingly, HGF/SF induces low-level phosphorylation and late nuclear translocation of STAT3, suggesting that Met triggers weak STAT3 activation. STAT3 signaling is involved in several Met-triggered biological responses, including morphogenesis and cell transformation (49, 50). Interestingly, HGF/SF-induced nuclear translocation of STAT3 depends on their prior trafficking from the peripheral endosome to the perinuclear endosome. In addition, Met kinase activity within the trafficking endosome is required for STAT3 nuclear translocation. Thus, Met endocytosis is not only required for Met degradation, but also favors signal transduction (48) (Fig. 1). It has been proposed that receptor trafficking in the endosome together with a signaling protein could be a mechanism supporting a weak signal (51). Other signaling proteins take advantage of the endosomal trafficking of Met. For instance, phosphorylated ERK can localize to focal adhesion complex upon HGF/SF treatment. This localization requires the trafficking of Met in the early endosome, which depends on PKCε activity. Localization of ERK to the focal adhesion complex could be involved in Met-triggered epithelial cell migration, since PKCε silencing inhibits wound healing (47).

Degradation through proteolytic cleavages

Caspase cleavage of Met during apoptosis

Besides ligand-dependent downregulation, novel mechanisms of Met degradation have recently been revealed. Some of these involve initial cleavages by cellular proteases that inactivate the receptor. The first evidence for this efficient downregulation mechanism was described in apoptotic cells, in which Met was found cleaved.

Apoptotic cell death is triggered by a variety of physiological and pathological stimuli. Its characteristic morphological features include cell rounding, membrane blebbing, cytoplasmic

condensation, and DNA fragmentation. During apoptosis, caspases (forming a family of cysteine-dependent aspartate-directed proteases) can cleave a wide range of substrates, thereby contributing to the morphological modifications characteristic of apoptosis (52). Caspases can also contribute to promoting cell death by inactivating survival mechanisms. They can notably cleave several RTKs, major mediators of the survival response (53). We have demonstrated that upon stress induction, the Met receptor is first cleaved at aspartic acid 1374 (mouse sequence) within a consensual DNID caspase site located in the extreme C-terminal tail. This initial cleavage removes only five amino acids of Met but favors a second cleavage occurring at aspartic acid 1000 within an ESVD motif located in the juxtamembrane domain. These sequential cleavages generate two main stable fragments: a 100 kDa membrane-anchored N-terminal fragment (p100 Met) containing the extracellular ligand-binding regions and a 40 kDa cytoplasmic C-terminal fragment (p40 Met) containing the tyrosine kinase domain. (54-56) (Fig. 2A). As a result of caspase cleavage, the level of full-length Met receptor decreases strongly during apoptosis, and separation of the extracellular ligand-binding domain from the kinase domain creates fragments unstimulable by the ligand. Thus, caspase cleavages participate in degrading the full-length Met receptor during apoptosis, and might thus prevent induction of a survival response by HGF/SF.

Caspase cleavages not only inactivate the receptor but also convert it to active fragments that can tip the survival/apoptosis balance in favor of the latter. On the one hand, we have shown that membrane-anchored p100 Met can bind HGF/SF and thus act as a decoy receptor (55). By reducing or preventing HGF/SF binding to full-length Met, the decoy might thus participate in decreasing the cell survival response during apoptosis. Secondly, we have demonstrated that ectopic expression of p40 Met can amplify cell death concomitantly with activation of caspase-3 (54). In addition, when the C-terminal caspase site of Met is mutated so as to inhibit generation of the p40 Met proapoptotic fragment, cell death induced by mild stress is prevented (56). Caspase-dependent cleavage of Met thus converts the survival receptor to a proapoptotic molecule involved in amplifying apoptosis (Fig. 2A).

The proapoptotic function mediated by p40 Met in the absence of ligand, contrasting with the anti-apoptotic function of full-length Met upon HGF/SF stimulation, is the hallmark of the emerging family of dependence receptors. Although a receptor is usually considered inactive until bound by its ligand, these receptors have been shown to trigger cell death in the absence of the ligand. Thus, expression of these receptors leads to dependence on the ligand for cell survival. Recent reports highlight the importance of dependence mechanisms during embryogenesis and tumorigenesis (57). The physiological relevance of the pro-apoptotic

function of the Met factor is unknown, but the strong involvement of Met in regulating the survival/apoptosis balance during development and cancer suggests that this mechanism could play important physiological roles.

Met shedding and degradation

The Met receptor, like other transmembrane proteins, is also processed by presenilin-regulated intramembrane proteolysis (PS-RIP). This proteolytic process involves an initial cleavage by membrane metolloproteases, called shedding. The involvement of ADAM10, known to cleave several membrane receptors, in this initial cleavage is suggested by the inhibition of Met cleavage upon ADAM10 silencing (58, 59). The initial cleavage generates a soluble N-terminal fragment (Met-NTF), which is shed into the extracellular space, and a membrane-anchored C-terminal fragment (Met-CTF) (25, 60-62). The latter is in turn cleaved at the membrane by the γ-secretase complex, of which presenilin is the catalytic subunit. The generated remnant intracellular domain of Met (Met-ICD) is released into the cytosol and degraded by the proteasome (63) (Fig 2B). Although various agents such as phorbol esters and suramin can promote Met shedding, the process occurs basally under normal conditions, and the generated fragment is efficiently degraded by the proteasome. Taken together, this regulated proteolysis decreases the half-life of the Met receptor, preventing its membrane accumulation. It is thus proposed to promote a basal, weak degradation of mature Met under steady-state conditions (64).

Downregulation of the Met receptor by PS-RIP contrasts with ligand-dependent degradation in that the proteolytic process is not activated by HGF/SF stimulation and does not require tyrosine kinase activity. This suggests the existence of two unconnected pathways involved in degrading Met according to its activation status. Yet recent evidence suggests that ligand-dependent and ligand-independent degradation of Met may share common features: we have demonstrated that the C-terminal fragment generated by ectodomain shedding can escape γ-secretase cleavage and be degraded directly via the lysosomal pathway (Fig. 2B). In contrast to canonical PS-RIP, this process requires internalization of the receptor and trafficking to the lysosomal compartment. Simultaneous inhibition of the proteasomal and lysosomal degradation pathways synergistically induces accumulation of C-terminal Met fragments, demonstrating that these pathways are complementary (Ancot et al, submitted). The degradation of Met C-terminal fragments in the lysosome after receptor internalization is thus reminiscent of HGF/SF-triggered degradation of full-length Met, but it does not require the

tyrosine kinase activity of Met, and no ubiquitination of the generated fragments was detected.

Met degradation by leucine-rich repeat containing proteins

Degradation through interaction with LRIG1

LRIG1 is a transmembrane leucine-rich repeat (LRR) and immunoglobulin-like domain containing protein. In addition to interacting with all four ErbB receptors and enhancing their ubiquitination and degradation, LRIG1 has been shown to interact with the Met receptor and to induce its downregulation in various cell lines(65). In contrast to degradation of the ErbB receptors, however, LRIG1-induced Met downregulation is ubiquitination independent and does not involve the E3 ubiquitin ligase CBL. The lysosomal compartment is involved in this downregulation, since the downregulation decreases when lysosome activity is inhibited (Fig 3A). Overexpression of LRIG1 in normal and tumoral epithelial cells represses Met expression and inhibits HGF/SF-induced responses, including cell growth and migration. LRIG1 thus appears as an efficient regulator of Met half-life and activity (65).

Decorin induces restricted phosphorylation and promotes degradation

Decorin is a member of the small leucine-rich proteoglycan family. Proteins of this family are known to interact with multiple RTKs. Decorin binds directly with high affinity (Kd≈1.5 nM) to the extracellular region of Met. Although it induces tyrosine phosphorylation of Met and causes efficient Met downregulation, it does not induce any biological responses in epithelial cells. Met downregulation is concomitant with receptor internalization, as in the case of HGF/SF-dependent degradation. Interestingly, the Met phosphorylation triggered by decorin is restricted to Y1234 and Y1235 in the kinase domain and Y1003 in the juxtamembrane domain. Y1349 in the C-terminal tail is not phosphorylated. This restricted phosphorylation is proposed to be sufficient to induce internalization and degradation of Met, through recruitment of CBL to Y1003, but not to allow induction of positive downstream signaling, since a tyrosine of the multi-substrate docking site is not phosphorylated (Fig 3B). Decorin also appears to induce degradation of Met through ectodomain shedding, as decorin treatment promotes the release Met-NTF into the extracellular medium and as this release is inhibited by the metalloprotease inhibitors TIMP2 and TIMP3 (66).

Interestingly, decorin-induced downregulation of Met is accompanied by proteasomal degradation of β-catenin and the Myc transcription factor. Accordingly, decorin induces Myc

phosphorylation at threonine residue 58, known to stimulate the ubiquitination of Myc and its subsequent degradation by the proteasome. Target genes of β-catenin and Myc, such as the cyclin D1 and CDK6 genes, also appear to be downregulated in response to decorin. The products of these genes are well-known regulators of cell cycle, suggesting that the cell growth arrest observed in response to decorin could be due to inhibition of these genes (67). Taken together, these results suggest that decorin-induced Met degradation might trigger a degradation process affecting a wider range of target proteins, including key actors of downstream signaling like β-catenin and the Myc transcription factor.

InlB of *Listeria* hijacks the endocytic machinery controlled by Met

The Gram-positive bacterium *Listeria monocytogenes* is an opportunistic pathogen found in food and capable notably of causing meningitis or abortions in pregnant women. The entry of *Listeria* into host cells, an important step in infection, is mediated by the listerial surface proteins InlA (internalin) and InlB. These internalins contain leucine-rich repeats and are capable of binding various host-cell membrane proteins. Through its LRR domain, InlB can notably bind with high affinity to the extracellular region of Met. The resolved structure of the InlB-Met complex reveals that the LRR domain of InlB interacts with the first immunoglobulin-like domain of the Met stalk and with the Met sema domain, two interaction regions distinct from the HGF/SF interaction region (68). Importantly, the presence of Met receptors at the host-cell surface is crucial for InlB-dependent internalization of the bacteria. InlB can also trigger the same response as HGF/SF, including receptor tyrosine phosphorylation, activation of downstream signaling pathways, Met receptor degradation, and finally biological responses such as scattering (69). The recently resolved structures of InlB and of the InlB-Met complex reveal a 2:2 stoichiometry with a back-to-back InlB dimer enabling direct contact between the stalk regions of two Met molecules (70). This dimerization allows activation of downstream signaling pathways, including phosphorylation of GAB1 and activation of PI3-kinase, required for InlB-mediated entry (71). This suggests that *Listeria* uses more than just Met binding to enter a host cell. Accordingly, the Met receptor has been shown to colocalize with *Listeria* during its endocytocis in clathrin-rich vesicles. This entry is accompanied by CBL recruitment and subsequent ubiquitination of Met. In addition, silencing of CBL or of various actors in RTK-mediated endosomal internalization, such as clathrin, dynamin, CIN85, and HRS, inhibits *Listeria* invasion (72). Altogether, these data demonstrate that Listeria hijacks the endocytic machinery controlled by Met through direct interaction with and activation of the RTK (Fig. 3C).

Three leucine-rich repeat proteins for three different consequences on Met

Although the three LRR-containing proteins LRIG1, decorin, and InlB are all able to bind the extracellular region of Met and to induce its degradation, they have distinct mechanisms of action and different functional consequences on downstream signaling. LRIG1 does not induce Met activation. Consequently, degradation of the receptor is independent of CBL and ubiquitination (65). In contrast, decorin induces restricted phosphorylation of Met, sufficient to induce Met internalization and degradation through recruitment of CBL, but insufficient to induce positive downstream signaling (66). Finally, InlB is a full agonist capable of triggering the various responses induced by HGF/SF, including both CBL- and ubiquitination-dependent degradation of Met and positive signaling (72). It would be tempting to propose that these distinct responses result from binding to different Met domains, but although InlB and HGF/SF do not interact with the same domain of Met, both can activate it fully. Conversely, the ability of decorin to interfere with HGF/SF-Met binding suggests a shared interaction domain, but decorin does not trigger the HGF/SF-induced responses. Perhaps the response depends on the strength of Met dimerization, strong dimerization being required for full activation of Met, including its downregulation, and weak dimerization leading to partial phophorylation and receptor degradation without positive signaling.

How to take advantage of Met degradation to fight cancer?

In many kinds of cancer, overexpression of the Met receptor has been observed. About half of all gastric tumors, for instance, overexpress Met. Met overexpression can result from various mechanisms, such as gene amplification as in gastric cancers (19) or increased transcription induced by oncogenes (20, 21) or hypoxia (22). Too much Met can also result from defective down-regulation. It has been shown, for instance, that a Met receptor mutated at the CBL juxtamembrane binding site Y1003 can induce constitutive scattering of epithelial cells and transformation of fibroblasts, probably because of its impaired downregulation and prolonged half-life (28). Interestingly, several intronic mutations resulting in deletion of exon 14, which includes the CBL-binding site, and more rarely mutation of Y1003 have been found in non-small-cell lung cancers (73-75) (www.sanger.ac.uk/genetics/CGP/cosmic/). The oncogenic fusion protein TPR-Met can also escape lysosomal degradation. Redirection of TPR-Met to the degradation pathway has been obtained through forced targeting of the oncogene to the plasma membrane or by adding the missing juxtamembrane domain containing the functional CBL binding site Y1003 (Fig. 4). Cells expressing the engineered degradable TPR-Met

display reduced anchorage-independent growth and tumorigenic capacity, especially in the presence of CBL overexpression (76). Altogether, these results demonstrate that transformation induced by Met results not only from aberrant kinase activation, but also from a defect in the lysosomal degradation pathway.

Along the same line, in addition to displaying constitutive tyrosine phosphorylation, Met having a D1246N or an M1268T mutation in its kinase domain exhibits increased endocytosis and recycling activity leading to a decreased level of degradation. Consequently, the mutant receptors accumulate in the signaling-competent endosomal compartments and induce the Rac1 GTPase and cell migration. Accordingly, blocking of endocytosis inhibits anchorage-independent growth and experimental tumorigenesis induced by the mutant receptors. The transforming properties of these active tyrosine kinase mutants thus result also from altered endocytic trafficking, which promotes recycling at the expense of degradation (77) (Fig. 4).

Recent study suggests that impaired-Met degradation is associated to other diseases. Indeed, autosomal dominant polycystic kidney disease (ADPKD) is a disorder caused by mutations at two loci, polycystin 1 and 2 (PKD1 and 2) and characterized by formation of multiple cysts in kidney that can lead to renal failure. In PKD1 deficient kidney cells, it has been shown that ligand-induced Met degradation is impaired and is associated to failure in its ubiquitination. Interestingly, the E3-ubiquitin ligase CBL was found sequestered in the Golgi apparatus in these cells, suggesting its inability to ubiquitinate Met. Finally, treatment with pharmacological inhibitor directed against the Met kinase results in inhibition of multiple cyst formation in kidney organ culture and in the mouse model of ADPKD (78). Thus, blockage of Met appears to be a potential novel therapeutic strategy to circumvent failure of the receptor degradation observed in this disease.

The fact that Met can be downregulated through multiple degradation pathways suggests an attractive strategy for circumventing its aberrant activation in cancer: forcing its degradation. Several monoclonal antibodies have been engineered to develop targeted therapy against the Met receptor. These antibodies are directed against the extracellular domain of Met, the aim being to hamper its interaction with HGF/SF-Met and its subsequent activation. Interestingly, the DN30 monoclonal antibody against extracellular Met, developed by Comoglio's team, displays an unexpected property: it induces efficient Met degradation accompanied by release of an extracellular N-terminal and an unstable intracellular C-teminal fragment (79). Actually, the antibody promotes Met PS-RIP with initial shedding effected mainly by ADAM10 and generation of the membrane-anchored Met-CTF, followed by γ-secretase cleavage and generation of the cytoplasmic Met-ICD (59, 63, 79). As observed under steady-state

conditions, forced downregulation of Met by DN30 involves degradation of Met-CTF in the lysosome and degradation of Met-ICD by the proteasome (63) (Ancot et al, submitted). Injection of DN30 in mice can inhibit growth of experimental tumors established from cells overexpressing Met, suggesting that artificial reactivation of a degradation pathway targeting an RTK is indeed an attractive therapeutic strategy. It has recently been shown that a one-arm version of anti-DN30 antibody, preventing undesirable dimerization of Met, can still induce PS-RIP and subsequent degradation of the receptor (80).

Likewise, Met degradation promoted by the leucine-rich repeat proteins decorin and LRIG1 prevents Met-induced cell transformation. Systemic delivery of decorin inhibits growth of tumor xenografts in a mouse model. Concomitantly, decorin treatment reduces the expression of Met and of the downstream signaling protein β-catenin in tumor samples. When fluorescently labeled decorin is administered, its distribution demonstrates its preferential association in vivo with the tumor xenograft and the kidneys (67). This suggests that decorin can target the tumor tissue specifically, likely through its interaction with the Met receptor. There is also evidence suggesting inhibition of cell transformation by the LLR protein LRIG1. MDCK normal epithelial cells overexpressing an activated form of ErbB2 (NeuT) display constitutive invasiveness (migration through Matrigel), further enhanced by HGF/SF. In these cells, expression of LRIG1 reduces Met and NeuT expression and abolishes invasion. Inhibition of RTK activity by LRIG1 thus appears to reverse cell transformation (65). Taken together, these results suggest that forced degradation of Met by physiological regulators of its stability can indeed avoid cell transformation and experimental tumorigenesis. This opens the way to novel strategies targeting the receptor.

Conclusion

In this review we highlight recent studies describing the multiple pathways of Met degradation. Interestingly, these data support novel views regarding the involvement of Met degradation in regulating the activity of the receptor. HGF/SF-dependent Met degradation is proposed as a means of attenuating the signaling induced by the activated receptor. This degradation within the intracellular lysosomal compartment requires Met internalization. However, internalization is involved in activation of sustained Met signaling, with Met remaining a signaling-competent receptor throughout the internalization process. This favors notably the redirection of signals to particular compartments such as the nucleus. Furthermore, the internalized Met receptor can escape degradation through active recycling and retargeting to the membrane. Thus, Met signaling definitely does not stop with

internalization of ligand-activated Met, since degradation is not the only possible destination of the receptor.

Besides the HGF/SF-dependent degradation of Met, many other degradation pathways are described. All of them occur without HGF/SF stimulation and thus cannot be viewed as mechanisms for attenuating induced signaling. Yet parts of these novel degradation pathways might act preventively to downregulate Met before its potential activation. For instance, caspase-dependent cleavages downregulate Met during apoptosis, preventing the potential induction of survival responses by the ligand-activated receptor. Likewise, Met degradation by γ-secretase under steady-state conditions prevents overexpression and aberrant activation of the receptor by decreasing the half-life of its mature membrane-anchored form. The physiological relevance of Met degradation induced by the leucine-rich repeat proteins decorin and LRIG1 is still elusive. Nevertheless, we can speculate that expression of these proteins might be associated with physiological conditions requiring dynamic downregulation of membrane Met, preventing its activation by HGF/SF. Decorin, it is worth noting, can interfere with HGF/SF-Met binding and thus might prevent ligand-dependent Met activation.

Interestingly, all these degradation pathways share common features with canonical HGF/SF-dependent downregulation, demonstrating that the molecular mechanisms governing Met degradation are exploited under many biological conditions. In this respect, the example of *Listeria monocytogenes* protein InlB is remarkable: activation of Met by InlB mimics perfectly the activation induced by HGF/SF, causing receptor internalization and the entry of bacteria into the cell. Lastly, failure to degrade Met is directly associated with its transforming activity in cancer cells while conversely, forced Met degradation is an attractive novel strategy for targeting the receptor.

Acknowledgments

This work was supported by the CNRS, the Institut Pasteur de Lille, and INSERM, and by grants from the "Ligue contre le Cancer, comité Nord", the “Association pour la Recherche sur le Cancer”, and the “Agence Nationale de la Recherche”, Young investigator Program. FA was supported by a fellowship from the “Association pour la Recherche sur le Cancer”, AL and GM by the “Agence Nationale pour la Recherche”, JL by a grant from the French Research and Technology Ministry.

References

1. Park, M., Dean, M., Kaul, K., Braun, M. J., Gonda, M. A., and Vande Woude, G. (1987) Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. *Proc Natl Acad Sci U S A* **84**, 6379-6383
2. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G. F. (2003) Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* **4**, 915-925
3. Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. (1995) Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376**, 768-771.
4. Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. (1995) Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* **373**, 699-702
5. Uehara, Y., Minowa, O., Mori, C., Shlota, K., Kuno, J., Noda, T., and Kitamura, N. (1995) Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature* **373**, 702-705
6. Maina, F., Hilton, M. C., Ponzetto, C., Davies, A. M., and Klein, R. (1997) Met receptor signaling is required for sensory nerve development and HGF promotes axonal growth and survival of sensory neurons. *Genes & development* **11**, 3341-3350.
7. Yant, J., Buluwela, L., Niranjan, B., Gusterson, B., and Kamalati, T. (1998) In vivo effects of hepatocyte growth factor/scatter factor on mouse mammary gland development. *Exp Cell Res* **241**, 476-481
8. Kawaida, K., Matsumoto, K., Shimazu, H., and Nakamura, T. (1994) Hepatocyte growth factor prevents acute renal failure and accelerates renal regeneration in mice. *Proc Natl Acad Sci U S A* **91**, 4357-4361
9. Borowiak, M., Garratt, A. N., Wustefeld, T., Strehle, M., Trautwein, C., and Birchmeier, C. (2004) Met provides essential signals for liver regeneration. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 10608-10613
10. Naldini, L., Weidner, K. M., Vigna, E., Gaudino, G. K., Bardelli, A., Ponzetto, C., Narsimhan, R. P., Hartmann, G., Zarnegar, R., Michalopoulos, G., Birchmeier, W., and Comoglio, P. M. (1991) Scatter factor and hepatocyte growth factor are undistinguishable ligands for the MET receptor. *EMBO J.* **10**, 2867-2878
11. Ferracini, R., Longati, P., Naldini, L., Vigna, E., and Comoglio, P. M. (1991) Identification of the major autophosphorylation site of the Met/hepatocyte growth factor receptor tyrosine kinase. *J Biol Chem* **266**, 19558-19564
12. Longati, P., Bardelli, A., Ponzetto, C., Naldini, L., and Comoglio, P. M. (1994) Tyrosines1234-1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). *Oncogene* **9**, 49-57
13. Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graaziani, A., Panayotou, G., and Comoglio, P. M. (1994) A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell* **77**, 261-271
14. Weidner, K. M., Sachs, M., and Birchmeier, W. (1995) Mutation of juxtamembrane tyrosine residue 1001 suppresses loss-of-function mutations of the met receptor in epithelial cells. *Proc. Natl. Acad. Sci. USA* **92**, 2597-2601
15. Tulasne, D., Paumelle, R., Weidner, K. M., Vandenbunder, B., and Fafeur, V. (1999) The multisubstrate docking site of the MET receptor is dispensable for MET-mediated RAS signaling and cell scattering. *Mol Biol Cell* **10**, 551-565
16. Park, M., Dean, M., Cooper, C. S., Schmidt, M., O'Brien, S. J., Blair, D. G., and Vande Woude, G. F. (1986) Mechanism of met oncogene activation. *Cell* **45**, 895-904

17. Schmidt, L., Duh, F. M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S. W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U. R., Feltis, J. T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C. J., Walther, M. M., Tsui, L. C., Geil, L., Orcutt, M. L., Stackhouse, T., Zbar, B., and et al. (1997) Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nat Genet* **16**, 68-73.
18. Ponzetto, C., Giordano, S., Peverali, F., Della Valle, G., Abate, M. L., Vaula, G., and Comoglio, P. M. (1991) c-met is amplified but not mutated in a cell line with an activated met tyrosine kinase. *Oncogene* **6**, 553-559
19. Di Renzo, M. F., Olivero, M., Giacomini, A., Porte, H., Chastre, E., Mirossay, L., Nordlinger, B., Bretti, S., Bottardi, S., Giordano, S., and et al. (1995) Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. *Clin Cancer Res* **1**, 147-154
20. Gambarotta, G., Boccaccio, C., Giordano, S., Ando, M., Stella, M. C., and Comoglio, P. M. (1996) Ets up-regulates MET transcription. *Oncogene* **13**, 1911-1917
21. Ivan, M., Bond, J. A., Prat, M., Comoglio, P. M., and Wynford-Thomas, D. (1997) Activated ras and ret oncogenes induce over-expression of c-met (hepatocyte growth factor receptor) in human thyroid epithelial cells. *Oncogene* **14**, 2417-2423
22. Pennacchietti, S., Michieli, P., Galluzzo, M., Mazzone, M., Giordano, S., and Comoglio, P. M. (2003) Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell* **3**, 347-361
23. Migliore, C., Petrelli, A., Ghiso, E., Corso, S., Capparuccia, L., Eramo, A., Comoglio, P. M., and Giordano, S. (2008) MicroRNAs impair MET-mediated invasive growth. *Cancer Res* **68**, 10128-10136
24. Yan, D., Dong Xda, E., Chen, X., Wang, L., Lu, C., Wang, J., Qu, J., and Tu, L. (2009) MicroRNA-1/206 targets c-Met and inhibits rhabdomyosarcoma development. *J Biol Chem* **284**, 29596-29604
25. Jeffers, M., Taylor, G. A., Weidner, K. M., Omura, S., and Vande-Woude, G. F. (1997) Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. *Molecular and cellular biology* **17**, 799-808
26. Abella, J. V., and Park, M. (2009) Breakdown of endocytosis in the oncogenic activation of receptor tyrosine kinases. *Am J Physiol Endocrinol Metab* **296**, E973-984
27. Czech, M. P. (2000) PIP2 and PIP3: complex roles at the cell surface. *Cell* **100**, 603-606
28. Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y., and Park, M. (2001) Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Mol Cell* **8**, 995-1004.
29. Carter, S., Urbe, S., and Clague, M. J. (2004) The met receptor degradation pathway: requirement for Lys48-linked polyubiquitin independent of proteasome activity. *J Biol Chem* **279**, 52835-52839
30. Abella, J. V., Peschard, P., Naujokas, M. A., Lin, T., Saucier, C., Urbe, S., and Park, M. (2005) Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. *Mol Cell Biol* **25**, 9632-9645
31. Take, H., Watanabe, S., Takeda, K., Yu, Z. X., Iwata, N., and Kajigaya, S. (2000) Cloning and characterization of a novel adaptor protein, CIN85, that interacts with c-Cbl. *Biochem Biophys Res Commun* **268**, 321-328
32. Petrelli, A., Gilestro, G. F., Lanzardo, S., Comoglio, P. M., Migone, N., and Giordano, S. (2002) The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. *Nature* **416**, 187-190

33. Soubeyran, P., Kowanetz, K., Szymkiewicz, I., Langdon, W. Y., and Dikic, I. (2002) Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors. *Nature* **416**, 183-187
34. Hammond, D. E., Carter, S., McCullough, J., Urbe, S., Vande Woude, G., and Clague, M. J. (2003) Endosomal dynamics of Met determine signaling output. *Mol Biol Cell* **14**, 1346-1354
35. Salcini, A. E., Chen, H., Iannolo, G., De Camilli, P., and Di Fiore, P. P. (1999) Epidermal growth factor pathway substrate 15, Eps15. *The international journal of biochemistry & cell biology* **31**, 805-809
36. de Melker, A. A., van der Horst, G., and Borst, J. (2004) Ubiquitin ligase activity of c-Cbl guides the epidermal growth factor receptor into clathrin-coated pits by two distinct modes of Eps15 recruitment. *J Biol Chem* **279**, 55465-55473
37. Parachoniak, C. A., and Park, M. (2009) Distinct recruitment of Eps15 via Its coiled-coil domain is required for efficient down-regulation of the met receptor tyrosine kinase. *J Biol Chem* **284**, 8382-8394
38. Raiborg, C., Bache, K. G., Mehlum, A., Stang, E., and Stenmark, H. (2001) Hrs recruits clathrin to early endosomes. *Embo J* **20**, 5008-5021
39. Gillooly, D. J., Raiborg, C., and Stenmark, H. (2003) Phosphatidylinositol 3-phosphate is found in microdomains of early endosomes. *Histochemistry and cell biology* **120**, 445-453
40. Mizuno, E., Kawahata, K., Okamoto, A., Kitamura, N., and Komada, M. (2004) Association with Hrs is required for the early endosomal localization, stability, and function of STAM. *Journal of biochemistry* **135**, 385-396
41. Komada, M., and Kitamura, N. (2005) The Hrs/STAM complex in the downregulation of receptor tyrosine kinases. *Journal of biochemistry* **137**, 1-8
42. Bache, K. G., Brech, A., Mehlum, A., and Stenmark, H. (2003) Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. *J Cell Biol* **162**, 435-442
43. Hammond, D. E., Urbe, S., Vande Woude, G. F., and Clague, M. J. (2001) Down-regulation of MET, the receptor for hepatocyte growth factor. *Oncogene* **20**, 2761-2770
44. Goormachtigh, G., Ji, Z., Le Goff, A., and Fafeur, V. (2011) Degradation of the GAB1 adaptor by the ubiquitin-proteasome pathway hampers HGF/SF-MET signaling. *Biochem Biophys Res Commun* **411**, 780-785
45. Parachoniak, C. A., Luo, Y., Abella, J. V., Keen, J. H., and Park, M. (2011) GGA3 functions as a switch to promote Met receptor recycling, essential for sustained ERK and cell migration. *Dev Cell* **20**, 751-763
46. Kermorgant, S., Zicha, D., and Parker, P. J. (2003) Protein kinase C controls microtubule-based traffic but not proteasomal degradation of c-Met. *J Biol Chem* **278**, 28921-28929
47. Kermorgant, S., Zicha, D., and Parker, P. J. (2004) PKC controls HGF-dependent c-Met traffic, signalling and cell migration. *Embo J* **23**, 3721-3734
48. Kermorgant, S., and Parker, P. J. (2008) Receptor trafficking controls weak signal delivery: a strategy used by c-Met for STAT3 nuclear accumulation. *J Cell Biol* **182**, 855-863
49. Boccaccio, C., Ando, M., Tamagnone, L., Bardelli, A., Michieli, P., Battistini, C., and Comoglio, P. M. (1998) Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. *Nature* **391**, 285-288.
50. Zhang, Y. W., Wang, L. M., Jove, R., and Vande Woude, G. F. (2002) Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis. *Oncogene* **21**, 217-226
51. McShane, M. P., and Zerial, M. (2008) Survival of the weakest: signaling aided by endosomes. *J Cell Biol* **182**, 823-825

52. Luthi, A. U., and Martin, S. J. (2007) The CASBAH: a searchable database of caspase substrates. *Cell Death Differ* **14**, 641-650
53. Ancot, F., Foveau, B., Lefebvre, J., Leroy, C., and Tulasne, D. (2009) Proteolytic cleavages give receptor tyrosine kinases the gift of ubiquity. *Oncogene* **28**, 2185-2195
54. Tulasne, D., Deheuninck, J., Lourenco, F. C., Lamballe, F., Ji, Z., Leroy, C., Puchois, E., Moumen, A., Maina, F., Mehlen, P., and Fafeur, V. (2004) Proapoptotic function of the MET tyrosine kinase receptor through caspase cleavage. *Mol Cell Biol* **24**, 10328-10339
55. Deheuninck, J., Foveau, B., Goormachtigh, G., Leroy, C., Ji, Z., Tulasne, D., and Fafeur, V. (2008) Caspase cleavage of the MET receptor generates an HGF interfering fragment. *Biochem Biophys Res Commun* **367**, 573-577
56. Foveau, B., Leroy, C., Ancot, F., Deheuninck, J., Ji, Z., Fafeur, V., and Tulasne, D. (2007) Amplification of apoptosis through sequential caspase cleavage of the MET tyrosine kinase receptor. *Cell Death Differ* **14**, 752-764
57. Goldschneider, D., and Mehlen, P. (2010) Dependence receptors: a new paradigm in cell signaling and cancer therapy. *Oncogene* **29**, 1865-1882
58. Kopitz, C., Gerg, M., Bandapalli, O. R., Ister, D., Pennington, C. J., Hauser, S., Flechsig, C., Krell, H. W., Antolovic, D., Brew, K., Nagase, H., Stangl, M., von Weyhern, C. W., Brucher, B. L., Brand, K., Coussens, L. M., Edwards, D. R., and Kruger, A. (2007) Tissue inhibitor of metalloproteinases-1 promotes liver metastasis by induction of hepatocyte growth factor signaling. *Cancer Res* **67**, 8615-8623
59. Schelter, F., Kobuch, J., Moss, M. L., Becherer, J. D., Comoglio, P. M., Boccaccio, C., and Kruger, A. (2010) A disintegrin and metalloproteinase-10 (ADAM-10) mediates DN30 antibody-induced shedding of the met surface receptor. *J Biol Chem* **285**, 26335-26340
60. Galvani, A. P., Cristiani, C., Carpinelli, P., Landonio, A., and Bertolero, F. (1995) Suramin modulates cellular levels of hepatocyte growth factor receptor by inducing shedding of a soluble form. *Biochem Pharmacol* **50**, 959-966
61. Prat, M., Crepaldi, T., Gandino, L., Giordano, S., Longati, P., and Comoglio, P. (1991) C-terminal truncated forms of Met, the hepatocyte growth factor receptor. *Mol Cell Biol* **11**, 5954-5962
62. Wajih, N., Walter, J., and Sane, D. C. (2002) Vascular origin of a soluble truncated form of the hepatocyte growth factor receptor (c-met). *Circ Res* **90**, 46-52
63. Foveau, B., Ancot, F., Leroy, C., Petrelli, A., Reiss, K., Vingtdeux, V., Giordano, S., Fafeur, V., and Tulasne, D. (2009) Downregulation of the Met Receptor Tyrosine Kinase by Presenilin-dependent Regulated Intramembrane Proteolysis. *Mol Biol Cell* **20**, 2494-2506
64. Trusolino, L., Bertotti, A., and Comoglio, P. M. (2010) MET signalling: principles and functions in development, organ regeneration and cancer. *Nat Rev Mol Cell Biol* **11**, 834-848
65. Shattuck, D. L., Miller, J. K., Laederich, M., Funes, M., Petersen, H., Carraway, K. L., 3rd, and Sweeney, C. (2007) LRIG1 is a novel negative regulator of the Met receptor and opposes Met and Her2 synergy. *Mol Cell Biol* **27**, 1934-1946
66. Goldoni, S., Humphries, A., Nystrom, A., Sattar, S., Owens, R. T., McQuillan, D. J., Ireton, K., and Iozzo, R. V. (2009) Decorin is a novel antagonistic ligand of the Met receptor. *J Cell Biol* **185**, 743-754
67. Buraschi, S., Pal, N., Tyler-Rubinstein, N., Owens, R. T., Neill, T., and Iozzo, R. V. (2010) Decorin antagonizes Met receptor activity and down-regulates {beta}-catenin and Myc levels. *J Biol Chem* **285**, 42075-42085
68. Niemann, H. H., Jager, V., Butler, P. J., van den Heuvel, J., Schmidt, S., Ferraris, D., Gherardi, E., and Heinz, D. W. (2007) Structure of the human receptor tyrosine kinase met in complex with the Listeria invasion protein InlB. *Cell* **130**, 235-246
69. Shen, Y., Naujokas, M., Park, M., and Ireton, K. (2000) InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. *Cell* **103**, 501-510

70. Ferraris, D. M., Gherardi, E., Di, Y., Heinz, D. W., and Niemann, H. H. (2010) Ligand-mediated dimerization of the Met receptor tyrosine kinase by the bacterial invasion protein InlB. *Journal of molecular biology* **395**, 522-532
71. Ireton, K., Payrastre, B., Chap, H., Ogawa, W., Sakaue, H., Kasuga, M., and Cossart, P. (1996) A role for phosphoinositide 3-kinase in bacterial invasion. *Science* **274**, 780-782
72. Veiga, E., and Cossart, P. (2005) Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells. *Nat Cell Biol* **7**, 894-900
73. Kong-Beltran, M., Seshagiri, S., Zha, J., Zhu, W., Bhawe, K., Mendoza, N., Holcomb, T., Pujara, K., Stinson, J., Fu, L., Severin, C., Rangell, L., Schwall, R., Amler, L., Wickramasinghe, D., and Yauch, R. (2006) Somatic mutations lead to an oncogenic deletion of met in lung cancer. *Cancer Res* **66**, 283-289
74. Ma, P. C., Jagadeeswaran, R., Jagadeesh, S., Tretiakova, M. S., Nallasura, V., Fox, E. A., Hansen, M., Schaefer, E., Naoki, K., Lader, A., Richards, W., Sugarbaker, D., Husain, A. N., Christensen, J. G., and Salgia, R. (2005) Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. *Cancer Res* **65**, 1479-1488
75. Onozato, R., Kosaka, T., Kuwano, H., Sekido, Y., Yatabe, Y., and Mitsudomi, T. (2009) Activation of MET by gene amplification or by splice mutations deleting the juxtamembrane domain in primary resected lung cancers. *J Thorac Oncol* **4**, 5-11
76. Mak, H. H., Peschard, P., Lin, T., Naujokas, M. A., Zuo, D., and Park, M. (2007) Oncogenic activation of the Met receptor tyrosine kinase fusion protein, Tpr-Met, involves exclusion from the endocytic degradative pathway. *Oncogene* **26**, 7213-7221
77. Joffre, C., Barrow, R., Menard, L., Calleja, V., Hart, I. R., and Kermorgant, S. (2011) A direct role for Met endocytosis in tumorigenesis. *Nat Cell Biol* **13**, 827-837
78. Qin, S., Taglienti, M., Nauli, S. M., Contrino, L., Takakura, A., Zhou, J., and Kreidberg, J. A. (2010) Failure to ubiquitinate c-Met leads to hyperactivation of mTOR signaling in a mouse model of autosomal dominant polycystic kidney disease. *The Journal of clinical investigation* **120**, 3617-3628
79. Petrelli, A., Circosta, P., Granziero, L., Mazzone, M., Pisacane, A., Fenoglio, S., Comoglio, P. M., and Giordano, S. (2006) Ab-induced ectodomain shedding mediates hepatocyte growth factor receptor down-regulation and hampers biological activity. *Proc Natl Acad Sci U S A* **103**, 5090-5095
80. Pacchiana, G., Chiriaco, C., Stella, M. C., Petronzelli, F., De Santis, R., Galluzzo, M., Carminati, P., Comoglio, P. M., Michieli, P., and Vigna, E. (2010) Monovalency unleashes the full therapeutic potential of the DN-30 anti-Met antibody. *J Biol Chem* **285**, 36149-36157

Figure comments:

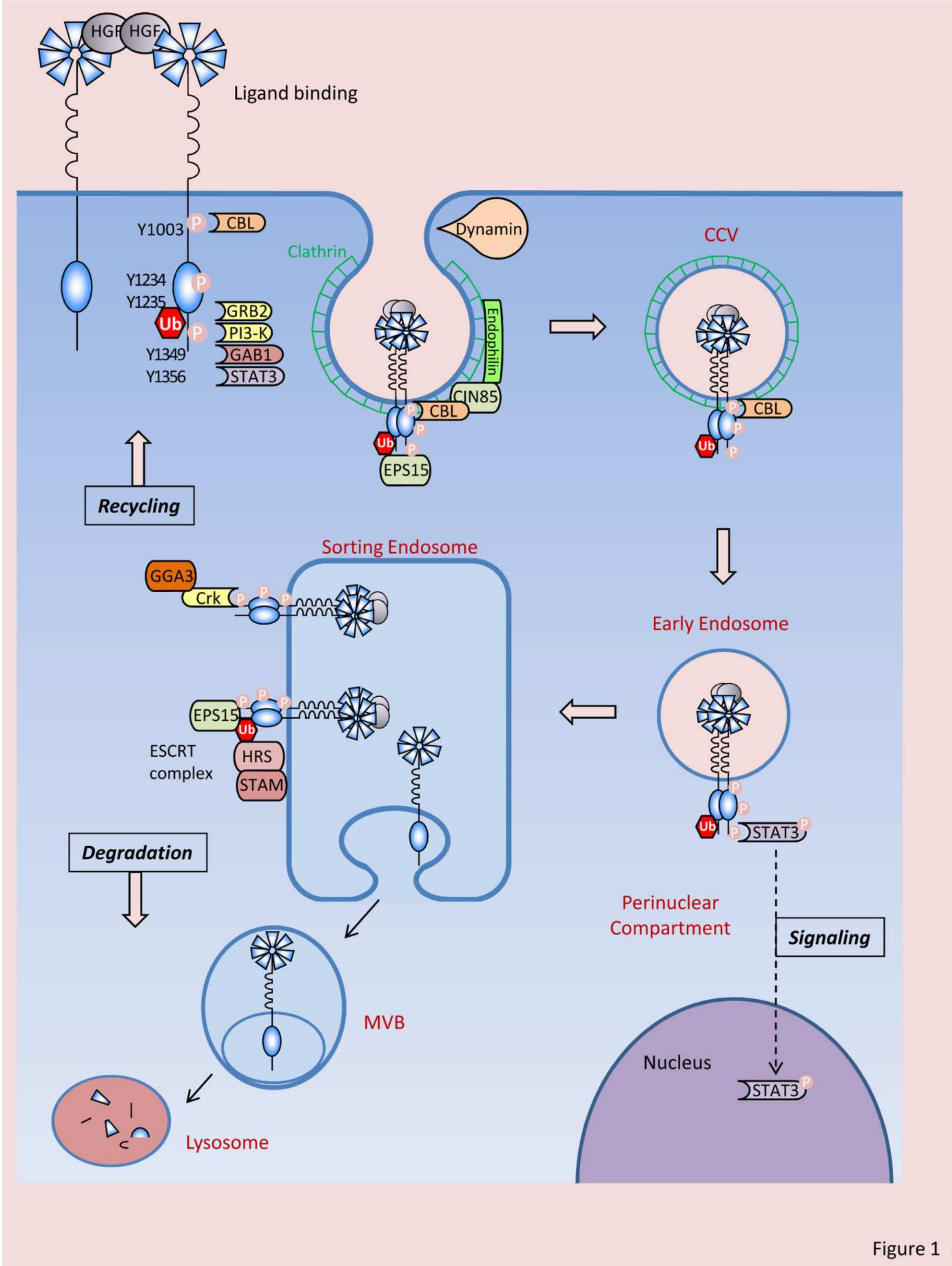


Figure 1: Signaling, recycling, and degradation of the HGF/SF-activated Met receptor

Upon HGF/SF binding, the Met receptor undergoes phosphorylation of the following tyrosine residues: Y1003 in the juxtamembrane domain, Y1234 and Y1235 in the kinase

domain, and Y1349 and Y1356 in the C-terminal multisubstrate docking site. The C-terminal phosphorylations lead to recruitment of signaling proteins, including GRB2, GAB1, PI3-K, and STAT3. Recruitment of CBL, notably through Y1003, leads to ubiquitination (Ub) of Met. The ubiquitinated receptor is targeted to clathrin-coated pit vesicles (CCVs). Invagination of the membrane and scission involve formation of a complex with Met including CBL, CIN85, endophilins, and EPS15. Dynamin is involved in vesicle scission from the membrane. Following trafficking in the early endosome, the vesicles fuse with the sorting endosome. The ubiquinated receptor molecules are retained through their interaction with the ubiquitn-interacting domain (UIM) containing the HRS protein. The endosomal sorting complex for transport (ESCRT) is then formed with STAM and EPS15, which initiates formation of the multivesicular body (MVB). Late MVBs are then targeted to the lysosome, where Met is degraded. In the sorting endosome, Met can also interact with GGA3 via the CRK adaptor, recruited by tyrosine-phosphorylated Met. This interaction is involved in recycling full-length Met to the plasma membrane. Within the endosomes, Met remains a signaling-competent receptor capable of inducing downstream signaling pathways. For instance, phosphorylated Met located within perinuclear endosomal vesicles can promote STAT3 nuclear translocation, a molecular mechanism proposed to support the weak initial STAT3 activation induced by Met.

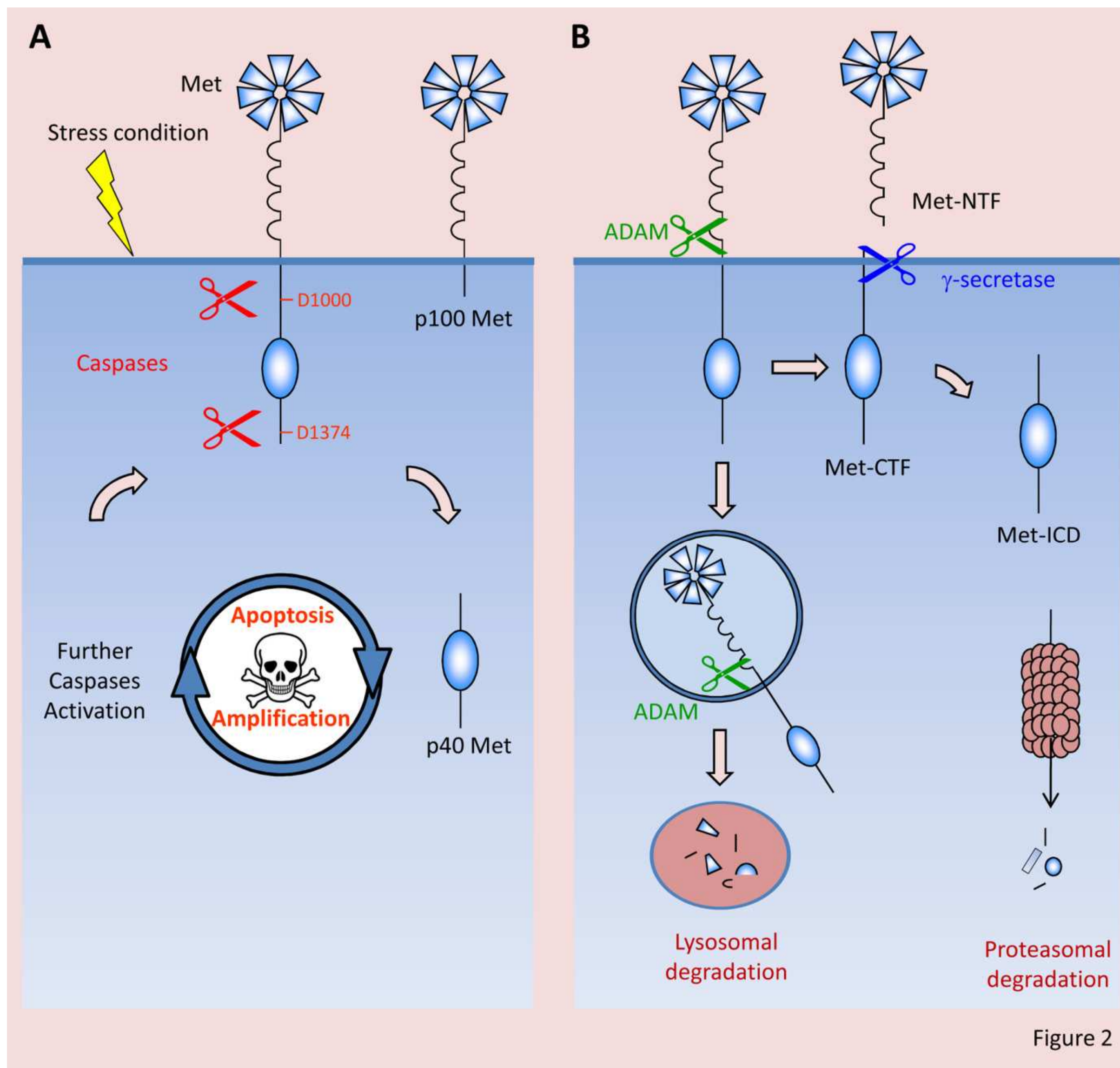


Figure 2: Met degradation through proteolytic cleavages

(A) In the absence of ligand and under conditions of stress, caspases can cleave Met at aspartic acid residue 1000 of the juxtamembrane region and aspartic acid residue 1374 of the C-terminal tail (mouse sequence). These cleavages split off the extracellular ligand-binding site from the intracellular kinase domain, generating two main fragments, p100 Met (still anchored to the membrane) and intracellular p40 Met. p100 Met conserves its ligand-binding capacity and can act as a decoy receptor for HGF/SF. p40 Met can induce caspase-3 activation and thus amplify cell death.

(B) Independently of ligand stimulation, the action of ADAM-family membrane metalloproteases causes Met to shed an extracellular N-terminal fragment (Met-NTF), thus generating a membrane-anchored C-terminal fragment (Met-CTF). After this initial cleavage, Met-CTF is cleaved at the membrane by the γ-secretase complex, generating a Met intracellular domain (Met-ICD). Both Met-CTF and Met-ICD are efficiently degraded. Met-

ICD is degraded by the proteasome, while Met-CTF can escape γ-secretase cleavage through internalization and trafficking to the lysosome and direct degradation therein. These cleavages and the subsequent degradation of Met fragments contribute to chronic degradation of Met under steady-state conditions.

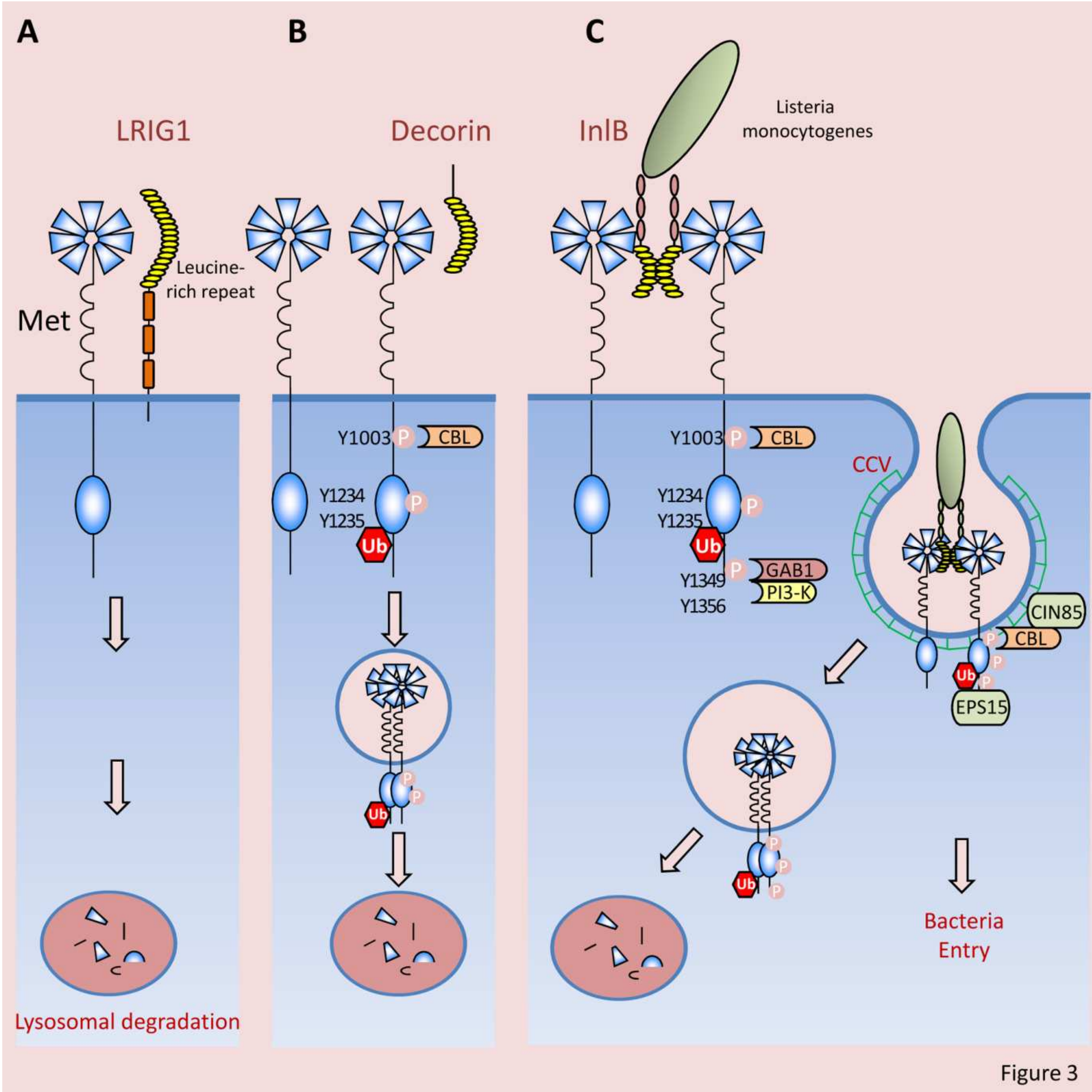


Figure 3: Degradation of Met by the leucin-rich repeat proteins LRIG1, decorin, and InlB

(A) LRIG1, a transmembrane leucine-rich repeat and immunoglobulin-like domain containing protein, induces downregulation of Met via a degradation process involving the lysosome. Although LRIG1 can induce ubiquitination and degradation of several EGF receptor-family members, Met degradation is independent of ubiquitination and of the E3 ubiquitin ligase CBL.

(B) Decorin, a member of the small leucine-rich proteoglycan family, can bind to the Met receptor with high affinity. This binding induces partial phosphorylation of the receptor (at Y1003 of the juxtamembrane domain and Y1234 and Y1235 of the kinase domain). In contrast, Y1349 of the C-terminal multisubstrate binding site appears not to be phosphorylated, which precludes activation of the downstream signaling pathways. Phosphorylated Y1003, however, can recruit the E3 ubiquitin ligase CBL, causing ubiquination (Ub), internalization, and degradation of Met in the lysosome.

(C) InlB of *Listeria monocytogenes*, a leucine-rich repeat containing protein, binds with high affinity to the extracellular domain of Met, forming a 2:2 complex with it. This leads to full tyrosine phosphorylation of Met (at residues 1003, 1234, 1235, 1349, and 1356) leading to recruitment of signaling proteins and activation of the downstream signaling pathways known to be induced by HGF/SF-activated Met. Recruitment of CBL leads to ubiquitination (Ub) of Met and further internalization into clathrin-coated pit vesicles (CCV) with involvement of the major components of the endocytic machinery, including CIN85 or EPS15. Internalization of Met together with InlB allows entry of the bacterium into the cell. The ubiquitinated receptor is then degraded in the lysosome.

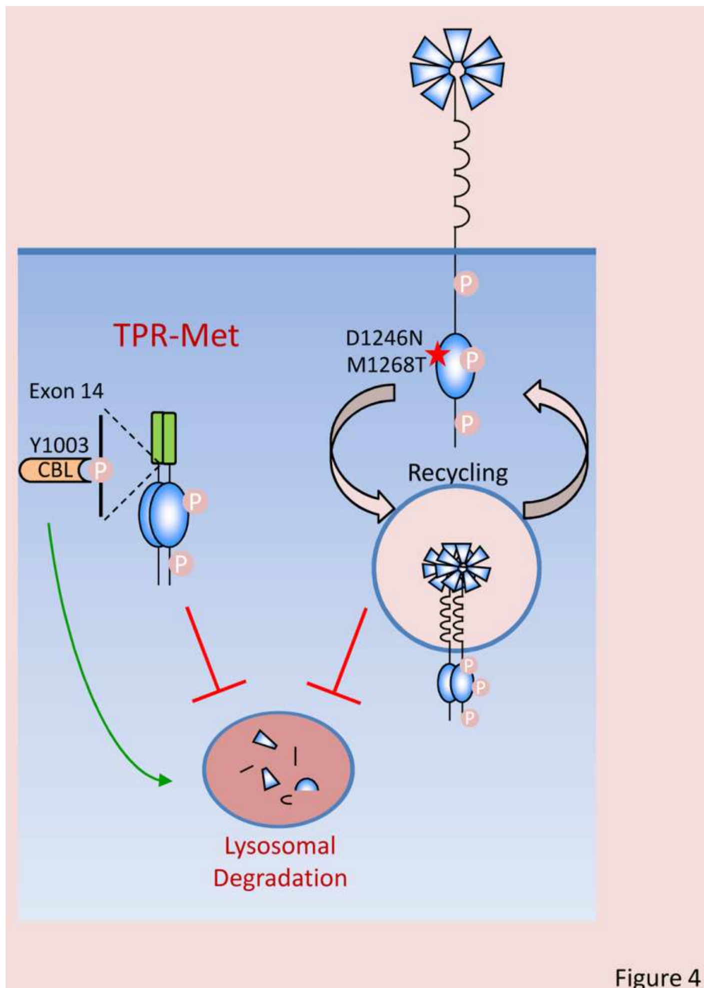


Figure 4: Activated forms of Met display resistance to degradation

Although the oncogenic fusion TPR-Met displays constitutive kinase activity, it can escape lysosomal degradation. Insertion of the missing juxtamembrane domain containing the CBL docking site restores degradation. The full-length Met receptor with an activating D1246N or M1268T mutation also displays reduced lysosomal degradation, due to more pronounced recycling of the active receptor to the plasma membrane.

B) PS-RIP

Comme cela a été abordé dans la revue ci-dessus (Lefebvre et al., 2010 en soumission), le récepteur Met est la cible de différentes protéases capable d'influencer ses fonctions biologiques. En réponse au ligand, Met est endocyté puis aiguillé vers la machinerie lysosomale pour y être dégradé. Cette dégradation participe à la signalisation de Met mais permet aussi de contrôler les réponses biologiques en diminuant la quantité de Met activé.

Il existe également une dégradation du récepteur Met qui opère en absence d'HGF/SF. Cette dégradation fait intervenir une seconde voie dite du « PS-RIP » (« presenilin-dependent regulated intramembrane proteolysis »). Ce mécanisme comporte deux clivages successifs. Le premier, en extracellulaire, aux abords de la membrane, est réalisé par les ADAM (A Disintegrin And Metalloproteinase). Cet élagage (*shedding*) libère dans le milieu extracellulaire un fragment N-terminal (« N-Terminal Fragment », NTF) et un fragment C-terminal qui reste ancré à la membrane (« C-Terminal Fragment », CTF). Ce fragment Met-CTF subit ensuite un deuxième clivage par le complexe γ-secrétase dont la sous-unité catalytique est la préséniline. Ce double clivage libère alors un domaine intracellulaire (ICD) qui est rapidement dégradé par le protéasome (Foveau *et al.*, 2009). En plus de la dégradation du fragment ICD par le protéasome, l'équipe a mis en évidence que le fragment CTF était pris en charge par la voie lysosomale. Cette voie de dégradation intervient suite à l'internalisation du récepteur. Celui-ci subit alors un premier un clivage par les ADAM, ce qui génère les fragments lyso-Met-NTF et lyso-Met-CTF. Ces fragments sont alors pris en charge par le lysosome. Ces deux mécanismes de dégradation constitutive agissent en synergie, limite la demi-vie du récepteur et réduit la quantité de récepteurs présents à la membrane. De manière intéressante, la dérégulation de ce mécanisme de dégradation par l'expression d'un récepteur non-clivable s'accompagne d'une quantité plus importante de récepteur à la membrane et d'une activité constitutive de celui-ci. A l'inverse, l'utilisation d'un anticorps (Le DN-30) qui induit la dégradation du récepteur Met en activant le mécanisme de PS-RIP, réduit la quantité de récepteurs à la membrane et diminue les capacités transformantes des cellules tumorales (Petrelli *et al.*, 2006).

Un autre mécanisme de dégradation du récepteur Met a été mis à jour par le laboratoire. En absence de ligand, le récepteur est clivé au cours de l'apoptose, une des formes de mort cellulaire. Ce clivage génère un fragment de 40 kDa, baptisé p40 Met et est médié par les caspases, une famille de protéases qui régule le bon déroulement du processus apoptotique. Avant de vous présenter les résultats de notre étude sur ce fragment, j'ai choisi d'introduire ici le déroulement du processus apoptotique.

VI) L'apoptose

Trois types de mort cellulaire ont été historiquement décrits. L'un dit « mort cellulaire programmée » regroupe l'apoptose et l'autophagie (morts cellulaires de type I et II) et fait intervenir des composés cellulaires pour l'initier et la contrôler. L'autre, la nécrose ou « mort cellulaire non programmée » (mort cellulaire de type III) est présentée classiquement comme une mort accidentelle en réponse à un stress important incompatible avec la survie cellulaire (Degterev and Yuan, 2008).

L'apoptose a été décrite pour la première fois en 1972 par John Kerr pour désigner une forme de mort cellulaire caractérisée par des modifications morphologiques typiques (Kerr *et al.*, 1972). La cellule est généralement arrondie et présente une réduction du volume cellulaire, un bourgeonnement de la surface membranaire, une condensation de la chromatine et une fragmentation du noyau (Wyllie *et al.*, 1980 ; Clarke, 1990) (Figure 11.A).

L'apoptose est basée sur l'existence d'un programme génétique capable de participer au développement embryonnaire. Il a été initialement démontré lors de l'étude de l'embryogénèse du nématode *Caenorhabditis elegans* qui, au cours de son développement, voit 131 cellules ni plus, ni moins, sur un total de 1090 cellules, mourir par apoptose (Figure 11.B) (Horvitz, 1999).

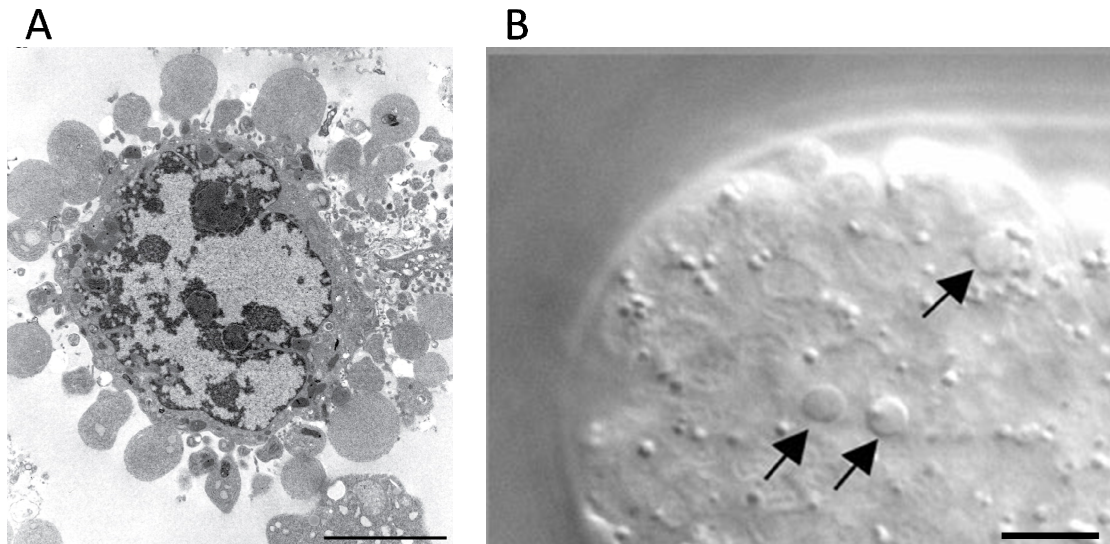


Figure 11 : Illustrations de cellules apoptotiques

(A) Micrographie en transmission de fibroblastes (NIH3T3) co-traités à la cycloheximideet au TNFα, pour induire l'apoptose (échelle 5µm) (Croft et al., 2005). Au niveau morphologique, on retrouve la présence de bourgeonnements membranaires, un phénotype typique retrouvé dans les cellules apoptotique.

(B) Image à contraste "Nomarski" d'un embryon de Caenorhabditis elegans (Stade comma). Les flèches indiquent trois cellules apoptotiques qui se distinguent par leur aspect arrondi. Echelle 5µm (source Wormbook).

De la même façon, l'apoptose joue un rôle prépondérant dans le développement embryonnaire des vertébrés et en assurant l'homéostasie tissulaire chez l'adulte (Krammer, 2000; Meier *et al.*, 2000) (Figure 12). Ainsi, chez l'embryon, l'élimination des cellules situées dans l'espace interdigité, au même titre que l'élimination des 131 cellules au cours du développement de *Caenorhabditis elegans,* est assuré par le processus apoptotique.

Au niveau cellulaire, la plupart des changements morphologiques sont le résultat de l'action de protéases activées pendant l'apoptose et qui pilotent le programme d'exécution : les caspases (Miura *et al.*, 1993 ; Yuan *et al.*, 1993). Dans cette partie, je décrirai tout d'abord les voies de signalisation contrôlant l'initiation de l'apoptose puis les conséquences de l'activation des caspases.

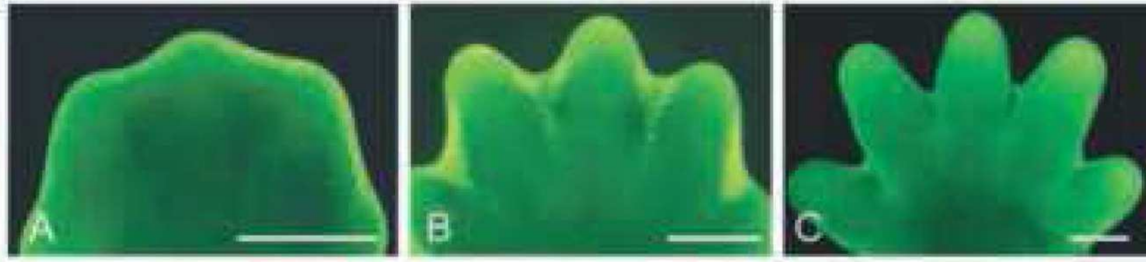


Figure 12 : Rôle de l'apoptose dans le développement du membre antérieur chez la souris

Suite à une coloration à l'acridine du membre antérieur, les cellules mortes apparaissent colorées en jaune. Embryon de souris à E12,5 / 13,5 / 14,5. (Wood et al., 2000).

A) La voie dite extrinsèque

La voie dite extrinsèque implique l'activation des récepteurs de mort. Ces récepteurs membranaires sont eux-mêmes activés par des ligands de mort comme FAS-L (Fas Ligand), le TNFα (Tumor Necrosis Factor) ou Apo2L/TRAIL (Apo-2 ligand ou Tumor Necrosis Related Apoptosis-Inducing Ligand) (Ashkenazi and Dixit, 1998). L'activation de ces récepteurs entraîne la formation de complexes multiprotéiques cytoplasmiques contenant la protéine FADD (Fas-Associated Death Domain). Cette protéine peut être recrutée directement par le récepteur de mort ou par l'intermédiaire de la protéine TRADD (TNFR-associated death domain). Les caspases 8 et 10 sont ensuite recrutées par FADD pour former le complexe DISC (Death-Inducing Signaling Complex) ce qui conduit à leur activation (Figure 13). Ces caspases vont alors pouvoir initier la cascade de clivage menant à l'activation des caspases effectrices (Galluzzi *et al.*). L'activation des caspases initiatrices et effectrices ainsi que les conséquences cellulaires sont traitées dans la partie C.

B) La voie dite intrinsèque

La voie dite intrinsèque ou mitochondriale est induite par différents stress cellulaires tels que l'exposition à des radiations UV, une irradiation γ, des dommages à l'ADN, ou encore une carence en facteurs de survie. De plus, la voie mitochondriale peut être activée par la plupart des agents chimio-thérapeutiques (Kaufmann and Earnshaw, 2000; Kroemer, 2003)

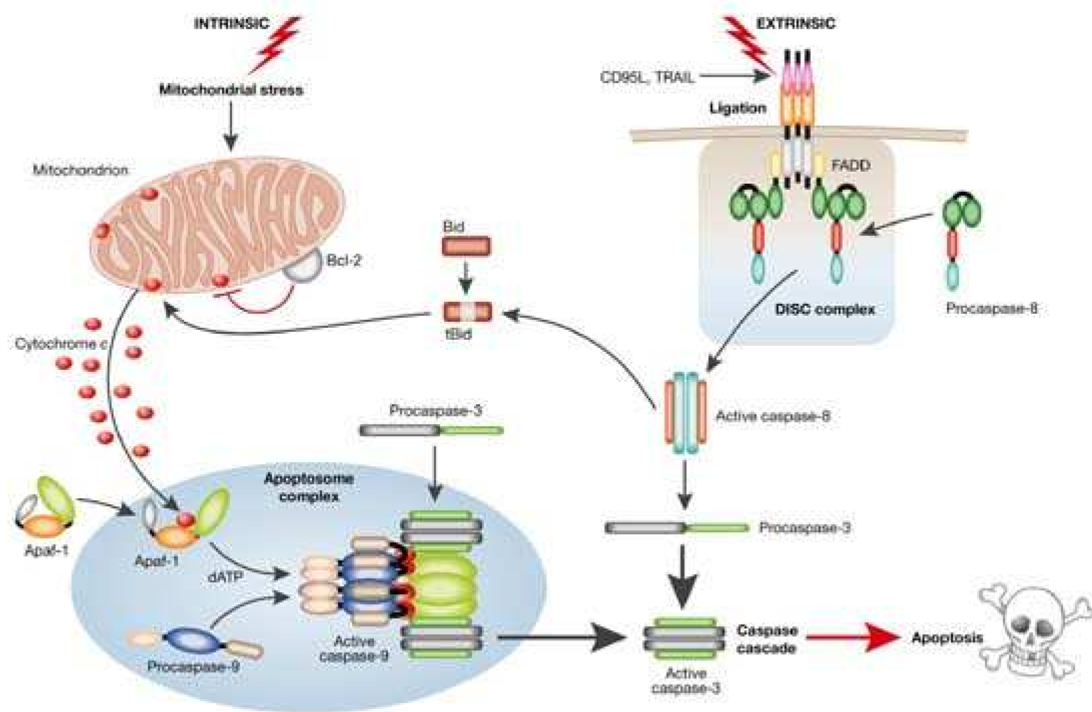


Figure 13 : Voies extrinsèque et intrinsèque de l'apoptose,
D'après (MacFarlane and Williams, 2004))

La voie mitochondriale fait intervenir la libération dans le cytoplasme de plusieurs protéines issues pour la majorité d'entre elles de l'espace intermembrannaire de la mitochondrie et dont la plus connue est le cytochrome ***c*****.** Celui-ci, participera à la formation d'un complexe multiprotéique appelé apoptotosome qui activera la caspase 9, une caspase initiatrice.

Les voies extrinsèques et intrinsèques sont connectées, comme l'indique la figure 13, avec notamment la caspase 8 issue de la voie extrinsèque qui est capable d'activer la protéine Bid qui initie alors la voie intrinsèque de l'apoptose.

D'autres protéines apoptogènes issues de l'espace inter-membranaire sont également relarguées par la mitochondrie. Pour la majorité d'entre elles, leur libération dans le cytoplasme fait intervenir la rupture de la membrane mitochondriale externe. Une présentation de l'organite, de ces fonctions ainsi que de sa structure est nécessaire afin de mieux comprendre ce mécanisme.

1) La mitochondrie

Les mitochondries ont été décrites pour la première fois par Kolliker en 1858 comme des structures d'aspect granulaire dans des cellules musculaires. En 1898, le terme mitochondrie est introduit par Benda, pour désigner ces éléments filamenteux (mitos) et granuleux (chondros).

Les mitochondries ont un diamètre d'environ 1µm et leur nombre varie selon le type cellulaire. Elles représentent environ 20% du volume d'un hépatocyte de rat et 30 à 40% du volume des cardiomyocytes (Loud, 1968; van der Klei *et al.*, 1994 ; Vendelin *et al.*, 2005). Les mitochondries ne sont pas des organites statiques puisqu'elles subissent des phénomènes de fission (aspect granulaire du réseau mitochondrial) ou au contraire de fusion (aspect filamenteux) ce qui explique leur hétérogénéité morphologique au sein d'une même cellule (Okamoto and Shaw, 2005). Les travaux de Palade et Sjostrand au début des années 50 ont permis de mettre en évidence la structure de la mitochondrie. Elle se compose de deux membranes dites membrane mitochondriale interne (IMM) et membrane mitochondriale externe (OMM). Elles sont très différentes dans leurs compositions, leurs fonctions et délimitent deux compartiments : l'espace inter-membranaire et la matrice qui renferment les crêtes mitochondriales. La membrane interne est subdivisée en deux parties : la membrane sous jacente, qui est séparée de la membrane externe par l'espace inter-membranaire, et les crêtes (cristae) comparables à des repliements aux morphologies variables et rattachées à la membrane interne par des structures appelées jonctions de crêtes. Par ses invaginations, la membrane interne possède une surface bien plus grande que la membrane externe (Perkins *et al.*, 1997) (Figure 14).

La membrane externe se caractérise par une forte perméabilité à toutes les molécules de 5 kDa ou moins grâce à la présence de porines. Elle contient également les translocases impliquées dans l'import des protéines (Translocase of the Outer Membrane, ou TOM). *A contrario*, la membrane interne, composée à 80% de protéines, se caractérise par une quasi-impermeabilité du fait de sa forte proportion en cardiolipides (20% des lipides totaux) qui entraine une stabilisation de la bicouche lipidique et une diminution de la fluidité membranaire. Le passage des protéines et autres métabolites à travers la membrane interne, est réalisé par des transporteurs et des échangeurs, ce qui explique sa grande richesse en protéines (Crompton, 1999 ; Nicholls, 2005)

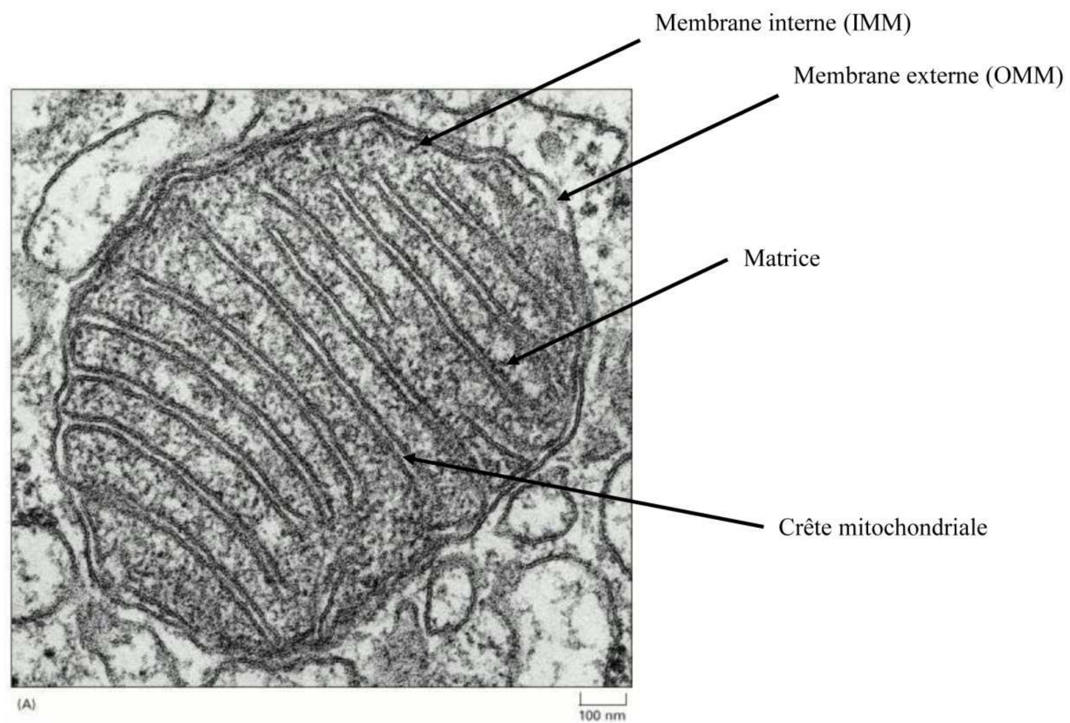


Figure 14 : Morphologie de la mitochondrie

Les mitochondries sont des organites intracellulaires caractéristiques des cellules eucaryotes et spécialisés dans la production de l'ATP. Cet organite cellulaire de la respiration serait issu, selon la théorie de Margulis (1981), d'un phénomène de symbiose réalisé il y a 1,5 milliards d'années entre une bactérie pourpre (α-protéobactérie) et une cellule eucaryote primitive. Cette hypothèse a été démontrée grâce à la découverte de similarités biochimiques et génétiques entre les bactéries et les mitochondries. Entre 1940 et 1950, les fonctions métaboliques des mitochondries ont été décrites. Outre son rôle dans le cycle de Krebs ou la β-oxydation des acides gras, la mitochondrie est apparue comme le premier fournisseur d'énergie à la cellule *via* le mécanisme de phosphorylation oxydative (OXPHOS) (Lehninger, 1964)

Au milieu des années 90, il est apparu que le cytochrome *c*, une molécule essentielle au mécanisme de phosphorylation oxydative participait également au mécanisme de mort cellulaire décrit vingt ans plus tôt par Kerr et ses collaborateurs appelé apoptose (Liu *et al.*, 1996). Il est alors apparu que la mitochondrie abritait une succession d'événements constituant une partie de la voie intrinsèque de l'apoptose.

2) Perméabilisation de la membrane mitochondriale externe et apoptose

Nous l'avons vu précédemment, au cours de l'apoptose, la mitochondrie libère plusieurs protéines de l'espace inter-membranaire vers le cytosol. Ces protéines participent directement au déroulement du processus apoptotique. Le relargage mitochondrial a été très étudié et les travaux de ces dernières années suggèrent que les mécanismes de perméabilisation de la mitochondrie sont multiples. Les deux grands mécanismes documentés ici sont :

1. La perméabilité de transition (PT), initiée au niveau de la membrane mitochondriale interne.

2. La formation de pores protéiques par les membres pro-apoptotiques de la famille des Bcl-2 au niveau de la membrane externe de la mitochondrie

a) Le pore de transition de perméabilité

L'existence d'une augmentation de la perméabilité mitochondriale a été mise en évidence dans les années 70. En effet, il a été observé qu'en présence de calcium, des mitochondries isolées perdent leur potentiel de membrane, gonflent et libèrent dans le milieu des composants matriciels (Hunter *et al.*, 1976). L'existence du pore a été révélée pour la première fois en 1989, en mesurant les courants ioniques transitant à travers les membranes par des expériences de patch-clamp (Petronilli *et al.*, 1989). Par la suite, la structure du pore de transition de perméabilité a été décrite. Ce pore est un complexe multi-protéique assemblé au niveau des jonctions entre les membranes mitochondriales interne et externe. Il est constitué de la porine VDAC (Voltage-Dependent Anion Channel) situé à la membrane externe, l'échangeur ATP/ADP ANT (Adenine Nucleotide Translocase) fixé à la membrane interne et la cyclophiline-D (Cyp-D), une protéine matricielle. D'autres protéines comme le récepteur périphérique aux benzodiazépines (PBR), l'hexokinase (HK) et la créatine kinase (CK) pourraient être associées au PTP (Brenner and Grimm, 2006) (Figure 15). Dans des conditions de survie, ce canal s'ouvre de manière transitoire assurant l'échange de métabolites entre le cytoplasme de la cellule et la matrice mitochondriale. En réponse à des messagers secondaires (ROS, Ca^{2+}), le canal adopte une conformation ouverte provoquant une équilibration des solutés de moins de 1500 Da entre les deux compartiments. L'ouverture

prolongée du canal provoque une dissipation du gradient de protons crée par la chaine de respiration, un découplage de la chaîne respiratoire conduisant à une hydrolyse de l'ATP, une entrée d'eau et de petits solutés entraînant un gonflement de la matrice mitochondriale. La dilatation de la matrice et l'excès de surface de la membrane interne par rapport à la membrane externe serait responsable de la rupture de cette dernière. Cette rupture entraîne alors le relargage des facteurs apoptotiques situés dans l'espace inter-membranaire (Massari and Azzone, 1972 ; Bernardi, 1999; Batandier *et al.*, 2004)

L'activité du PTP peut être modulée par une variété d'agents pharmacologiques qui activent ou inhibent l'ouverture du pore en réponse à un stimulus donné. Ainsi, il y a quelques années, l'équipe de Martin Crompton rapporte que l'utilisation de la cyclosporine-A, un agent aux propriétés immunosuppressives inhibait de manière spécifique l'ouverture du PTP (Crompton *et al.*, 1988). Par la suite, il a été établi que la cyclosporine-A fixe la cyclophiline D, inhibant ainsi son interaction avec d'autres composantes protéiques du pore (Davidson and Halestrap, 1990; Halestrap and Davidson, 1990). Depuis, de nombreux travaux ont permis de mettre en évidence que le PTP joue un rôle central dans l'initiation la mort cellulaire par la voie intrinsèque.

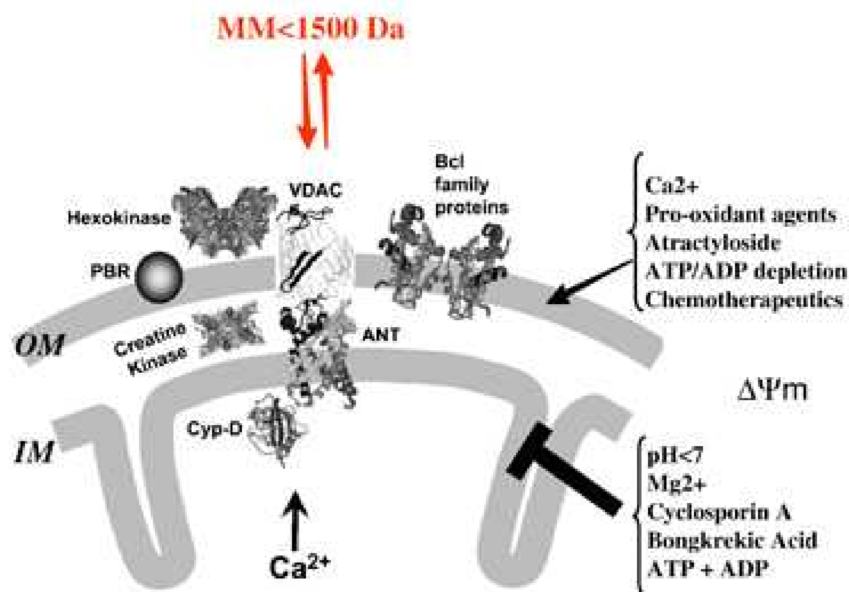


Figure 15 : Composition du pore de perméabilité de transition
D'après (Brenner and Grimm, 2006)

b) Formation de canaux protéiques Bax/Bak

Le premier membre de la famille BCL-2 a été découvert dans des lymphomes folliculaires à cellules B. Ce cancer est lié à la translocation des chromosomes 14 et 18. Il en résulte une juxtaposition du promoteur qui régule l'expression du gène codant les chaînes lourdes des immunoglobulines et du gène codant la protéine BCL-2. Cette translocation a pour effet de créer une surexpression de BCL-2 qui est associée à la survie cellulaire dans les tissus exprimant normalement le gène des immunoglobulines. En fonction des domaines BH (BCL-2 homology) qui les composent, les membres de cette famille ont été répartis en trois sous-groupes les membres (i) anti-apoptotiques, (ii) pro-apoptotiques à plusieurs domaine BH et (iii) les pro-apoptotiques BH3-only (Figure 16).

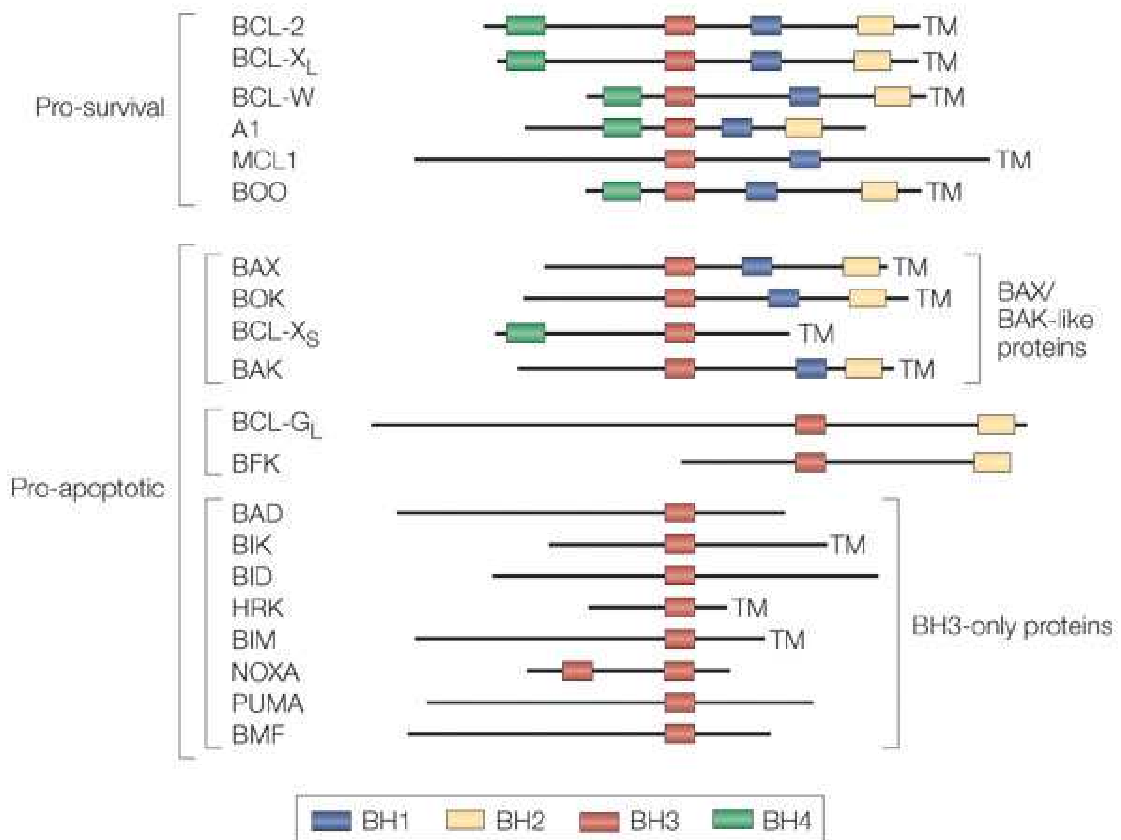


Figure 16 : La famille BCL2

Les domaines BH 1 à 4 sont indiqués en bleu, jaune, rouge et vert. En région C-terminale, la présence du domaine transmembranaire permet une localisation de la protéine dans la membrane mitochondriale, le réticulum endoplasmique ou la membrane nucléaire externe. Les protéines regroupées dans l'encart gris sont des protéines appartenant à la famille BCL2 (BAX/BAK-like proteins) mais dont les propriétés sont encore mal connues (Youle and Strasser, 2008).

Les 6 membres de la première catégorie baptisé Bcl-2-like, partagent une homologie de séquence pour 4 domaines BH noté BH1, BH2, BH3 et BH4 (Bcl-2 Homology). Ces protéines anti-apoptotiques favorisent la survie cellulaire en neutralisant l'activité des protéines Bak-like et, de ce fait, elles protègent les cellules de nombreux stimuli apoptogènes et de la perméabilisation des mitochondries.

Les membres de la sous-famille Bak-like sont pro-apoptotiques et possèdent les domaines BH1, BH2, BH3. Activées pendant l'apoptose, les protéines Bax et Bak sont directement impliquées dans la perméabilisation mitochondriale par leur capacité à former des pores au niveau de la membrane mitochondriale.

Enfin, il existe une sous-catégorie de protéines ne possédant que le domaine BH3 qui, à lui seul, est responsable de leurs propriétés apoptotiques. Les protéines BH3-only, qui jouent le rôle de senseurs, sont capables d'activer les protéines Bax-like après avoir intégré un signal de stress. En condition de survie, les BH3-only sont maintenues inactives par des mécanismes agissant au niveau transcriptionel ou post-traductionnel empêchant ainsi une mort inappropriée (Strasser *et al.*, 2000 ; Puthalakath and Strasser, 2002). Par contre, la transcription de Noxa et Puma est activée en réponse à un stress génotoxique, tandis qu'en réponse à une privation en facteurs de croissance, Bad est déphosphorylée et libérée de la protéine 14-3-3 sous sa forme active et Bid est activée par un clivage par les caspases pour former Bidt (Bid tronqué), la forme active de la protéine.

Activées pendant l'apoptose les BH3-only vont activent Bax/Bak qui sont impliquées dans la libération du cytochrome *c*. Le mode d'activation de Bax/Bak est encore débattu et deux modèles ont été proposés. Le modèle d'activation directe postule qu'il existe une liaison et une activation directes de Bax/Bak par certains BH3-only (BAD, Bidt, Puma). Dans ce modèle, les BH3-only sont répartis en deux sous-groupes. Les « activateurs » tels que Bim et Bid, qui sont normalement séquestrés par les protéines anti-apoptotiques de type Bcl-2, et les « sensibilisateurs » qui ne peuvent lier que les protéines anti-apoptotiques. En condition de stress, ces sensibilisateurs sont activés et neutraliseraient les protéines anti-apoptotiques, ce qui conduirait à la libération de tous les « activateurs » et à l'activation directe de Bak/Bax. En revanche, le «modèle indirect» postule qu'en condition de mort, la totalité des protéines BH3-only doit être engagée pour inhiber l'ensemble des protéines anti-apoptotiques de type Bcl-2, libérant ainsi Bax et Bak (Kaufmann *et al.*). Dans ce modèle, Bim est considéré comme

un puissant inducteur de l'apoptose puisqu'il est capable d'antagoniser les 5 protéines anti-apoptotiques (Willis *et al.*, 2007) (Figure 17).

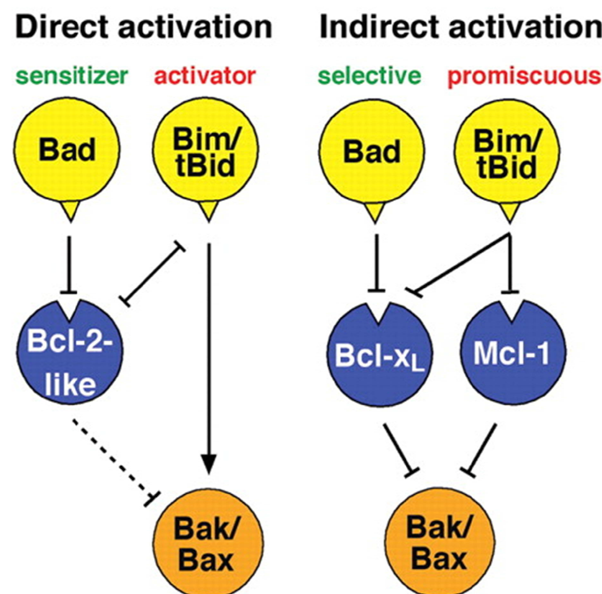


Figure 17 : Modèles de l'activation de Bax et Bak par les BH3-only
D'après (Willis et al., 2007)

Les interactions entre protéines BH3-only et Bcl-2-like sont rendues possibles par la structure en hélice α du domaine BH3 qui est capable de se loger dans la poche hydrophobe formée par les domaines BH1, 2, 3 des Bcl-2-like (Figure 18).

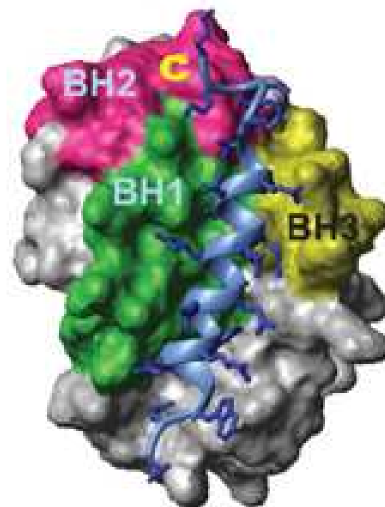


Figure 18 : Structure tridimensionnelle du complexe Bcl-XL/Bad
Le domaine BH3 de Bad possède une structure en hélice α (en bleu) capable de s'insérer dans la poche hydrophobe de Bcl-XL formée par les domaines BH1 à 3. Le domaine BH4 permettrait la stabilisation de la poche hydrophobe (Cory et al., 2003).

Quel que soit le modèle, les protéines Bax et Bak, libérées de leurs répresseurs, sont alors activées. Présentes au niveau de la membrane mitochondriale, elles vont pouvoir former des multimères qui aboutissent à la formation des pores à la surface externe de la mitochondrie ce qui permet la libération des protéines mitochondriales qui participe à la signalisation du processus apoptotique. Récemment, les réarrangements structuraux permettant l'activation de Bax et Bak ont été décrits (Kim *et al.*, 2009; Yao and Marassi, 2009) (Figure 19).

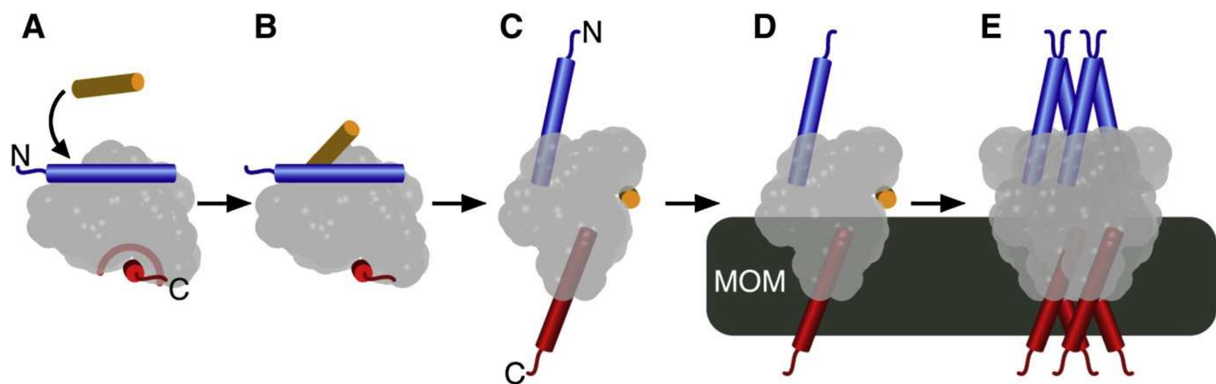


Figure 19 : Modèle d'activation de Bax par une protéine BH3-only

A) Sous sa forme inactive, Bax (en gris) est une protéine cytoplasmique dont le site de liaison au domaine BH3 (représenté par le demi-cercle rouge) est auto-inhibé par son l'hélice α9 (représentée par le cylindre rouge). L'hélice α1 (représentée par le cylindre bleu) de Bax forme avec l'hélice α6 un site d'interaction transitoire pour le domaine BH3 de la protéine BH3-only (représenté le cylindre jaune).

B) Le domaine BH3 de la protéine BH3-only (représenté en jaune) interagit avec les hélices α1/α6 et induit un changement conformationnel de Bax.

C) Le changement conformationnel de Bax libère la poche hydrophobe dans laquelle le BH3 va pouvoir s'insérer.

D) et E) La conformation activée de Bax permet son insertion et sa multimérisation dans la membrane externe de la mitochondrie (Kim et al., 2009; Yao and Marassi, 2009).

3) La boîte de Pandore

a) L'apoptosome

Pendant le processus apoptotique, plusieurs protéines sont libérées de l'espace inter-membranaire mitochondrial vers le cytosol. Le cytochrome *c* assure, en conditions normales, le transport des électrons entre les complexes III et IV de la chaine respiratoire au niveau de la membrane interne de la mitochondrie. Le cytochrome *c* est associé à la membrane interne coté espace inter-membranaire par une interaction avec la cardiolipine. Au cours de l'apoptose,

l'oxydation de la cardiolipine entraîne une perte de l'interaction électrostatique avec le cytochrome *c* et la libération de celui-ci dans l'espace inter-membranaire (Ow *et al.*, 2008).

Après la perméabilisation de la mitochondrie, le cytochrome *c* est ensuite relargué de l'espace inter-membranaire vers le cytosol. S'ensuit alors une association du cytochrome *c* avec la molécule adaptatrice Apaf-1 et de l'ATP pour constituer le cœur d'un complexe multimoléculaire d'environ 1 MDa, l'apoptosome (Tait and Green, ; Riedl and Salvesen, 2007). Le recrutement de la procaspase-9 au sein de ce complexe moléculaire est la première étape d'une cascade d'activation conduisant à l'activation des caspases effectrices (Figure 20).

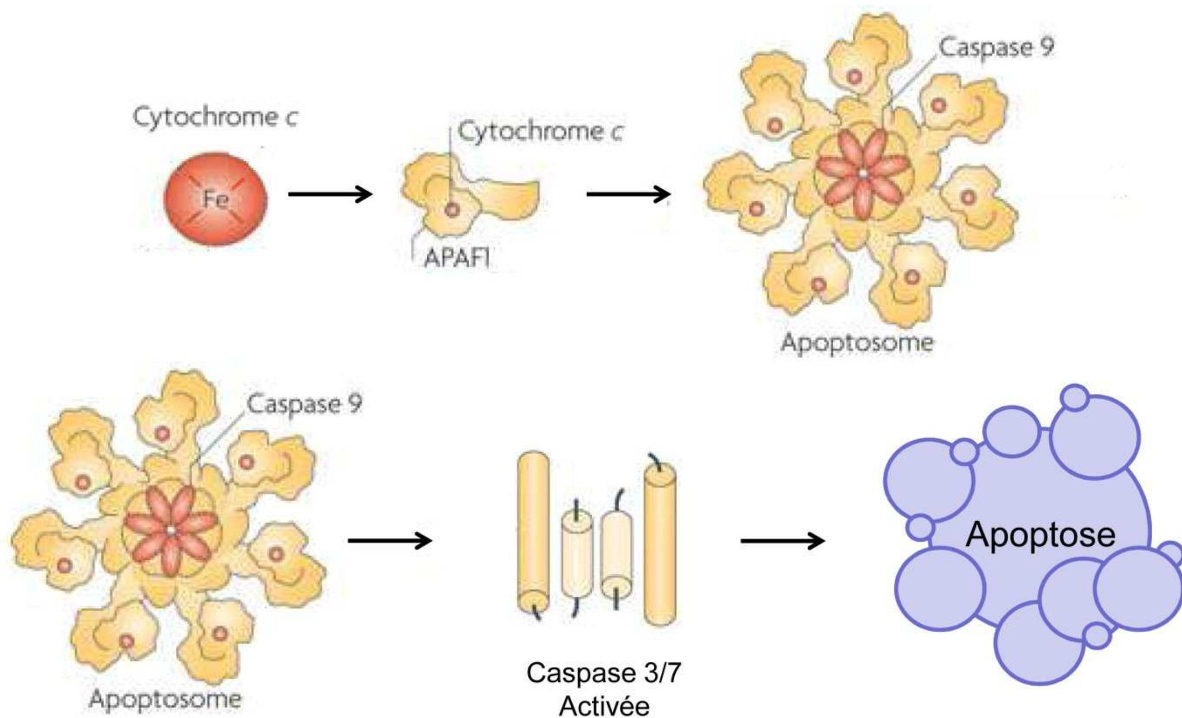


Figure 20 : Formation de l'apoptosome

Le cytochrome c libéré par la mitochondrie. En presence d'ATP, Apaf-1 fixe le cytochrome c qui à son tour fixe les pro-caspase 9, formant un complexe appelé "Apoptosome". Activée dans complexe, la caspases 9 active à son tour les caspases effetrices 3/7 ce qui conduit à l'apoptose de la cellule (Tait and Green).

b) Les autres facteurs mitochondriaux apoptogènes

SMAC/Diablo (Second Mitochondrial Activator of Caspases / Direct IAP Binding protein of Low PI) relarguée de l'espace inter-membranaire vers le cytosol interagit et neutralise l'action des IAPs (Inhibitors of Apoptosis Proteins), des inhibiteurs des caspases situés dans le cytosol (Vaux and Silke, 2003).

OMI/HTRA-2, par son activité sérine protéase, est impliqué dans la dégradation de plusieurs substrats comme les éléments du cytosquelette (actine, α- et β-tubuline, vimentine) mais va également interagir avec les IAPs (Hegde *et al.*, 2002; Vande Walle *et al.*, 2008).

AIF (Apoptosis Inducing Factor) est une oxydo-réductase jouant un rôle dans la phosphorylation oxydative et qui possède à la fois des séquences d'adressage mitochondrial et nucléaire (Susin *et al.*, 2000). En condition de survie, AIF est ancrée à la membrane interne mitochondriale par l'intermédiaire d'un domaine transmembranaire situé en région N-terminale. Au cours de l'apoptose, le clivage en aval de cette région, opéré par la calpaïne, libère AIF sous sa forme active dans l'espace inter-membranaire puis vers le cytosol. AIF est alors capable de se transloquer au noyau pour participer à la condensation et à la fragmentation de la chromatine (Susin *et al.*, 1996; Susin *et al.*, 1999).

L'endonucléase G subit également une translocation mitochondrio-nucléaire où elle induit la fragmentation de l'ADN (Li *et al.*, 2001; van Loo *et al.*, 2001). L'extinction du gène cps-6, codant l'homologue de l'endonucléase G chez *Caenorhabditis elegans*, entraîne un retard de l'apoptose pendant le développement des embryons (Parrish *et al.*, 2001). De plus, les études réalisées par Zhang montre que les cellules issues de souris hétérozygotes pour l'endonucléase G présentent une résistance à l'apoptose tandis que les blastocystes des embryons homozygotes sont anormaux et meurent précocement pendant l'embryogenèse, soulignant l'importance de l'endonucléase pendant le développement (E 2,5-3,5) (Zhang *et al.*, 2003). Cependant, son rôle au cours de l'apoptose reste controversé puisque le même KO réalisé par une équipe différente n'entraîne pas de létalité et les cellules issues de ces souris ne présentent pas de retard de dégradation de l'ADN pendant l'apoptose (Irvine *et al.*, 2005).

Les voies AIF et de l'endonucléase G sont indépendantes des caspases puisqu'elles ne nécessitent aucun intermédiaire pour provoquer l'apoptose nucléaire : ils interviendraient dans la voie apoptotique indépendante des caspases (Figure 21).

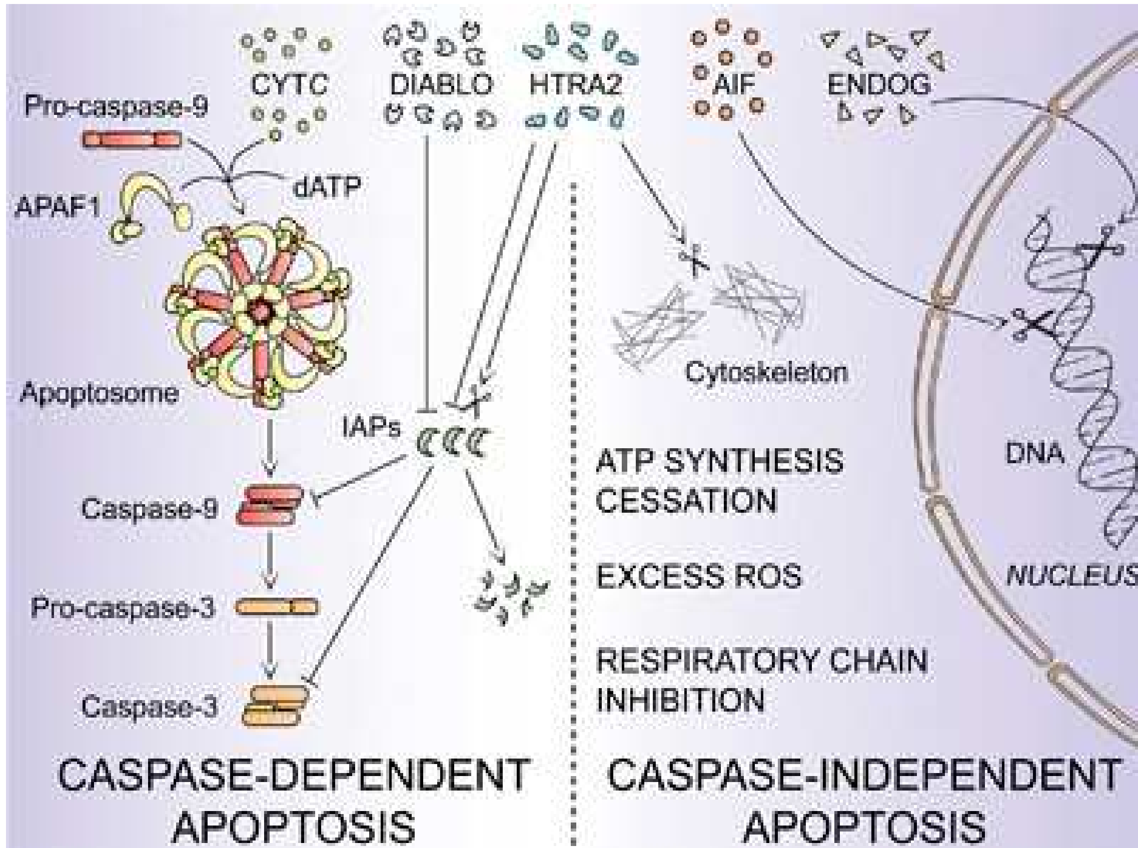


Figure 21 : Mécanismes d'action des facteurs mitochondriaux libérés pendant l'apoptose *D'après (Galluzzi et al.)*

Contrairement aux protéines citées ci dessus, la protéine ARTS (Sept4-i2, Apoptosis-related protein in the TGF-beta signaling pathway), une protéine localisée sur la membrane externe de la mitochondrie est relarguée précocement par la mitochondrie indépendamment de l'activation des caspases suite à un traitement apoptogène. Cette translocation vers le cytosol, qui précède celle du cytochrome *c*, participe à l'inhibition de la protéine XIAP (une protéine inhibitrice des caspases) et donc à l'activation précoce des caspases (Edison *et al.*).

C) Les caspases

1) Classification des caspases

A ce jour, on dénombre 14 caspases chez les Mammifères. En fonction de leur structure et leurs substrats, elles ont été divisées en trois catégories. Le premier groupe, composé des caspases 1, 4 et 5, n'est pas impliqué dans le processus apoptotique mais contrôle les réponses inflammatoires. Le deuxième et le troisième groupe regroupent les

caspases initiatrices et les caspases effectrices de l'apoptose qui comprennent respectivement les caspases 2, 9, 8, 10 et les caspases 3, 6, et 7. L'activation des caspases opère de façon hiérarchique. Ainsi, les caspases effectrices sont activées par les caspases initiatrices, elles-mêmes activées par la voie intrinsèque ou extrinsèque (Fuentes-Prior and Salvesen, 2004).

2) Structure et activation

Ces protéases qui participent au démantèlement de la cellule apoptotique possèdent une structure très conservée. Elles sont exprimées sous la forme de précurseurs inactifs appelés procaspases ou zymogènes. Les caspases effectrices ou initiatrices possèdent une grande sous-unité et une petite sous-unité. La grande sous-unité contient le résidu cystéine conservé nécessaire à l'activité catalytique à l'origine de la spécificité de cette famille de protéases et de son nom (aspartic-acid-specific cystein proteases) (Rotonda *et al.*, 1996).

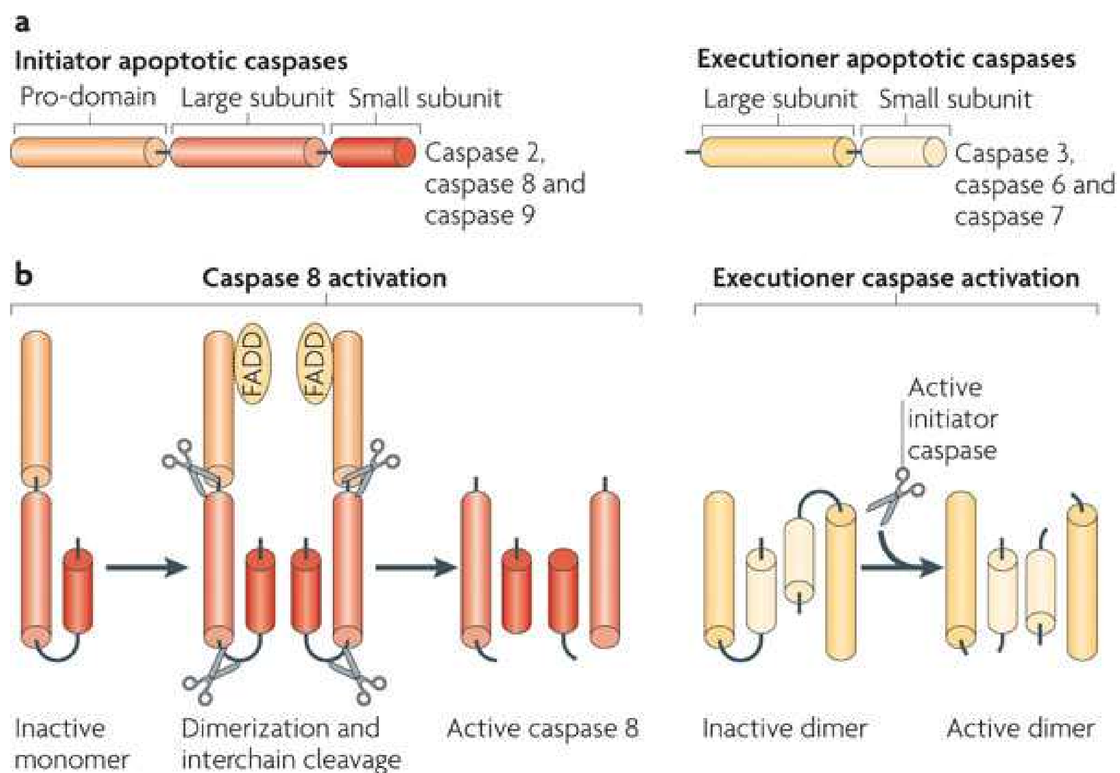


Figure 22 : Activation des caspases initiatrices et effectrices de l'apoptose
Chez les Mammifères, les voies de signalisation intrinsèque et extrinsèque permettent l'activation des protéases initiatrices et effectrices de l'apoptose (Tait and Green).

Les caspases initiatrices 8 et 10 possèdent en plus au niveau de l'extremité N-terminale un long pro-domaine contenant un domaine DED (Death Effector Domain) pouvant interagir avec la protéine FADD tandis que la caspase 9 possède un domaine CARD

(CApases Recruitment Domain) impliqué dans l'interaction avec la protéine Apaf-1 (Hofmann *et al.*, 1997) (Figure 22).

Les interactions induites entre les caspases initiatrices et leurs sous-unités régulatrices (FADD ou Apaf-1) entraînent une concentration des formes zymogènes ce qui permet leur activation. Ce mécanisme d'activation par proximité induite intervient par exemple lors de la multimérisation des récepteurs de mort ou lors de la formation de (Muzio *et al.*, 1998; Algeciras-Schimnich *et al.*, 2002). Sous leurs formes activées, les caspases s'organisent alors en tétramères composés de deux hétérodimères formés par la petite et la grande sous-unité (Figure 22).

Les caspases initiatrices activent ensuite par protéolyse les caspases effectrices de l'apoptose constituant ainsi le deuxième mécanisme d'activation des caspases. Les caspases effectrices sont alors capables de cliver un grand nombre de substrats protéiques par la reconnaissance d'un motif X-X-X-D. Le clivage a lieu après l'acide aspartique C-terminal (P1) du motif tandis que sa spécificité est déterminée par les trois résidus P2, P3 et P4 situés immédiatement en amont (notés X) (Thornberry *et al.*, 1997) (Figure 23).

Spécificités de substrat des caspases Humaines					
Groupe	Enzyme	**P4**	**P3**	**P2**	**P1**
Groupe I	Caspase-1	W	E	H	D
	Caspase-4	W/L	E	H	D
	Caspase-5	W/L	E	H	D
Groupe II	Caspase-3	D	E	V	D
	Caspase-7	D	E	V	D
	Caspase-2	D	E	H	D
Groupe III	Caspase-6	V	E	H	D
	Caspase-8	L	E	T	D
	Caspase-9	L	E	H	D
	Caspase-10	L	E	X	D

Figure 23 : Spécificité des substrats protéiques des caspases
Les caspases initiatrices activent par protéolyse les caspases effectrices de l'apoptose constituant ainsi le deuxième mécanisme d'activation des caspases. Les caspases sont capables de cliver un grand nombre de substrats protéiques par la reconnaissance d'un motif tetrapeptidique dans lequel un Aspartate occupe systématiquement la position P1. Les résidus en position P2, P3 et P4 déterminent la spécificité de substrat de la caspase et sont précisés dans le tableau pour 9 des 14 caspases humaines classées en fonction de leur groupe (Thornberry et al., 1997).

3) Conséquences fonctionnelles des clivages par les caspases

A ce jour, près de 400 substrats des caspases, ont été identifiés et regroupés depuis quelques années dans des bases de données (Luthi and Martin, 2007). L'ensemble de ces substrats regroupe des protéines structurales (cytoplasmiques et nucléaires), des protéines jouant un rôle dans le métabolisme ou la réparation de l'ADN, des protéines impliquées dans des voies de signalisation, l'expression de gènes, ou encore le contrôle du cycle cellulaire (Fischer *et al.*, 2003). Dans la plupart des cas, le clivage de ces protéines conduit à une perte de leur fonction biologique. Cependant, dans certains cas, ces clivages peuvent se traduire par une activation ou à une réorientation de la fonction primaire des protéines-cibles.

a) Clivage perte de fonction

Le phénotype des cellules apoptotiques est lié à l'action des caspases. Activées, elles sont capables de dégrader des constituants cellulaires. Ainsi, la caspase 6 est responsable du clivage des lamines nucléaires A/C et B1 qui assurent l'intégrité de l'enveloppe nucléaire (Ruchaud *et al.*, 2002). Ce clivage des lamines entraîne une déstructuration du noyau et est essentiel au bon déroulement du processus apoptotique puisque l'expression de formes non-clivables des lamines A/C entraîne un retard dans la condensation de la chromatine. Pour autant, ce retard n'inhibe pas pour autant le processus apoptotique (Rao *et al.*, 1996). De la même façon, les caspases sont impliquées dans la désorganisation du cytosquelette puisqu'elles sont capables de cliver les constituants des filaments intermédiaires comme la kératine-18 qui forme alors des agrégats (Caulin *et al.*, 1997 ; Schutte *et al.*, 2004) Au cours de l'apoptose, la signalisation anti-apoptotique est également interrompue et ciblé par les caspases. Ainsi la protéine Akt, capable d'induire la survie en phosphorylant la protéine Bad, n'est plus capable d'exercer ses fonctions de survie puisqu'elle est également un substrat de la caspase 3 pendant l'apoptose (Widmann *et al.*, 1998; Jahani-Asl *et al.*, 2007). Dans d'autres cas, la protéine ciblée par les caspases génère des produits de clivage actifs capables d'intervenir dans la signalisation pro-apoptotique.

b) Clivage gain de fonction

Nous l'avons vu précédemment, au cours de l'apoptose, les inhibiteurs des caspases comme IAP peuvent être inactivés par Omi/Htra2, une protéase relarguée par la mitochondrie. Cependant, d'autres travaux, ont montré que cet inhibiteur de l'activité apoptotique pouvait être la cible des caspases. Son clivage génère alors un fragment stable porteur d'une activité pro-apoptotique (Clem *et al.*, 2001). Cette reconversion de la fonction primaire de la protéine substrat n'est pas unique. En effet, les protéines anti-apoptotiques Bcl-2 et Bcl-XL subissent des clivages par les caspases en région N-terminale, ce qui génère des fragments débarrassés du domaine BH4 et dont les structures s'approchent ainsi des protéines Bak-like (voir Figure 16). Les études des fragments ΔNterm-Bcl2/Bcl-XL ont montré qu'ils étaient capables d'induire le relargage du cytochrome *c* et/ou d'accélérer le processus apoptotique lorsqu'il est engagé. Pour ΔNtermBcl-XL, sa propriété pro-apoptotique serait lié à sa capacité à former des pores dans des membranes lipidiques d'origine synthétiques (Fujita *et al.*, 1998 ; Kirsch *et al.*, 1999; Basanez *et al.*, 2001).

De façon intéressante, pour certaines sérines-thréonines kinases, l'activité kinase est impliquée dans l'apoptose en plus de fonctions dans la survie cellulaire. Après clivage par les caspases, les activités des fragments générés ne sont plus inhibées suite à l'élimination de domaines régulateurs (Jakobi, 2004). Par exemple, les fragments issus du clivage des kinases ROCK 1 (Rho-associated protein kinase 1), Mst (Mammalian STE20-like protein kinase) 1 et 2 ont une activité kinase constitutive et portent une activité pro-apoptotique. Pour Mst-1, le rôle apoptotique du fragment est la conséquence de sa relocalisation nucléaire (alors que la forme non clivée est exclusivement cytoplasmique). Sa capacité à phosphoryler l'histone 2B participe à la compaction de la chromatine et donc au processus apoptotique (Anand *et al.*, 2008). Pour ROCK1, dont le rôle est de maintenir le système actine-myosine, son clivage par la caspase-3 lui permet de phosphoryler les chaînes légères de la myosine provoquant ainsi le phénotype typique de bourgeonnement membranaire des cellules apoptotiques (Sebbagh *et al.*, 2001).

c) Cas des récepteurs à dépendance

Il est classiquement admis, que dans des conditions physiologiques, un récepteur n'est activé qu'après son interaction avec son ligand. Cependant, au cours des dernières années, un nouveau concept avancé par P. Mehlen et B.E. Bredesen, a émergé, suggérant que certains récepteurs dits « à dépendance » peuvent délivrer deux messages. En présence de leur ligand, ces récepteurs relayent un signal positif classique, induisant par exemple une réponse de survie, de prolifération ou de migration cellulaire. En revanche, en absence de leur ligand, ces récepteurs ne sont pas inactifs mais capables d'induire la mort par apoptose. Par cette double signalisation, ils imposent donc aux cellules un état de dépendance aux ligands.

C'est avec l'étude du récepteur $p75^{NTR}$ que sont apparues les prémices du concept de récepteur à dépendance. Ce récepteur a tout d'abord été décrit sous le nom de récepteur au NGF ($p75^{NGF}$). Depuis, il a été démontré qu'il était capable de se lier avec une faible affinité à toutes les neurotrophines (NT), le NGF (Nerve growth factor), le BDNF (Brain Derived Neurotrohpic Factor), et les NT 3 et 4. Chacune des ces neurotrophines peut également se lier spécifiquement à un des récepteurs tyrosine kinase Trk (Goldschneider and Mehlen). Dans le cas de TrkA, son association avec $p75^{NTR}$ permet d'augmenter l'affinité de TrkA pour le NGF. $p75^{NTR}$ peut également modifier la spécificité des récepteurs Trk. En effet, TrkB est capable de lier le BDNF et NT-3 tandis que la co-expression de $p75^{NTR}$ limite TrkB à la

fixation au BDNF. Inversement, la co-expression de $p75^{NTR}$ avec TrkC diminue la spécificité de ce dernier envers NT-3 (Hempstead *et al.*, 1991). De manière intéressante $p75^{NTR}$ partage des homologies de structure avec la famille des récepteurs au TNF et il a été proposé qu'il puisse induire un signal de mort en absence du NGF. Cette théorie a été étayée par différentes études qui montrent que l'extinction de *p75*^{NTR} entraîne une augmentation de la quantité de neurones cholinergiques et une hyperinnervation de l'hippocampe (Yeo *et al.*, 1997; Naumann *et al.*, 2002). Enfin, les souris issues d'un croisement entre des souris hémizygotes pour le gène *ngf,* qui présentent une réduction du nombre de neurones cholinergiques avec des individus $p75^{NTR\ -/-}$, présentent alors une restauration partielle du nombre de ces cellules, suggérant ainsi que l'expression de $p75^{NTR}$ par les cellules les rendent dépendantes au NGF. Cependant, le concept de récepteur à dépendance a vraiment émergé avec l'étude des récepteurs DCC (Deleted in Colorectal Cancer) et UNC5H2 (Uncoordinated gene 5), deux récepteurs transmembranaires décrits comme étant les récepteurs à la nétrine-1.

Le couple DCC/nétrine-1 joue un rôle prépondérant dans le développement du système nerveux central. En effet, les souris *dcc*$^{-/-}$ présentent au niveau de la plaque du plancher du tube neural une perte des projections commissurales. Ces projections font partie d'une catégorie de fibres axonales qui traversent la ligne médiane du tube neural afin de mettre en communication les hémisphères gauche et droit du cerveau. Ces résultats rejoignent également les données obtenues *in vitro* montrant l'implication du couple DCC/nétrine-1 dans l'extension des axones, l'orientation du cône neuronal et la migration neuronale (de la Torre *et al.*, 1997; Fazeli *et al.*, 1997). De manière intéressante, chez l'adulte, le gène codant DCC est fréquemment délété dans les carcinomes colorectaux (Fearon and Pierceall, 1995) tandis que sa réexpression dans les cellules HT-29 (une lignée issue d'un cancer du colon) diminue leur croissance et abolit leur tumorigénicité (Velcich *et al.*, 1999). De manière similaire, une autre étude montre que l'expression de DCC en absence de nétrine-1 dans plusieurs autres lignées cancéreuses provoque leur mort par apoptose tandis que l'ajout de la nétrine-1 permet de les protéger de cette mort (Mehlen *et al.*, 1998). *In vivo*, la surexpression de la nétrine-1 dans l'épithélium intestinal conduit à une inhibition de 50 % de la mort cellulaire programmée et à un développement significatif d'hyperplasies (Mazelin *et al.*, 2004; Bernet and Mehlen, 2005). Ces données renforcent donc l'hypothèse selon laquelle les récepteurs à dépendance auraient un rôle de suppresseur de tumeurs en plus de leur rôle dans le développement.

Au niveau moléculaire, l'absence du ligand du récepteur à dépendance considéré favorise son clivage par les caspases. Ces clivages mènent alors à la libération d'un fragment

pro-apoptotique ou à l'exposition d'un domaine pro-apoptotique (nommé ADD pour Addiction Dependence Domain) (Goldschneider and Mehlen).

En l'absence de nétrine-1, DCC recrute et active la caspase 9. Classiquement, l'activation de la caspase 9 nécessite le relargage du cytochrome *c* et la formation de l'apoptosome. Dans le cas de DCC, cette activation se fait indépendamment de ces événements et définit ainsi un mécanisme original d'induction de l'apoptose par la caspase 9 (Forcet *et al.*, 2001). UNC5H2, interagit constitutivement avec la DAPK, une kinase capable d'induire l'apoptose. La fixation de la nétrine-1 par UNC5H2 induit l'oligomérisation des récepteurs tandis que la DAPK est maintenue sous sa forme inactive par une phosphorylation inhibitrice. En absence de son ligand, nétrine-1, UNC5H2 et la DAPK forment un complexe avec PP2A (protein phosphatase 2A), une phosphatase capable de déphosphoryler la DAPK, libèrant ainsi son activité apoptotique. Dans le même temps, UNC5H2 est la cible de la caspase 3 au niveau de sa région juxtamembraire. Ce clivage génère alors un fragment stable lié à la DAPK qui, sous sa forme active, est alors capable d'induire l'apoptose (Guenebeaud *et al.*).

Cet état de dépendance imposé aux cellules, contrôlé par un récepteur membranaire, s'est récemment élargi à la superfamille des RTK avec l'intégration de Met, TrkC, Ret, ALK et EphA4 (Bordeaux *et al.*, 2000; Furge *et al.*, 2000; Mourali *et al.*, 2006; Tauszig-Delamasure *et al.*, 2007). Ces nouveaux membres de la famille des récepteurs à dependance partagent également des activités liées au développement du système nerveux et à la progression tumorale. De façon générale, Met, Ret et TrkC subissent en absence de leur ligand un double clivage par les caspases, ce qui génère un fragment stable qui possède une activité apoptotique Cependant le mécanisme de fonctionnement de ces fragments est encore méconnu.

Les différentes caractéristiques des clivages par les caspases des récepteurs à dépendance de type tyrosine kinase et leurs conséquences fonctionnelles ont fait l'objet d'une revue où je signe troisième auteur. La revue est consultable en annexe.

d) Met, un substrat des caspases

Suite à un stress apoptotique et en absence de son ligand, le récepteur Met est la cible des caspases. En effet, l'équipe où j'ai réalisé ma thèse a montré que Met est clivé de manière séquentielle au niveau de son extrémité C-terminale, ce qui favorise un deuxième clivage dans le domaine juxtamembranaire intracellulaire, au niveau de l'acide aspartique 1002.Un fragment de 40 kDa, qui correspond au domaine kinase de Met, est alors libéré dans le cytoplasme. Il a été nommé p40 Met. L'autre fragment p100 Met reste quant à lui ancré à la membrane (Tulasne *et al.*, 2004; Foveau *et al.*, 2007).

Ce clivage empêche toute activation potentielle par l'HGF/SF puisque le domaine de liaison au ligand est séparé du domaine kinase. Ainsi, le clivage du récepteur Met prévient la réponse de survie normalement induite par son ligand. D'ailleurs, ces deux mécanismes ne peuvent co-exister. En effet, en présence d'HGF/SF, la phosphorylation de la tyrosine 1003 masque le site de clivage juxtamembranaire, ce qui empêche le clivage de Met par la caspase 3 (Deheuninck *et al.*, 2009).

Pour autant, le rôle de Met dans l'apoptose ne s'arrête pas à l'inactivation du récepteur. En effet, les fragments p100 Met et p40 Met participent à l'amplification de la mort cellulaire. Ainsi, p100 Met est encore capable d'interagir avec l'HGF/SF et sa surexpression prévient l'activation des voies de signalisation induites par Met en réponse au ligand. Le fragment p100 Met agit comme un récepteur leurre capable d'empêcher la réponse de survie induite par l'HGF/SF et de favoriser la mort cellulaire (Deheuninck *et al.*, 2008). Le second fragment, p40 Met, est quant à lui capable d'induire seul l'apoptose dans différents types cellulaires lorsqu'il est exprimé de façon ectopique. De manière endogène, ce fragment est stable et contribuerait directement à l'amplification de la mort cellulaire (Figure 24) (Tulasne *et al.*, 2004; Foveau *et al.*, 2007).

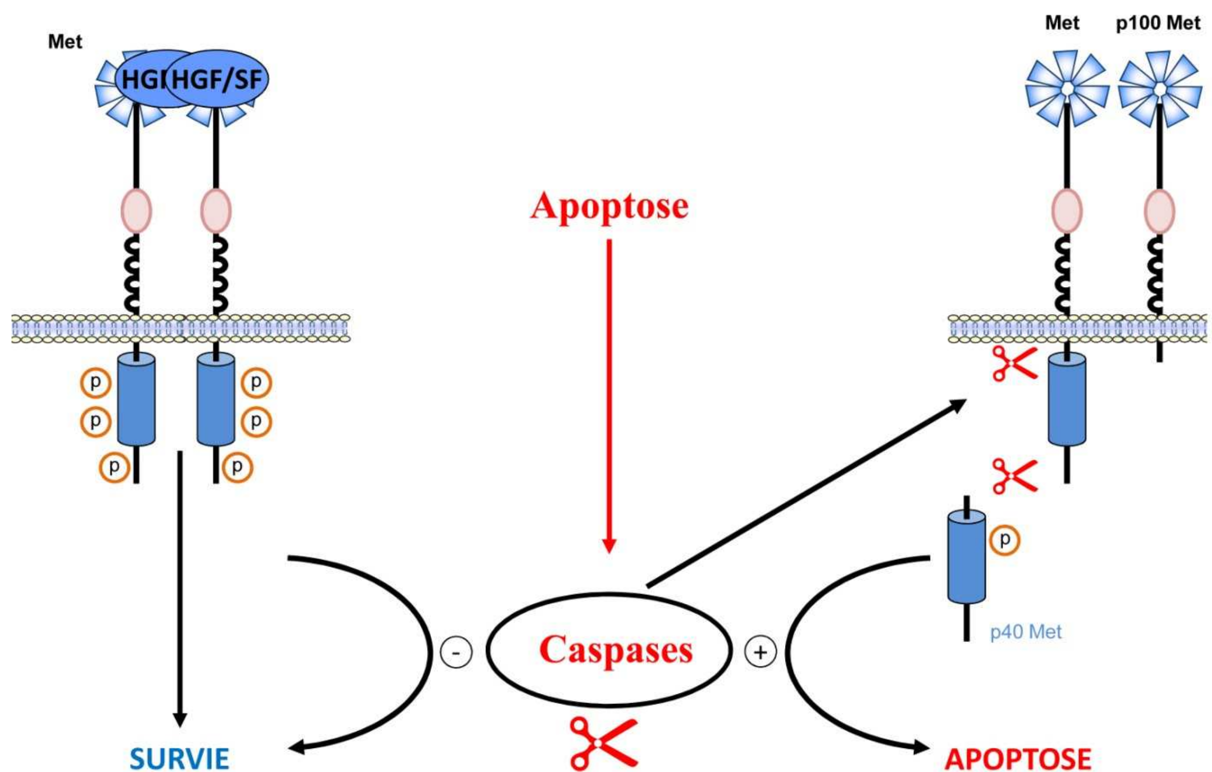


Figure 24 : Dualité fonctionnelle du récepteur Met

Par ses fonctions apoptotiques, son implication dans le développement et les cancers, le récepteur Met présente donc des caractéristiques fonctionnelles comparables avec le fonctionnement des récepteurs à dépendance.

En utilisant une construction exprimant une version de p40 Met dépourvue de son site de liaison à l'ATP, les travaux du laboratoire suggéraient que les propriétés du fragment p40 Met étaient dépendantes de son activité kinase. A l'époque, cette observation était cohérente avec les nombreux exemples de la littérature où le clivage d'une kinase pendant l'apoptose libère l'activité enzymatique de la protéine cible.

L'objectif de ma thèse a donc été de déterminer comment Met était capable de participer à l'apoptose par l'intermédiaire de son émissaire p40 Met.

RESULTATS

I) p40 Met a rendez-vous avec la mitochondrie

Grâce aux outils mis en place au cours de mon Master, j'ai pu démontrer que p40 Met ne possède pas d'activité kinase lorsqu'il est produit par une cellule apoptotique ou lorsqu'il est surexprimé à l'aide d'un système de surexpression de type Tet-On. Contrairement à ce qui était attendu, p40 Met n'a donc pas la capacité de contrôler des voies de signalisation induites en aval de son activité kinase. De plus, d'autres travaux du laboratoire ont montré que p40 Met n'est pas capable d'interagir avec les caspases 9 et 3, comme c'est le cas pour le récepteur à dépendance DCC pour qui cette interaction confère l'activité pro-apoptotique (Forcet *et al.*, 2001).

Mes travaux menés ont également montré que p40 Met se localise à la mitochondrie. A partir de ces constats, nous avons alors envisagé que p40 puisse médier ses fonctions apoptotiques en participant à l'activation de la voie mitochondriale de l'apoptose.

Les données sur le mode de fonctionnement de p40 Met ont été insérées dans la Publication 2.

Publication 2: Caspase-dependent fragment of Met receptor favors apoptosis through the intrinsic pathway

Lefebvre J., Muharram G., Leroy C., Kerrouche Z., Ichim G., Tauszig-Delamasure S., Chotteau-Lelièvre A., Brenner C., Mehlen P. and Tulasne D

Soumis à Journal of Cell Biology

Caspase-generated fragment of the Met receptor favors apoptosis via the intrinsic pathway

Jonathan LEFEBVRE[1], Ghaffar MUHARRAM[1], Catherine LEROY[1], Zoulika KHERROUCHE[1], Gabriel ICHIM[2], Servane TAUSZIG-DELAMASURE[2], Anne CHOTTEAU-LELIEVRE[1], Catherine BRENNER[3], Patrick MEHLEN[2] and David TULASNE[1].

[1] CNRS UMR 8161, Institut de Biologie de Lille - Institut Pasteur de Lille - Université de Lille 1 – Université de Lille 2, Lille 59021, France.

[2] Apoptosis, Cancer and Development Laboratory- Equipe labellisée "La Ligue", Centre de Cancérologie de Lyon, INSERM U1052-CNRS UMR5286, Université de Lyon, Centre Léon Bérard, Lyon 69008, France.

[3] INSERM UMR S-769, Université Paris-Sud 11, 92296 Châtenay-Malabry, France.

Keywords: c-Met, receptor tyrosine kinase, hepatocyte growth factor/scatter factor, caspase, apoptosis, dependence receptor, mitochondrial permeabilization, Bcl-2, BH3.

Running title: Mitochondrial apoptosis induced by Met cleavage

Number of characters (except methods and references): 32 652

Corresponding Author: David Tulasne, CNRS UMR 8161, Institut de Biologie de Lille - Institut Pasteur de Lille, 1 Rue Pr Calmette, BP447, 59021 Lille, France

Abstract

The receptor tyrosine kinase Met and its ligand, the hepatocyte growth factor, are essential to embryonic development, whereas deregulation of Met signaling is associated with tumorigenesis. While ligand-activated Met promotes survival, caspase-dependent generation of the p40 Met fragment leads to apoptosis induction, hallmark of the dependence receptor. We show that although p40 Met contains the entire kinase domain, it amplifies apoptosis independently of kinase activity. In cell cultures and mouse liver undergoing apoptosis, the fragment shows a mitochondrial localization, required for p40 Met-induced cell death. Interestingly, p40 Met exhibits a BH3-like domain overlapping with its ATP-binding site and required for the apoptotic response. It induces mitochondrial permeabilization, while Met silencing delays this response. This demonstrates the involvement of receptor cleavage in regulating mitochondrial cell death. The Met dependence receptor thus displays overlapping kinase and BH3 domains, the former involved in survival, the latter in cell death via the intrinsic apoptosis pathway.

Introduction

Members of the emerging functional family of dependence receptors can mediate both efficient cell survival (after ligand binding) and apoptotic cell death (in the absence of ligand). Expression of these bifunctional receptors leads to ligand-dependent cell survival. To date, more than 15 dependence receptors have been identified, belonging to several membrane receptor families: single-pass type I receptors (DCC, UNC5), receptor tyrosine kinases (RET, TRKA, TRKC, EphA4, IGF1R, InsulinR), integrins ($\alpha v\beta 3$), and multi-pass membrane proteins (Patched). Most dependence receptors are substrates of caspases, the cysteine aspartyl-specific proteases that mediate apoptosis. The cytoplasmic or membrane-anchored fragments generated by these cleavages are direct inducers of apoptosis (Mehlen and Bredesen, 2011).

The receptor tyrosine kinase (RTK) Met is classified as a dependence receptor because it strongly induces cell survival in the presence of ligand and favors apoptosis when cleaved by caspase in the absence of ligand (Tulasne and Foveau, 2008). The Met receptor, expressed predominantly at the surface of cells of epithelial origin, is activated by its stromal high-affinity ligand, the hepatocyte growth factor/scatter factor (HGF/SF). Ligand-activated Met stimulates proliferation, scattering, invasion, and morphogenesis of epithelial cells, acts as an angiogenic factor, and has chemoattractant and neurotrophic activities (Trusolino et al., 2010). Met is also a survival receptor, protecting a number of cell types against toxicity and apoptosis (Tulasne and Foveau, 2008). The Met receptor and its ligand are essential to embryonic development, since mouse mutants affected in either ligand or receptor show defects in placenta, liver, muscle, and neuron formation (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). The severe size reduction of the liver in these mice results from massive apoptosis, highlighting the involvement of HGF/SF-Met signaling in hepatocyte survival (Schmidt et al., 1995).

Upon HGF/SF binding, the Met receptor dimerizes and its tyrosine kinase activity stimulates its ATP-dependent autophosphorylation. Intracellular tyrosine phosphorylation allows recruitment of cytoplasmic proteins involved in activating multiple intracellular signaling pathways (Longati et al., 1994) (Ponzetto et al., 1994). As in the case of other RTKs, the PI3K–Akt and RAS-ERK signaling pathways play a central role in the anti-apoptotic responses induced by activated Met in numerous cell types in response to various apoptotic inducers (Tulasne and Foveau, 2008). The apoptotic process is notably regulated by proteins of the Bcl-2 family, whose action affects the intrinsic apoptosis pathway involving mitochondrial outer membrane permeabilization (MOMP), formation of the apoptosome, and activation of caspase 9. In many studies, HGF/SF-induced survival responses have been found to correlate with increased expression of the anti-apoptotic Bcl-X_L and Bcl-2 proteins, which inhibit mitochondria-dependent apoptosis (Fan et al., 1998; Yamamoto et al., 2001). Upon HGF/SF stimulation, furthermore, Akt activated downstream from PI3K phosphorylates the pro-apoptotic BH3-only protein Bad, causing its inactivation and thereby preventing apoptosis (Liu, 1999). HGF/SF can

also induce expression of the genes encoding the anti-apoptotic factors TRAF-2 and cIAP (Fan et al., 2005).

We have shown that, in addition to the well-known involvement of ligand-activated Met in cell survival, the Met receptor can directly favor apoptosis. Under stress conditions, activated caspases can cleave Met at aspartate residues 1374 (within the DNID motif of its C-terminal tail) and 1000 (within the ESVD motif of the juxtamembrane region) (mouse sequence) (Foveau et al., 2007; Tulasne et al., 2004), thus generating an intracellular 40-kDa fragment containing the kinase domain. This p40 Met fragment can trigger apoptosis in epithelial cells. Expression of a caspase-uncleavable Met causes resistance to apoptosis, indicating that p40 Met participates in apoptotic signaling. In response to HGF/SF, induction of the survival signaling pathway and phosphorylation of Met near the juxtamembrane caspase site inhibit the action of caspase and thus prevent the generation of p40 Met through cleavage (Deheuninck et al., 2009). Met thus displays the hallmark of dependence receptors: the ability to trigger a pro-apoptotic response in the absence of ligand and an anti-apoptotic response in its presence.

Although the survival signaling pathways induced by membrane receptors, including dependence receptors, have been extensively studied and are well described, the relevant pro-apoptotic signaling pathways have been deciphered only recently. Caspase cleavages of DCC, Patched, and UNC5 release fragments containing addiction or dependence domains (ADD), i.e. interacting domains promoting recruitment of pro-apoptotic factors. These recruitments lead to caspase activation, a mechanism reminiscent of the extrinsic apoptosis pathway, i.e. apoptosis induced by “death receptors” (Goldschneider and Mehlen, 2010).

The mechanisms by which tyrosine-kinase-family dependence receptors promote apoptosis are unknown. We show here that p40 Met favors apoptosis independently of its kinase activity and that the fragment localizes to the mitochondria, triggering permeabilization of these organelles independently of caspase activity. Thus, unlike previously described dependence receptors, Met amplifies apoptosis via the intrinsic pathway.

Results

Expression of p40 Met amplifies epithelial cell apoptosis

We have previously shown that during apoptosis, Met is cleaved by caspases at aspartic acid residues 1000 and 1374, giving rise to a 40-kDa fragment (Foveau et al., 2007; Tulasne et al., 2004) (Fig. 1A). For specific detection of p40 Met generation during apoptosis (to the exclusion of full-length Met), we developed a custom polyclonal antibody against the neo-epitope created by juxtamembrane cleavage (anti-p40 Met). MCF-10A human mammary epithelial cells were treated for an increasing period of time with the apoptosis inducer staurosporin, and Met expression was evaluated with an antibody against its kinase domain or with the custom anti-p40 Met antibody. While anti-Met kinase recognized both full-length Met and p40 Met generated upon staurosporin treatment, the anti-p40 Met detected only a 40-kDa band during apoptosis (Fig. 1B). This 40-kDa band was indeed the Met fragment, since a Met-targeting siRNA abolished its detection during apoptosis (Fig. 1C). The p40 Met fragment detected with either antibody first appeared 2 h after staurosporin addition, simultaneously with the cleaved PARP fragment. This indicates that p40 Met generation occurs early in the apoptotic process.

We previously showed that transient transfection of p40 Met in epithelial cell induces activation of caspase 3 in about fifteen percent of the transfected cells (Foveau et al., 2007). To further evaluate the involvement of p40 Met expression in epithelial cells, we generated Tet-on inducible clones of MDCK normal canine kidney epithelial cells expressing the mouse Met fragment and used an anti-mouse Met antibody that does not detect the endogenous Met of these cells (Fig. S1A). In clone a, doxycycline (Doxy) was found to induce p40 Met expression in a dose-dependent manner (Fig. S1B). Expression was highest 12 h after the start of induction (Fig. S1C). When apoptosis was induced with TNF-α/cycloheximide (TNF-CHX) (Fig. 2A,B) or anisomycin (Fig. S2A), p40 Met expression was found to accelerate apoptosis, as evaluated by PARP and caspase 3 cleavage, notably after 2 to 4 hours of TNF-CHX treatment. In contrast, inducible expression of p40 Met did not induce caspase activation on its own. After 4 h of TNF-CHX stimulation, the relative caspase activity was twice as high in p40 Met-expressing cells as in control cells, while no caspase activity increase was observed in the absence of apoptosis induction (Fig. 2C). This tallies with the level of cell death, as evaluated by trypan blue staining (Fig. 2D). Similar amplification of apoptosis was observed in a second p40 Met-expressing clone (clone b) (Fig. S2B,D). In contrast, wild-type MDCK cells and Tet-on inducible MDCK cells expressing a kinase-dead p40 Met (mutated at lysine 1108) displayed no amplification (Fig. S2C,E,F). Thus, the p40 Met fragment generated by caspase cleavage early in the apoptotic process can accelerate apoptotic cell death when overexpressed. It is noteworthy that in transiently transfected cells, p40 Met can induce apoptosis on its own, probably because of the initial stress induced by cell transfection.

The p40 Met fragment is not a constitutively active kinase

The p40 Met fragment contains the whole Met kinase domain. We have previously shown that overexpression of the fragment in transiently transfected cells can lead to its tyrosine phosphorylation (see Fig. S5 C). Furthermore, mutation of lysine 1108 of the kinase domain, known to be involved in ATP binding, has been shown to abolish the apoptotic effects of p40 Met in both transiently transfected cells and the inducible model presented above. We thus hypothesized that the apoptotic effects of p40 Met might depend on its tyrosine kinase activity (Tulasne et al., 2004). Therefore, we first determined the phosphorylation status of the fragment during apoptosis, using antibodies against the phosphorylated tyrosines of the kinase domain, hallmarks of Met kinase activation. In contrast to transient transfection experiments where p40 Met was found to be phosphorylated, we failed to detect any tyrosine phosphorylation of the fragment in extracts of whole cells undergoing apoptosis or even in fragment-enriched immunoprecipitates obtained with the custom anti-p40 Met antibody (Fig. 3A). We obtained similar results in a time course experiment of apoptosis (Fig. S3). In contrast, HGF/SF treatment induced efficient tyrosine phosphorylation of full-length Met. In p40 Met Tet-on inducible MDCK epithelial cells, likewise, we observed neither p40 Met tyrosine phosphorylation nor kinase activity, evaluated by in vitro incorporation of ATP (Fig. 3B). In contrast, the TPR-Met oncogene, a constitutively dimerized and activated form of Met produced by fusion of the dimerization domain of TPR to the kinase domain of Met, displayed proper phosphorylation and kinase activity, while its kinase-dead version did not. Hence, although p40 Met contains the entire kinase domain, the fragment generated during apoptosis is not a constitutively active kinase.

To test whether the action of p40 Met depends on its kinase activity, we determined the effects of ATP-mimetics targeting selectively the Met kinase, i.e. PHA-665752 and SU-11274, on p40 Met-induced apoptosis. While both Met inhibitors prevented the HGF/SF-induced survival response, as evaluated by Met and PARP cleavage (Fig. S4), they failed to inhibit the apoptosis-enhancing activity of ectopically expressed p40 Met, as evaluated by immunofluorescence monitoring of caspase 3 cleavage (Fig. 3C). Taken together our results show that, although the survival response induced by ligand-activated Met requires kinase activity, apoptosis enhancement by p40 Met does not.

The subcellular localization of p40 Met governs its pro-apoptotic action

The p40 Met fragment, because it lacks the transmembrane domain, should be absent from the membrane. To see how the localization of p40 Met might affect its pro-apoptotic action, we fused the 15amino acids of the myristoylation site of SRC, responsible for its membrane anchorage, with p40 Met to generate SMS-p40 Met (Fig. S5A). Immunofluorescence staining revealed a dual localization of p40 Met, both cytoplasmic and nuclear, whereas SMS-p40 Met was detected mainly at the plasma membrane (Fig. 4A). Cell death, evaluated by cleaved caspase 3 staining in MDCK cells, appeared drastically reduced in SMS-p40 Met- versus p40 Met-expressing cells. Thus, restoring the membrane localization of the Met kinase domain inhibits its pro-apoptotic action (Fig. 4B).

Mitochondrial localization of p40 Met

In subcellular fractionation experiments, p40 Met generated in staurosporin-treated MCF-10A cells was found in both the nuclear and mitochondrial fractions, but not in the cytoplasmic fraction (Fig. 5A). The effectiveness of purification was attested by the enrichement of Cox IV in the mitochondrial, Nuc p62 in nuclear and GAPDH in cytoplasm fractions, respectively. This was confirmed by immunofluorescence detection of transiently expressed p40 Met, which displayed nuclear and punctate intracytoplasmic staining, the latter colocalizing sharply with the mitochondria (Fig.5B).

To see if p40 Met localizes to the mitochondria in vivo, we examined generation of the fragment in the mouse liver, an epithelial organ expressing Met and in which the ligand-activated receptor induces cell survival. Mice were treated with anti-Fas antibody (Jo2 antibody), known to induce fulminant hepatic failure and massive apoptosis via activation of the death receptor pathway (Ogasawara et al., 1993). Liver mitochondria were purified and analyzed by western blotting (Fig. 5C). The effectiveness of purification was attested by the presence of abundant Cox IV in the isolated mitochondria, whereas GAPDH was mainly detected in whole-cell extracts. Monitoring of caspase 3 activation in whole liver extract confirmed the strong apoptosis-inducing action of Jo2. Full-length Met was present in the whole liver extract, where its level decreased upon Jo2 treatment, but it was never detected in the purified mitochondria. In contrast, the p40 Met fragment, specifically detected with the custom antibody, was undetectable in the whole-cell extract but present in the purified mitochondria, exclusively under apoptotic conditions. A fragment of similar size was observed in apoptotic MCF-10A cells (Fig. 5C). Thus, p40 Met is generated in vivo in the apoptotic mouse liver and localizes predominantly to the mitochondria.

We performed sequential N-terminal deletions of p40 Met (Fig. 6A and Fig. S5B) and examined the subcellular localization of the fragments obtained. While p40 Met deleted of the sequence extending to Glu1009 or Ser1072 displayed an unchanged nuclear and mitochondrial localization, the fragment truncated up to Ile1180 displayed an exclusively mitochondrial localization (Fig. 6B). Thus, as previously reported for engineered intracellular Met fragments, the region between Ser1072 and Ile1180 of the Met kinase domain is necessary for translocation of Met to the nucleus (Pozner-Moulis et al., 2006; Matteucci et al., 2009).

The apoptosis-enhancing response induced by p40 Met depends on a BH3 domain

Although p40 Met is not an active kinase, its lysine 1108, well known for its involvement in Met kinase activity, is required for the fragment's pro-apoptotic action (Tulasne et al., 2004). K1108 might thus belong to another functional domain, involved in the p40 Met-triggered apoptotic response. Interestingly, alignment of the sequence surrounding K1108 with the Bcl-2-family BH3-only proteins revealed the presence of a potential BH3 domain, with a conserved leucine and a conserved aspartic

acid residue (Fig. 7A). This conserved signature is necessary for the pro-apoptotic functions of Bcl-2-family proteins such as Bid or Bik (Lomonosova and Chinnadurai, 2008) and of BH3-like domain containing proteins such as the ErbB2 and ErbB4 receptor tyrosine kinases (Naresh et al., 2006; Strohecker et al., 2008). Cell death, as evaluated by immunofluorescence monitoring of caspase 3 cleavage in MDCK cells (Fig. 7B) and by estimating clonogenicity in HEK293 cells (Fig. 7C), was abolished or drastically reduced in cells expressing a p40 Met fragment mutated at L1110, D1115, or both (p40 Met 2E). In contrast to mutation of residue K1108, mutation of residue L1110 and/or D1115 did not abolish the forced tyrosine phosphorylation of p40 Met observed in transiently transfected cells. These mutations thus appear not to affect Met kinase activity (Fig. S5). BH3-only proteins trigger cell death through their interaction with - and inhibition of - the anti-apoptotic Bcl-2 family members. Like other BH3-only proteins, our results demonstrate that p40 Met was found to co-immunoprecipite with Bcl-2, the prototype anti-apoptotic Bcl-2-family member. Mutation K1108A inside the BH3 domain decreased this interaction (Fig 7D). Taken together, these results suggest that the BH3-like domain of p40 Met is involved in its pro-apoptotic response.

p40 Met induces apoptosis through mitochondrial permeabilization

Caspase 3 activation induced by p40 Met transfection was efficiently inhibited by cyclosporin A, known to inhibit the permeability transition pore complex (PTPC) (Brenner and Grimm, 2006) (Fig. 8A,B). Interestingly, although the pan-caspase inhibitor (zVAD) prevented caspase 3 activation as expected, it failed to inhibit the nuclear condensation induced by p40 Met (Figure 8A). These data suggest an involvement of mitochondrial permeabilization in p40 Met-induced apoptosis. We therefore next evaluated mitochondrial permeabilization on the basis of cytochrome *c* release in human MCF-10A cells, where cytochrome *c* is easily detectable with specific antibodies. The p40 Met fragment was found to induce cytochrome *c* release in about 15% of the transfected cells (Fig. 8C,D).
Cells transfected with p40 Met 2E, mutated in the BH3 domain, displayed decreased mitochondrial permeabilization. It is noteworthy that mutations outside the BH3 domain, such as the H1104D mutation (called H1124D in the human sequence) characterized in cancer and known to promote Met kinase activity (Schmidt et al., 1997), did not inhibit mitochondrial release (Fig. 8C). In p40 Met-transfected cells, treatment with a pan-caspase inhibitor did not prevent cytochrome *c* release or nuclear condensation, a hallmark of apoptosis, but on the contrary increased these phenomena. The mitochondrial permeabilization induced by the fragment thus occurs independently of caspase activation. This suggests that p40 Met may act at an early step of apoptosis to induce release from the mitochondria, and that the caspase inhibitor might prevent later events in apoptosis. Accordingly, treatment of MDCK cells with cyclosporin A inhibits both caspase activation and nuclear condensation (Fig. 8C,D). Thus, p40 Met-induced mitochondrial permeabilization occurs upstream from caspase activation and can lead to apoptosis, including caspase 3 activation and nuclear condensation.

The Met receptor is involved in both ligand-dependent survival and mitochondrial permeabilization

To evaluate the involvement of Met in the mitochondrial permealization induced during epithelial cell apoptosis, synthesis of the receptor was prevented in MCF-10A cells with siRNA and apoptosis was induced with staurosporin. Under control conditions, induction of apoptosis led to p40 Met generation (Fig. 9A) and to cytochrome *c* release (monitored by immunofluorescence) in more than 50% of the cells after 3 h of treatment (Fig. 9 B,C). In keeping with the survival response induced by ligand-activated Met, HGF/SF prevented p40 Met generation (Fig. 9A) and decreased cytochrome *c* release by half (Fig. 9B). A pool of three siRNAs against *c-met* efficiently inhibited the synthesis of both the full-length receptor and the p40 Met fragment (Fig. 9A). Under these conditions, cytochrome *c* release was again reduced by half. Hence, Met is indeed required for efficient mitochondrial release. As expected in the absence of its receptor, HGF/SF failed to prevent cytochrome *c* release upon Met silencing (Fig. 9B). Similar results were obtained with the individual Met-targeting siRNAs of the pool (data not shown). Thus, under conditions of stress and in agreement with the dependence paradigm, the Met receptor is required for ligand-triggered cell survival but also participates, in the absence of its ligand and via its p40 Met fragment, in mitochondrial permeabilization.

Discussion

During apoptosis, the p40 Met fragment is generated by caspase cleavage of the full-length receptor within the C-terminal tail and in the juxtamembrane domain, releasing an intracellular fragment. Although p40 Met is generated by caspases, we detected its expression from 2 h after the start of apoptosis induction, at a moment when caspases are not fully activated. The p40 Met fragment was detected with a custom antibody recognizing the sequence immediately downstream from aspartic acid 1000, confirming that cleavage occurs at this site. The generation of p40 Met early in the apoptotic process and its pro-apoptotic action when overexpressed suggest that the fragment contributes to apoptotic signaling. This hypothesis was confirmed with p40 Met Tet-on inducible MDCK epithelial cells, where apoptosis is accelerated upon fragment expression. Altogether, our results suggest that p40 Met is an amplifier of apoptosis in the absence of the Met ligand.

In caspase-targeted kinases, cleavage can lead to either activation or inactivation of kinase activity, and this modulation has a direct impact on apoptosis (Kurokawa and Kornbluth, 2009). For instance, caspase cleavage of ROCK1 leads to constitutive kinase activation, phosphorylation of myosin light chains, and subsequent membrane blebbing, characteristic of apoptosis (Coleman et al., 2001; Sebbagh et al., 2001). In contrast, caspase cleavage abolishes activation of the Akt kinase, preventing the pro-survival responses it triggers (Bachelder et al., 2001). We initially demonstrated that the p40 Met fragment includes the entire kinase domain and that mutation of lysine residue 1108, required for ATP binding and thus kinase activity, is sufficient to abolish its pro-apoptotic action

(Tulasne et al., 2004). Yet the endogenous fragment generated during apoptosis and in Tet-on inducible MDCK cells displays neither phosphorylation nor kinase activity. In addition, potent specific inhibitors of Met kinase activity do not affect p40 Met-induced cell death. Hence, caspase cleavages of Met do not generate a constitutively active kinase fragment.

We have shown that K1108 belongs to a BH3-like domain found in BH3-only proteins and characterized by the presence of two conserved residues: L1110 and D1115. Like the K1108A mutation, mutation of these conserved residues abolishes p40 Met-induced apoptosis. In addition, like several BH3-only proteins, p40 Met can interact with the anti-apoptotic Bcl-2 protein, and a mutation within the BH3 domain inhibits this interaction. Hence, the kinase domain of Met contains a functional BH3 domain containing the K1108 residue, necessary for both kinase activity and BH3 function. The two domains thus overlap.

Consistently with its mitochondrial localization and with the presence of a BH3-like domain, p40 Met can induce MOMP, as shown by monitoring cytochrome *c* release. As a pan-caspase inhibitor does not affect this release or the resulting nuclear damage, p40 Met must control MOMP independently of caspase activation, although its own generation requires caspase cleavages. The nuclear damage observed when p40 Met is expressed could be due to other released mitochondrial factors, such as endoG or AIF, well known to disrupt nuclear organization during apoptosis (Kroemer et al., 2007). Preventing mitochondrial release with cyclosporin A, which inhibits the permeability transition pore (PTP), abolishes both the caspase activation and the nuclear damage caused by p40 Met, suggesting that MOMP triggered by the overexpressed fragment is an initial event leading to full apoptosis. Several BH3-only proteins acquire pro-apoptotic activity when caspase-cleaved. For instance, caspase cleavage of the canonical BH3-only protein Bad generates a truncated form (tBad) that can interact preferentially with Bcl-X_L and inhibit its pro-survival function, causing more efficient MOMP (Condorelli et al., 2001). We propose that p40 Met acts as a BH3-only protein by inactivating anti-apoptotic factors like Bcl-2 and thus causing mitochondrial permeabilization.

Interestingly, the intracellular regions of both ErbB4 and the orphan receptor ErbB2 contain a BH3 sequence involved in association with the anti-apoptotic protein Bcl-2 or Bcl-X_L, respectively (Naresh et al., 2006; Strohecker et al., 2008). Like that of Met, this motif is required for the MOMP induced by cleavage-generated ErbB4 and ErbB2 fragments. On the basis of these properties, these fragments are viewed as apoptosis-regulating BH3-like-only proteins. Yet unlike Met, ErbB2 and ErbB4 do not possess all the features of dependence receptors. In the case of ErbB4, the pro-apoptotic fragment of is generated by constitutive gamma-secretase cleavage, and caspase cleavage of this receptor has not been reported so far. In the case of ErbB2, caspase cleavage is indeed responsible for generation of the BH3-containing fragment, suggesting that this process might be associated with amplification of cell death. To date, ErbB2 is an orphan receptor, so it has been impossible thus far to investigate whether the key step of dependence, i.e. classical ligand stimulation leading to survival, actually occurs with this receptor. Taken together, our results show that Met, ErbB2, and ErbB4

contain a common functional BH3-like domain, indicating that a pro-apoptotic function is not exceptional among RTKs. Yet only in the case of the Met receptor has this domain been directly linked to the dependence receptor paradigm.

We have previously shown that the CBL binding site and the caspase cleavage site overlap within the Met juxtamembrane domain. Consequently, ligand-dependent phosphorylation of tyrosine residue 1001 of the CBL binding site prevents caspase cleavage at the previous residue, aspartic acid 1000 (Deheuninck et al., 2009). These events are thus mutually exclusive. Interestingly, we demonstrate here that the BH3 motif of the Met kinase domain overlaps with the ATP-binding site, whereas those of ErbB2 and ErbB4 are both outside the kinase domain. This suggests that kinase activity and BH3 function might also be mutually exclusive: in response to ligand-stimulation, kinase activation would prevent both caspase cleavage and BH3 function, while in the absence of ligand and under stress, caspase cleavage of Met could prevent kinase activation but would release a BH3-containing fragment. Taken together, our data shed new light on the molecular mechanisms underlying the dependence receptor concept, according to which a receptor can trigger opposite responses, and the response elicited depends solely on whether its ligand is present or not.

The survival response induced by ligand-stimulated Met notably involves increased expression of the anti-apoptotic Bcl-X_L and Bcl-2 proteins, leading to inhibition of mitochondria-dependent apoptosis (Fan et al., 1998; Yamamoto et al., 2001). In the absence of ligand and under stress, in contrast, p40 Met acts as a BH3-only protein promoting mitochondria-dependent apoptosis. Thus, the Met receptor can affect intrinsic apoptosis in opposite ways according to the cellular conditions, tipping the survival/apoptosis balance one way or the other.

The apoptotic responses induced by several dependence receptors have recently been elucidated. For instance, the dependence receptor DCC associates with pro-caspase 3 when its ligand is present. Upon ligand withdrawal, DCC is cleaved by a first round of local caspase activation, this leading to exposure of an addiction or dependence domain (ADD) present in the membrane-anchored receptor fragment. The receptor then associates indirectly with caspase 9. These interactions lead to activation of caspase 9 and then caspase 3 (Forcet et al., 2001; Liu et al., 2002). In the case of Patched, ligand withdrawal initiates its caspase cleavage, allowing recruitment of DRAL, TUCAN, and caspase 9 to the membrane-bound fragment. Formation of this complex leads to caspase 9 activation and subsequent caspase 3 activation. These mechanisms are reminiscent of those triggered by death receptors such as FAS which, by recruiting the death-inducing signaling complex (DISC), control caspase 8 activation (Mille et al., 2009). This has led to the view that Patched and DDC dependence receptors trigger extrinsic apoptosis. We demonstrate here that upon Met cleavage, the p40 Met fragment can translocate to the mitochondria and promote permeabilization of this organelle, causing caspase activation which amplifies apoptotic cell death. The Met dependence receptor thus triggers apoptosis via the intrinsic pathway. Interestingly, it’s the membrane-anchored fragments of DCC and Patched that induce apoptosis, whereas the pro-apoptotic p40 Met fragment is no longer linked to the

membrane and can localize to the mitochondria. This suggests that the subcellular localization of the ADD-containing fragment may determine the apoptotic pathway, with membrane-anchored ADD inducing extrinsic apoptosis and membrane-dissociated ADD inducing intrinsic apoptosis. For RTKs such as Ret and TRKC, the ADD domain is in the cytoplasmic fragment, whereas for Alk and EphA4, it remains anchored to the membrane (Goldschneider and Mehlen, 2010). This suggests that these RTKs may cause dependence through distinct apoptotic mechanisms.

In transformed cells, the survival/apoptosis balance is often deregulated in favor of survival. The Met receptor may contribute to this imbalance through its aberrant activation leading to survival, together with other cellular responses such as growth or invasion (Trusolino et al., 2010). Many strategies are developed to prevent Met activation, such as the use of an ATP mimetic targeting Met kinase activity or direct silencing of Met expression (Comoglio et al., 2008). Interestingly, we show here that Met kinase inhibitors (such as PHA-665752) efficiently prevent Met-dependent survival without affecting p40 Met-induced apoptosis. In contrast, silencing of Met expression with siRNA inhibits both ligand-dependent survival and p40 Met-dependent mitochondrial release. Our findings thus reveal that Met kinase inhibitors offer the advantage of preserving the pro-apoptotic action of Met, which could further participate in causing the death of treated transformed cells.

Materials and methods

Cytokines, drugs, and cell cultures

Human recombinant HGF/SF and TNFα were purchased from Peprotech (Rocky Hill, NJ, USA). The Met kinase inhibitors PHA-665752 and SU-11274 were purchased from Promega (Madison, WI, USA) and Biomol Research Laboratories (Philadelphia, PA, USA), respectively. Anisomycin and the caspase inhibitor zVAD-FMK were purchased from Calbiochem (San Diego, CA, USA). Staurosporin, doxycycline, and cyclosporin A were purchased from Sigma (St Louis, MO, USA). Cycloheximide was purchased from ICN (Irvine, CA, USA).

HEK-293, HEK293T, Madin-Darby canine kidney (MDCK) epithelial cells, and p40 Met Tet-on inducible MDCK epithelial cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) or fetal bovine serum tetracycline free (Clontech, Mountain View, CA, USA) and 1% antibiotics (penicillin 5000 U/ml - Streptomycin 5000 μg/ml, Invitrogen). MCF-10A spontaneously immortalized human mammary epithelial cells were cultured in DMEM and HAM's F12 vol/vol (Invitrogen) supplemented with 5% horse serum (Invitrogen), 500 ng/ml hydrocortisone (Calbiochem), 20 ng/ml epidermal growth factor (Peprotech), 10 μg/ml insulin (Sigma), and 100 ng/ml cholera toxin (Calbiochem) and 1% antibiotics. Cells were cultured at 37°C in a water-saturated 5% CO_2 atmosphere.

Antibodies

Mouse monoclonal antibody against the C-terminal domain of mouse Met (B-2), goat polyclonal antibody against the C-terminal domain of human actin (C-11), rabbit polyclonal against the C-terminal domain of human PARP-1 (H250), and antibody against ERK2 (C14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody against the kinase domain of Met (3D4), Green-fluorescent Alexa fluor 488 conjugated anti-mouse IgG (H+L) and red-fluorescent Alexa fluor 594 conjugated anti-rabbit IgG (H+L) were purchased from Invitrogen. Mouse antibody against cytochrome *c* oxidase subunit 4 (20E8) and nucleoporin p62 were purchased from Abcam (Cambridge, MA, USA). Rabbit polyclonal antibody against activated caspase 3 (Asp 275, #9661) and rabbit antibody against the phosphorylated tyrosine (Y 1234/1235) of the Met kinase domain (#3126) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibodies against the Flag and HA epitopes were purchased from Sigma. Custom polyclonal antibody against p40 Met was produced by Covalab (Villeurbanne, France). Briefly, rabbits were first injected twice with bridging peptide (NH2-NESVDYRATFPC-COOH, corresponding to the uncleaved sequence of Met) coupled to keyhole limpet hemocyanin without adjuvant. Secondly, rabbits were injected twice with neo-peptide (NH2-YRATFPC-COOH, corresponding to the neo-epitope generated by caspase cleavage (Tulasne et al., 2004), coupled to ovalbumin with Freund's

incomplete adjuvant. The antibodies were immunoaffinity purified first by passage of the sera through a bridging peptide sepharose column and then by passage of the flow-through through the Met neo-peptide sepharose column. The Jo2 antibody (Purified NA/LE) against mouse CD95 (the Fas receptor) was purchased from BD Pharmingen (San Diego, CA, USA). Peroxidase-conjugated antibodies against rabbit and mouse IgGs were purchased from Jackson Immunoresearch Labs (West Grove, PA, USA).

Plasmid constructs

The pSTAR vectors encoding wild-type and K1108A p40 Met were constructed as follows. Mouse Met fragments (wild-type and K1108A) were amplified by PCR with the following primers 5'-CAGAATTCATGTACAGAGCTACTTTTCCAG-3' containing the EcoRI restriction site and 5'-GTGGATCCTCAATCAATGTTGTCTTGGGATG-3' containing the BamHI restriction site. The PCR products were inserted into pSTAR (Zeng et al., 1998) between the EcoRI and BamHI restriction sites.

The N-terminally deleted vectors were constructed as follows. Mouse Met fragments were amplified by PCR with the following forward primers: 5'-CTACTTTTCCAGGATCCCAGTTTCCC-3'(Q1009), 5'-GTAGTGATTGGATCCAGCAGCC-3'(S1072), 5'-CTGACTGAGGGATCCATCATG-3'(I1180) containing the BamHI restriction site and the reverse primer 5'-GGGCCCTCTAGACCCCTAGCCATC-3' containing an XbaI restriction site. The PCR products were inserted into pcDNA3-flag between the BamHI and XbaI restriction sites.

The pCMV plasmid constructs encoding HA-tagged wild-type and kinase-dead p40 Met were made as follows. Mouse Met fragments (wild-type and K1108A) were amplified by PCR with the following primers 5'-CCCGAATTCAATACAGAGCTACTTTTCCAGAA-3' containing an EcoRI restriction site and 5'-GTACCTCGAGCCCCTCTCAATCAATGTTGTCTTGGG-3'. The PCR product was inserted into pCMV-Ha from Clontech.

The p40 Met fragments mutated in BH3 (L1110E and D1115E) and at H1104D mutated were created with the QuickChange site-directed mutagenesis system of Stratagene, using pcDNA3 Flag p40 Met WT as a template and the following primers: 5'-GTGCTGTGAAATCCGAGAATAGAATCACAG-3 and 5'-CTGTGATTCTATTCTCGGATTTGACAGCAC-3' or 5'-GAATAGAATCACAGAGATAGAAGAGGTCTC-3' and 5'-GAGACCTCTTCTATCTCTGTGATTCTATTC-3' or 5'-GACGGAAAGAAAATTGACTGTGCTGTGAAATCC-3' and GGATTTCACAGCACAGTCAATTTTCTTTCCGTC-3'. The amplified sequences were subcloned into pcDNA3 Flag between the BamHI and XhoI restriction sites and the presence of the mutation was checkes by sequencing.

SMS-p40 Met was constructed as follows. The mouse Met fragment was amplified by PCR with the following primers 5'-CTGGCGGCCGCCTACAGAGCTACTTTTCCAGAAGACCAG-3' containing a NotI restriction site and 5'-TACCTCTAGATCAATCAATGTTGTCTTGGGATGGCAAC-3' containing an XbaI restriction site. The PCR product was subcloned into pcDNA3-SMS between the NotI and XbaI restriction sites. Plasmid pcDNA3-SMS was constructed as follows. A vector containing v-src was amplified by PCR with the following primers 5'-GAATTAATTGCGGCCGCATTGAAAGCGTTAAC-3' containing a NotI restriction site and 5'GACGTGAAGCTTACCACCATGGGGAG-3' containing a HindIII restriction site. The PCR product was subcloned into an empty pcDNA3 vector between the HindIII and NotI restriction sites.

Transfections

Transfections of HEK293 and MDCK cells with polyethyleneamine (PEI) Exgen 500 (Euromedex) and Lipofectamine (Invitrogen) reagents were performed as previously described (Foveau et al., 2007). HEK293T cells were transiently transfected by the CaCl2 method. MCF-10A cells were plated on glass coverslips in 12-well plates (100,000 per well). The next day the cells were transfected with Jet Prime (Polyplus Transfection, Illkirch, France; 0.75 µg DNA/1 µl Jet Prime reagent/75 µl Jet Prime buffer in 1 mL serum-containing medium). After 4 h, the transfection medium was replaced with serum-containing medium.

RNA interference

MCF-10A cells were harvested. 200,000 cells were incubated with 3 µL Lipofectamine 2000 (Invitrogen) mixed with 3 nM of a pool of three stealth siRNAs (Invitrogen) targeting Met [5′-CCAUUUCAACUGAGUUUGCUGUUAA-3′, 5′-UCCAGAAGAUCAGUUUCCUAAUUCA-3′, 5′CCGAGGGAAUCAUCAUGAAAGAUUU-3']. The cells were then plated in a 6-well plate in complete medium.

Western blotting and immunoprecipitation

Western blotting were performed as previously described (Foveau et al., 2009; Foveau et al., 2007). For co-immunoprecipitation in HEK 293T, cells were harvested 24 h after $CaCl_2$ transfection and lysed in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH8.0) at 4°C for 1 h. For co-immunoprecipitation in MCF-10A , cells were harvested 24 h after serum starved. The next day, the cells were treated or not with staurosporin and lysed in PY buffer (50mM NaCl, 5mM EDTA, 1% Triton X-100, 20mM Tris-HCL pH 7.4).

Immunofluorescence staining

MDCK and MCF-10A cells were plated on glass coverslips in 12-well plates (100,000 per well) and transiently transfected the next day as described above. After transfection, the cells were washed

and fixed in 4% PFA at room temperature. The cells were washed and permeabilized with PBS containing 0.5% Triton X-100 for 10 min at room temperature and blocked in 0.2% casein for 30 min. Primary antibodies were incubated for 1 h at room temperature. Combinations of antibodies were used (mouse anti-C-terminal domain of mouse Met (0.4 µg/mL) with rabbit anti-cleaved caspase 3 (1:250); mouse anti-cytochrome *c* (5 µg/mL) with rabbit anti-Flag (1 µg/mL)). The cells were washed with PBS and incubated for 60 min with a combination of Alexa Fluor-conjugated secondary antibodies (green-fluorescent Alexa Fluor 488 conjugated anti-mouse IgG (H+L) and red-fluorescent Alexa Fluor 594 conjugated anti-rabbit IgG (H+L) diluted to 2 µg/ml. The cells were washed with PBS and the nuclei counterstained with Hoechst 33258. Coverslips were mounted with Glycergel mounting medium (Dako, Carpenteria, CA). Fluorescence was examined in oil immersion at 21°C. For fluorescence microscopy, slides were observed in an Axion Imager Z1 (Carl Zeiss), numerical aperture: ECL-PLAN NEOFLUAR 40x NA 1.3, with a monochrome Zeiss AxioCam MRm camera and the Axiovision acquisition software. For confocal fluorescence microscopy, slides were observed in a LSM 710 Laser Scanning Microscope (Carl Zeiss), numerical aperture PLAN-APOCHROMAT 63x NA 1.4, with the ZEN acquisition software.

Mitochondrial staining was performed 24 h after transfection. Cells were stained with 100 nM MitoTracker (Invitrogen) for 30min at 37°C and coverslips were immersed in methanol:acetone (1:1) at -20°C for 10 minutes. Cells were washed, 0.2% casein was added and incubation was carried out for 30 min to block nonspecific binding. Staining was performed as described above.

In vitro kinase assays

Proteins were immunoprecipitated as described above. Immune complexes were washed four times with ice-cold lysis PY buffer and once with kinase buffer (20 mM MOPS pH: 7.2, 7.5 mM $MgCl_2$, 25 mM glycerophosphate, 5 mM EGTA, 1 mM Na_3VO_4, 1 mM DTT). Each immunoprecipitate was incubated for 30 min at 30°C with 20 µl kinase reaction buffer (kinase buffer supplemented with 50 µM ATP and 10 µM (10 µCi) [γ-^{33}P] ATP (2000 Ci/mmol) (Perkin Elmer, San Jose, CA, USA). Kinase reactions were stopped by addition of 10 µl Laemmli sample buffer (5x). The phosphorylated proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis, dried, and incorporation of [γ-^{33}P] was visualized by autoradiography on hyperfilm-MP (Amersham, Arlington Heights, IL, USA).

Subcellular fractionation

MCF-10A cells were collected, washed in PBS, resuspended in buffer (10 mM Tris pH 7.6, 250 mM sucrose, 10 mM KCl, 0.15 mM $MgCl_2$) and lysed by Dounce homogenization. The suspension was centrifuged at 850 *g* for 5 min at 4°C to collect the nuclear fraction. The resulting supernatant was centrifuged at 20,000 *g* for 15 min at 4°C to pellet the mitochondria. The cytosolic fraction (supernatant) was then obtained by centrifugation at 100,000 *g* for 1 h at 4°C, and cytosolic proteins

were precipitated overnight with acetone at -20°C. Each fraction was resuspended in PY buffer and the same amount of protein was solubilized in Laemmli sample buffer.

Isolation of mouse liver mitochondria

Male C57BL/6 mice weighing 19-20 g, having fasted overnight, were injected intraperitoneally with 4 µg anti-Fas antibody (Jo2-antibody) per mouse or saline buffer (Wang et al., 2005). Mice were sacrificed by cerebral dislocation and livers were quickly removed and placed in ice-cold sucrose medium A (0.3 M sucrose, 5 mM TES, pH7.2, 1 mM EGTA, 1mg/mL BSA) (Belzacq et al., 2003). The livers were minced and homogenized with a Potter-Elvehjem homogenizer with a loose-fitting pestle. Nuclei and cell debris were removed by centrifugation at 760 *g* for 10 min at 4°C. Then the supernatant was centrifuged at 8700 *g* for 10 min at 4°C to obtain the mitochondrial pellet and resuspended in 300 µL medium A. To separate intact from broken mitochondria, the organelles were layered on Percoll gradients (10 min at 8800 *g*) consisting of a layer at 18%, a layer at 30%, and a layer at 60% Percoll (w/v) in medium B (0.3 M saccharose, 10 mM TES, 0.2 mM EGTA, 1 mg/mL BSA, pH 6.9). Then mitochondria-enriched fractions were collected from the 30%/60% interface and washed (10 min at 6800 *g*) with medium A and the pellet was resuspended in 500 µl medium A. Protein concentrations were determined by the BCA protein assay (ThermoFisher Scientific, Rockford, IL, USA).

Caspase activity

Adherent MDCK cells, detached with trypsin, and cells in suspension, collected after elimination of the medium by centrifugation, were lysed in PY buffer without protease inhibitor. 50 µL aliquots all containing the same amount of protein were transferred to a 96-well plate and 50 µL assay buffer (100 mM Hepes, 10% Sucrose, 10 mM DTT, 500 µM EDTA) was added. After 30 min of incubation at 37°C, the caspase substrate DEVD-AFC was added to each well and the fluorescence was monitored every 10 min for 3 h (excitation=395 nm, emission=510 nm) with a fluorimeter (BMG Labtech FLUOstar OPTIMA). The slopes of the activity curves were calculated and expressed with respect to the untreated control.

Colony formation assay

For the colony formation assay, 6 x10^5 HEK293 cells were plated in 6-well plates and the next day, transient transfections were performed using the PEI Exgen 500 procedure with 1 µg DNA/well. Two days after transfection, the cells were split into three 100-mm dishes containing DMEM-10% FCS, and the next day the medium was supplemented with 1.1 mg/ml G418 (Gibco). After 10 days, the cells were counted.

Acknowledgments

We thank the Microscopy-Imaging-Cytometry Facility of the Lille Pasteur Campus (MICPal) and the BioImaging Center Lille Nord-de-France for access to instruments and technical advice. This work was supported by the CNRS, the Institut Pasteur de Lille, and INSERM, and by grants from the the "Ligue contre le Cancer, comité Nord", the “Association pour la Recherche sur le Cancer”, and the “Agence Nationale de la Recherche”, Young investigator Program. JL was supported by a grant from the French Research and Technology Ministry, GM by the “Agence Nationale pour la Recherche”. We would like to thank Cindy Gallerne, Christophe Lemaire (INSERM UMR S-769, Paris, France), and Patrick Dumont (CNRS, UMR8161, IBL, Lille, France).

Conflict of Interest

The authors declare to have no conflict of interest.

References

Bachelder, R.E., M.A. Wendt, N. Fujita, T. Tsuruo, and A.M. Mercurio. 2001. The cleavage of Akt/protein kinase B by death receptor signaling is an important event in detachment-induced apoptosis. *J Biol Chem*. 276:34702-7.

Belzacq, A.S., H.L. Vieira, F. Verrier, G. Vandecasteele, I. Cohen, M.C. Prevost, E. Larquet, F. Pariselli, P.X. Petit, A. Kahn, R. Rizzuto, C. Brenner, and G. Kroemer. 2003. Bcl-2 and Bax modulate adenine nucleotide translocase activity. *Cancer Res*. 63:541-6.

Bladt, F., D. Riethmacher, S. Isenmann, A. Aguzzi, and C. Birchmeier. 1995. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature*. 376:768-71.

Brenner, C., and S. Grimm. 2006. The permeability transition pore complex in cancer cell death. *Oncogene*. 25:4744-56.

Coleman, M.L., E.A. Sahai, M. Yeo, M. Bosch, A. Dewar, and M.F. Olson. 2001. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nature cell biology*. 3:339-45.

Comoglio, P.M., S. Giordano, and L. Trusolino. 2008. Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nat Rev Drug Discov*. 7:504-16.

Condorelli, F., P. Salomoni, S. Cotteret, V. Cesi, S.M. Srinivasula, E.S. Alnemri, and B. Calabretta. 2001. Caspase cleavage enhances the apoptosis-inducing effects of BAD. *Mol Cell Biol*. 21:3025-36.

Deheuninck, J., G. Goormachtigh, B. Foveau, Z. Ji, C. Leroy, F. Ancot, V. Villeret, D. Tulasne, and V. Fafeur. 2009. Phosphorylation of the MET receptor on juxtamembrane tyrosine residue 1001 inhibits its caspase-dependent cleavage. *Cell Signal*. 21:1455-63.

Fan, S., M. Gao, Q. Meng, J.J. Laterra, M.H. Symons, S. Coniglio, R.G. Pestell, I.D. Goldberg, and E.M. Rosen. 2005. Role of NF-kappaB signaling in hepatocyte growth factor/scatter factor-mediated cell protection. *Oncogene*. 24:1749-66.

Fan, S., J.A. Wang, R.Q. Yuan, S. Rockwell, J. Andres, A. Zlatapolskiy, I.D. Goldberg, and E.M. Rosen. 1998. Scatter factor protects epithelial and carcinoma cells against apoptosis induced by DNA-damaging agents. *Oncogene*. 17:131-41.

Forcet, C., X. Ye, L. Granger, V. Corset, H. Shin, D.E. Bredesen, and P. Mehlen. 2001. The dependence receptor DCC (deleted in colorectal cancer) defines an alternative mechanism for caspase activation. *Proc Natl Acad Sci U S A*. 98:3416-21.

Foveau, B., F. Ancot, C. Leroy, A. Petrelli, K. Reiss, V. Vingtdeux, S. Giordano, V. Fafeur, and D. Tulasne. 2009. Downregulation of the Met Receptor Tyrosine Kinase by Presenilin-dependent Regulated Intramembrane Proteolysis. *Mol Biol Cell*. 20:2494-506.

Foveau, B., C. Leroy, F. Ancot, J. Deheuninck, Z. Ji, V. Fafeur, and D. Tulasne. 2007. Amplification of apoptosis through sequential caspase cleavage of the MET tyrosine kinase receptor. *Cell Death Differ*. 14:752-64.

Goldschneider, D., and P. Mehlen. 2010. Dependence receptors: a new paradigm in cell signaling and cancer therapy. *Oncogene*. 29:1865-82.

Kroemer, G., L. Galluzzi, and C. Brenner. 2007. Mitochondrial membrane permeabilization in cell death. *Physiol Rev*. 87:99-163.

Kurokawa, M., and S. Kornbluth. 2009. Caspases and kinases in a death grip. *Cell*. 138:838-54.

Liu, J., F. Yao, R. Wu, M. Morgan, A. Thorburn, R.L. Finley, Jr., and Y.Q. Chen. 2002. Mediation of the DCC apoptotic signal by DIP13 alpha. *J Biol Chem*. 277:26281-5.

Liu, Y. 1999. Hepatocyte growth factor promotes renal epithelial cell survival by dual mechanisms. *Am J Physiol*. 277:F624-33.

Lomonosova, E., and G. Chinnadurai. 2008. BH3-only proteins in apoptosis and beyond: an overview. *Oncogene*. 27 Suppl 1:S2-19.

Longati, P., A. Bardelli, C. Ponzetto, L. Naldini, and P.M. Comoglio. 1994. Tyrosines1234-1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). *Oncogene*. 9:49-57.

Matteucci, E., P. Bendinelli, and M.A. Desiderio. 2009. Nuclear localization of active HGF receptor Met in aggressive MDA-MB231 breast carcinoma cells. *Carcinogenesis*. 30:937-45.

Mehlen, P., and D.E. Bredesen. 2011. Dependence receptors: from basic research to drug development. *Sci Signal*. 4:mr2.

Mille, F., C. Thibert, J. Fombonne, N. Rama, C. Guix, H. Hayashi, V. Corset, J.C. Reed, and P. Mehlen. 2009. The Patched dependence receptor triggers apoptosis through a DRAL-caspase-9 complex. *Nat Cell Biol*. 11:739-46.

Naresh, A., W. Long, G.A. Vidal, W.C. Wimley, L. Marrero, C.I. Sartor, S. Tovey, T.G. Cooke, J.M. Bartlett, and F.E. Jones. 2006. The ERBB4/HER4 intracellular domain 4ICD is a BH3-only protein promoting apoptosis of breast cancer cells. *Cancer Res*. 66:6412-20.

Ogasawara, J., R. Watanabe-Fukunaga, M. Adachi, A. Matsuzawa, T. Kasugai, Y. Kitamura, N. Itoh, T. Suda, and S. Nagata. 1993. Lethal effect of the anti-Fas antibody in mice. *Nature*. 364:806-9.

Ponzetto, C., A. Bardelli, Z. Zhen, F. Maina, P. dalla Zonca, S. Giordano, A. Graaziani, G. Panayotou, and P.M. Comoglio. 1994. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell*. 77:261-271.

Pozner-Moulis, S., D.J. Pappas, and D.L. Rimm. 2006. Met, the hepatocyte growth factor receptor, localizes to the nucleus in cells at low density. *Cancer Res*. 66:7976-82.

Schmidt, C., F. Bladt, S. Goedecke, V. Brinkmann, W. Zschiesche, M. Sharpe, E. Gherardi, and C. Birchmeier. 1995. Scatter factor/hepatocyte growth factor is essential for liver development. *Nature*. 373:699-702.

Schmidt, L., F.M. Duh, F. Chen, T. Kishida, G. Glenn, P. Choyke, S.W. Scherer, Z. Zhuang, I. Lubensky, M. Dean, R. Allikmets, A. Chidambaram, U.R. Bergerheim, J.T. Feltis, C. Casadevall, A. Zamarron, M. Bernues, S. Richard, C.J. Lips, M.M. Walther, L.C. Tsui, L. Geil, M.L. Orcutt, T. Stackhouse, B. Zbar, and et al. 1997. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nat Genet*. 16:68-73.

Sebbagh, M., C. Renvoize, J. Hamelin, N. Riche, J. Bertoglio, and J. Breard. 2001. Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nature cell biology*. 3:346-52.

Strohecker, A.M., F. Yehiely, F. Chen, and V.L. Cryns. 2008. Caspase cleavage of HER-2 releases a Bad-like cell death effector. *J Biol Chem*. 283:18269-82.

Trusolino, L., A. Bertotti, and P.M. Comoglio. 2010. MET signalling: principles and functions in development, organ regeneration and cancer. *Nat Rev Mol Cell Biol*. 11:834-48.

Tulasne, D., J. Deheuninck, F.C. Lourenco, F. Lamballe, Z. Ji, C. Leroy, E. Puchois, A. Moumen, F. Maina, P. Mehlen, and V. Fafeur. 2004. Proapoptotic function of the MET tyrosine kinase receptor through caspase cleavage. *Mol Cell Biol*. 24:10328-39.
Tulasne, D., and B. Foveau. 2008. The shadow of death on the MET tyrosine kinase receptor. *Cell Death Differ*. 15:427-34.
Uehara, Y., O. Minowa, C. Mori, K. Shlota, J. Kuno, T. Noda, and N. Kitamura. 1995. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature*. 373:702-705.
Wang, X., Y. Lu, and A.I. Cederbaum. 2005. Induction of cytochrome P450 2E1 increases hepatotoxicity caused by Fas agonistic Jo2 antibody in mice. *Hepatology*. 42:400-10.
Yamamoto, K., R. Morishita, S. Hayashi, H. Matsushita, H. Nakagami, A. Moriguchi, K. Matsumoto, T. Nakamura, Y. Kaneda, and T. Ogihara. 2001. Contribution of Bcl-2, but not Bcl-xL and Bax, to antiapoptotic actions of hepatocyte growth factor in hypoxia-conditioned human endothelial cells. *Hypertension*. 37:1341-8.
Zeng, Q., Y.H. Tan, and W. Hong. 1998. A single plasmid vector (pSTAR) mediating efficient tetracycline-induced gene expression. *Anal Biochem*. 259:187-94.

Figure legends

Figure 1: Specific detection of p40 Met during apoptosis

(A) Schematic representation of p40 Met generated by caspase cleavage of the full-length Met receptor during apoptosis. The neo-epitope produced by caspase cleavage of Met can be detected with a home-made antibody. (B) MCF-10A epithelial cells were serum starved overnight and treated for the indicated times with staurosporin (Stauro; 1 µM). (C) MCF-10A epithelial cells were transfected for 24 h with a control siRNA (lane 1 and 2) or a pool of 3 siRNAs targeting Met, starved overnight, and treated for 4 h with staurosporin. (B, C) For each condition the same amount of proteins was resolved by 10% SDS-PAGE and analyzed by western blotting with antibodies against the kinase domain of Met, against p40 Met (custom antibody against the neo-epitope generated by caspase cleavage), or against PARP. To assess loading an anti-actin or an anti-ERK2 antibody was used.

Figure 2: Consequence of p40 Met expression during the apoptotic response

(A,B,C,D) p40 Met Tet-on inducible MDCK epithelial cells (clone a) and wild-type MDCK cells were starved, stimulated or not overnight (16 h) with 0.2 µg/ml doxycycline (Doxy), and treated for the indicated time with 30 ng/ml TNFα and 10 µg/ml cycloheximide (CHX). (A) Pictures of Tet-On p40 Met MDCK epithelial cells (clone a). (B) Proteins were resolved by 12% SDS-PAGE and analyzed by western blotting with an anti-mouse Met antibody, an anti-PARP antibody, an anti-cleaved caspase 3 antibody, and an anti-ERK2 antibody to assess loading. (C) Relative caspase activity was determined from the same amount of proteins (n=3; ±standard deviation). (D) Cells were stained with trypan blue. The percentage of dead cells stained in blue was determined (n=3, ±standard deviation).

Figure 3: p40 Met phosphorylation and kinase activity

(A) MCF-10A epithelial cells were starved overnight and treated 4h with 1 µM staurosporin (Stauro) or 15 min with 30 ng/ml HGF/SF. For each condition, the same amount of protein was

immunoprecipitated with antibody against the kinase domain of Met or against p40 Met. Immunoprecipitated proteins and whole-cell extracts were resolved by 10% SDS-PAGE and analyzed by western blotting with antibody against the kinase domain of Met or against the phosphorylated tyrosine residues of the Met kinase domain. (B) MDCK epithelial cells were transfected with a vector expressing the fusion protein TPR-Met or kinase-dead TPR-Met (mutation K1108A). WT or kinase-dead (K1108A) p40 Met Tet-on MDCK epithelial cells were starved and stimulated or not overnight with 0.2 µg/ml doxycycline. Proteins were immunoprecipitated with anti-mouse Met antibody or anti-human Met antibody. Immunoprecipitated proteins were resolved by 12% SDS-PAGE and analyzed by western blotting with an antibody against the kinase domain of Met or against the phosphorylated tyrosine residues of the Met kinase domain. (C) MDCK epithelial cells were transiently transfected with a vector expressing Flag-tagged wild-type p40 Met and treated or not with 0.1 µM PHA-665752. Twenty-four hours after transfection, the nuclei were stained with Hoechst (blue staining), p40 Met was specifically labeled with an anti-flag antibody (green staining), and an anti-cleaved caspase 3 antibody was used to detect apoptotic cells (red staining). An overlay of the three stains is shown (merge). Cells were observed by fluorescence confocal microscopy. White arrows indicate representative transfected cells expressing p40 Met. The percentage of caspase 3-positive cells among the Met-expressing cells was determined. At least 200 cells were counted per well (n=3; ±standard deviation).

Figure 4: Effect of p40 Met localization on its apoptotic action

(A) MDCK epithelial cells were transfected with a vector expressing Flag-tagged WT p40 Met or SMS-p40 Met. Twenty-four hours after transfection, the nuclei were stained with Hoechst (blue staining) and immunofluorescence staining was performed with an anti-mouse Met antibody (green staining) and an anti-cleaved caspase 3 antibody (red staining). Cells were observed by fluorescence confocal microscopy. White arrows indicate representative transfected cells expressing p40 Met. (B) The percentage of caspase 3-positive cells among the Met-expressing cells was determined. At least 200 cells were counted per well (n=3; ±standard deviation).

Figure 5: Subcellular localization of p40 Met

(A) MCF-10A epithelial cells were starved overnight and treated for 4 h with 1 µM staurosporin (Stauro). After treatment, the cells were fractionated into a nuclear, a cytosolic, and a mitochondrial fraction. The same amount of proteins from the whole-cell lysates and the different fractions was resolved by 12% SDS-PAGE and analyzed by western blotting with antibodies against p40 Met, the nuclear protein Nup 62, the cytosolic protein GAPDH, or the inner mitochondrial membrane protein Cox IV. (B) MDCK epithelial cells were transfected with a vector expressing Flag-tagged p40 Met. Twenty-four hours after transfection, the nuclei were stained with Hoechst (blue staining), the mitochondria were stained with MitoTracker (Red), and immunofluorescence staining was performed

with an anti-flag antibody (green staining). Cells were observed by fluorescence confocal microscopy. (C) Mice were injected with saline (ø) or Jo2 antibody at the indicated time. After treatment, their livers were removed and the mitochondria were isolated. The same amount of proteins from the liver and the purified mitochondria was resolved by 15% or 10% SDS-PAGE and analyzed by western blotting with antibodies against p40 Met, the kinase domain of Met, the cytosolic protein GAPDH, or the inner mitochondrial membrane protein Cox IV. The same amount of MCF-10A whole-cell lysate, treated or not for 4 h with 1 μM anisomycin, was used as a control for p40 Met detection. Arrows indicate p40 Met and a non-specific band (NS).

Figure 6: p40 Met localizes to both the mitochondria and the nucleus

(A) Schematic representation of p40 Met tagged N-terminally deleted version, as indicated generated by mutagenesis. (B) MDCK epithelial cells were transfected with a vector expressing Flag-tagged wild-type p40 Met or a Flag-tagged N-terminally deleted version, as indicated. Twenty-four hours after transfection, the nuclei were stained with Hoechst (blue staining), the mitochondria were stained with MitoTracker (Red), and immunofluorescence staining was performed with an anti-Flag antibody (green staining). The cells were observed by fluorescence confocal microscopy.

Figure 7: Characterization of the p40 Met BH3 domain

(A) Alignment of BH3-only and BH3-only-like proteins with the sequences of human and mouse p40 Met. Their accession numbers and amino acid coordinates are indicated. Φ represents a hydrophobic residue (Φ_2 is usually leucine); Σ is a small residue (G, A, S), Z is usually an acidic residue; Γ is a hydrophilic residue (N, H, D or Y) as adapted from Lomonosova and Chinnadurai (2008). (B) MDCK epithelial cells were transiently transfected with a vector expressing Flag-tagged wild-type p40 Met, K1108A p40 Met, or p40 Met mutated at two conserved residues of the BH3 site. The conserved Leu^{1110} and Asp^{1115} in mouse p40 Met were mutated to glutamic acid in combination (L1110E/D1115E, named 2E). Twenty-four hours after transfection, the nuclei were stained with Hoechst (blue staining) and immunofluorescence straining was performed with an anti-Flag antibody (green staining) and an anti-cleaved caspase 3 antibody (red staining). The percentage of caspase 3 positive cells among the Met-expressing cells was determined. At least 200 cells were counted per well in three different wells (n=3; ±standard deviation). (C) HEK293 cells were transiently transfected with a vector expressing Flag-tagged p40 Met wild type, K1108A or mutated on conserved residue of BH3 site, either alone (L1110E or D1115E) or in combination (L1110E/D1115E, named 2E). After selection, cells were counted. Results are shown as percentages of surviving cells, using transfection with the empty vector as reference (n=3; ±standard deviation). (D) HEK293T cells were transiently transfected with a vector expressing HA-tagged WT or K1108A p40 Met and/or a vector expressing Bcl-2. Twenty-four hours after transfection, the cells were lysed and proteins were

immunoprecipitated with anti-HA antibody. The immunoprecipitated proteins and whole cell lysate (WCL) were resolved by 10% SDS-PAGE and analyzed by western blotting with antibodies against HA or Bcl-2.

Figure 8: Cytochrome *c* release induced by p40 Met

(A,B) MDCK epithelial cells were transiently transfected with a vector expressing Flag-tagged wild-type, kinase-dead, or 2E p40 Met and treated or not with 2.5 µM cyclosporin A (CSA) or 20 µM caspase inhibitor zVAD-FMK. Twenty-four hours after transfection, the nuclei were stained with Hoechst (blue staining), and immunofluorescence staining was performed with an anti-anti-mouse Met antibody (green staining) and an anti-cleaved caspase 3 antibody (red staining). An overlay of the three stains is shown (merge). Cells were observed by fluorescence microscopy. White arrows indicate representative apoptotic cells expressing p40 Met. The percentage of activated caspase 3 among the Met-transfected cells was determined. At least 200 cells were counted per well (n=3; ±standard deviation). (C,D) MCF-10A epithelial cells were transiently transfected with a vector expressing Flag-tagged wild-type, H1104D, or 2E p40 Met and treated or not with 2.5 µM cyclosporin A (CSA) or 20 µM caspase inhibitor zVAD-FMK. Twenty-four hours after transfection, the nuclei were detected with Hoechst (blue staining) and immunofluorescence staining was performed with an anti-Flag antibody (red staining) and an anti-cytochrome *c* antibody (green staining). Cells were observed by fluorescence confocal microscopy. White arrows indicate representative transfected cells expressing p40 Met. The percentage of Met-transfected cells displaying cytochrome *c* release was determined. At least 200 cells were counted per well (n=3; ±standard deviation).

Figure 9: Effect of Met silencing on mitochondrial release

(A,B,C) MCF-10A epithelial cells were transfected with a control siRNA or a pool of 3 Met-targeting siRNAs for 24 h and starved overnight. Then the cells were treated for 2 h with HGF/SF (30 ng/ml) before staurosporin (1 µM) treatment at the indicated time. (A) Cells were lysed, proteins were resolved by 10% SDS-PAGE and analyzed by western blotting with antibodies against the kinase domain of Met and against ERK2. (B,C) For each condition, the nuclei were stained with Hoechst (blue staining) and immunofluorescence was performed with an anti-cytochrome *c* antibody (green staining). Cells were observed by fluorescence microscopy. (B) The percentage of cells displaying cytochrome *c* release was determined (200 cells were counted per well; n=3; ±standard deviation).

Supplementary data

Figure S1: (A) MDCK Tet-on p40 Met inducible epithelial cells (clone a) or wild type MDCK epithelial cells were starved and stimulated or not with 0.2 µg/ml doxycylin overnight or treated for the indicated time with 30 ng/ml TNFα and 10 µg/ml cycloheximide (CHX). (B) WT p40 Met Tet-on inducible MDCK epithelial cells (clone a) were starved and stimulated or not overnight with different concentrations of doxycyline. (C) p40 Met Tet-on inducible MDCK epithelial cells was starved and stimulated or not with 0.2µg/ml doxycyline (Doxy) at indicated times. (A, B, C) Proteins were resolved by 12% SDS-PAGE and analyzed by Western blotting using an anti-mouse Met antibody,an anti-cleaved caspase 3 antibody and an anti-ERK2 antibody to assess the loading.

Figure S2: (A,B) WT p40 Met (clone a and clone b) Tet-on inducible MDCK epithelial cells and (C) wild-type MDCK cells were starved and stimulated or not overnight with 0.2 µg/ml doxycyline and treated for the indicated time with 50 µM anisomycin. (D) WT p40 Met (clone b) or (E) kinase-dead p40 Met (K1108A) Tet-on inducible MDCK epithelial cells and (F) wild-type MDCK cells were starved, stimulated or not overnight with 0.2 µg/ml doxycyline, and treated for the indicated time with 30 ng/ml TNFα and 10 µg/ml cycloheximide (CHX). Proteins were resolved by 12% SDS-PAGE and analyzed by western blotting with anti-mouse Met antibody, anti-PARP antibody, anti-cleaved caspase 3 antibody, and anti-ERK2 antibodies.

Figure S3: MCF-10A cells were cultured for three days and treated for the indicated time with 50 µM anisomycin or for 15 min with 30 ng/ml HGF/SF. Proteins were resolved by 10% SDS-PAGE and analyzed by western blotting with antibodies against the kinase domain of Met, the phosphorylated tyrosine residues of the Met kinase domain and ERK2.

Figure S4: MCF-10A epithelial cells were starved and treated overnight with the Met kinase inhibitor PHA-665752 (0.1 µM) or SU-11274 (2.5 µM). Cells were treated or not for 6 h with 30 ng/ml HGF/SF and for 4 h with 50 µM Anisomycin (Aniso). For each condition the same amount of protein was resolved by 10% SDS-PAGE and analyzed by western blotting with antibodies against the kinase domain of Met or PARP. To assess loading, an anti-ERK2 antibody was used.

Figure S5:

(A) HEK 293 cells were transfected or not with an empty vector or a vector expressing Flag-tagged wild-type p40 Met (p40 Met), kinase-dead p40 Met (p40 Met K1108A), or p40 Met fused to the Src myristylation site (SMS-p40 Met). Cell extracts were resolved by 12% SDS-PAGE and analyzed by western blotting with an anti-mouse Met antibody to assess expression from the transfected vector.

(B) HEK 293 cells were transfected with a vector expressing Flag-tagged wild-type 40 Met or a Flag-tagged N-terminally deleted version schematically presented. Twenty-four hours after transfection, the cells were lysed and the protein mixture was resolved by 12% SDS-PAGE and analyzed by western blotting with antibodies against the kinase domain of Met and against ERK2.
(C) Cell extracts prepared 48 h after transfection of HEK 293 (Fig. 7C) were resolved by 12% SDS-PAGE and analyzed by western blotting with anti-mouse Met and anti-phospho Met antibodies.

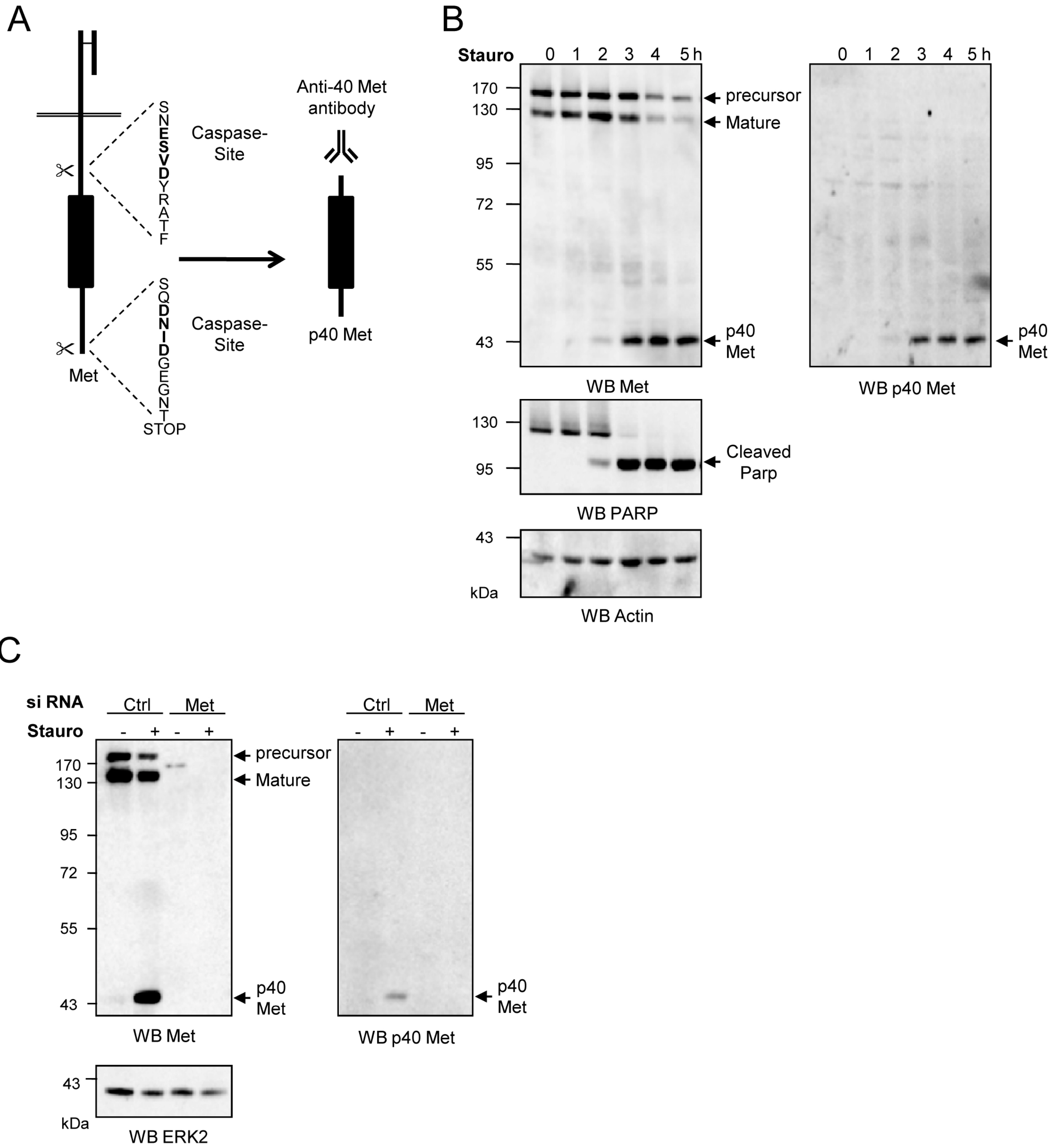


Figure 1

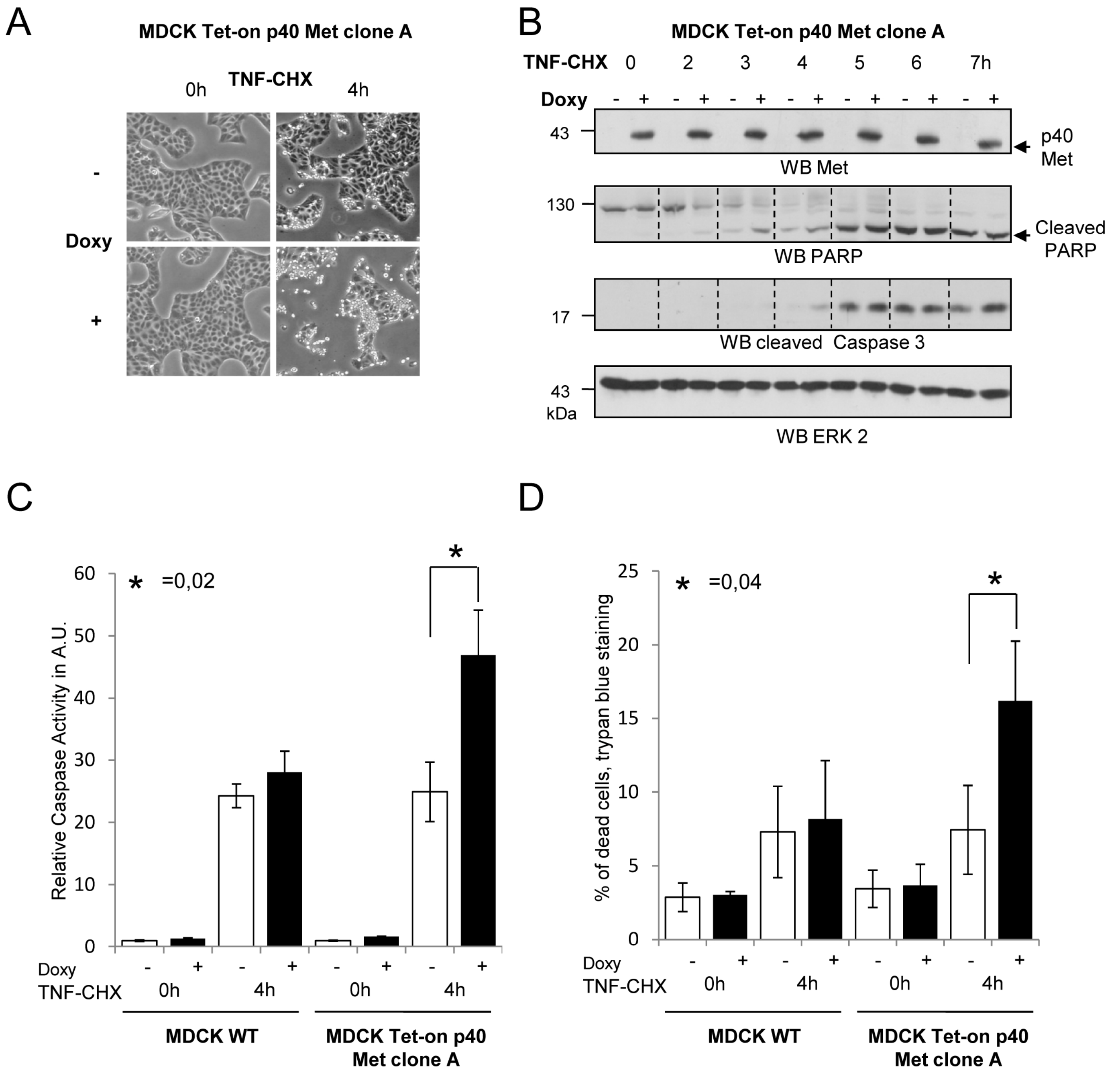


Figure 2

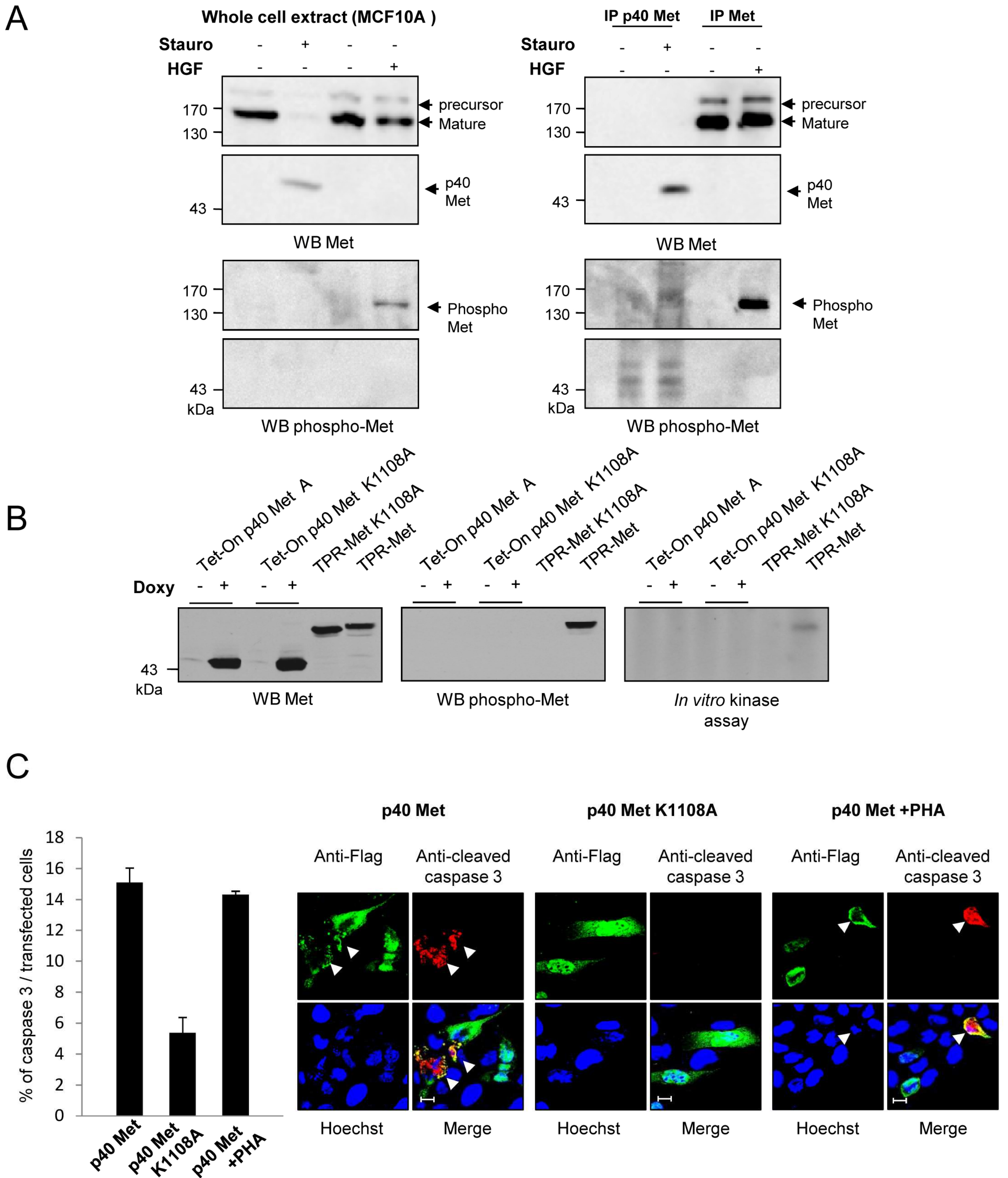
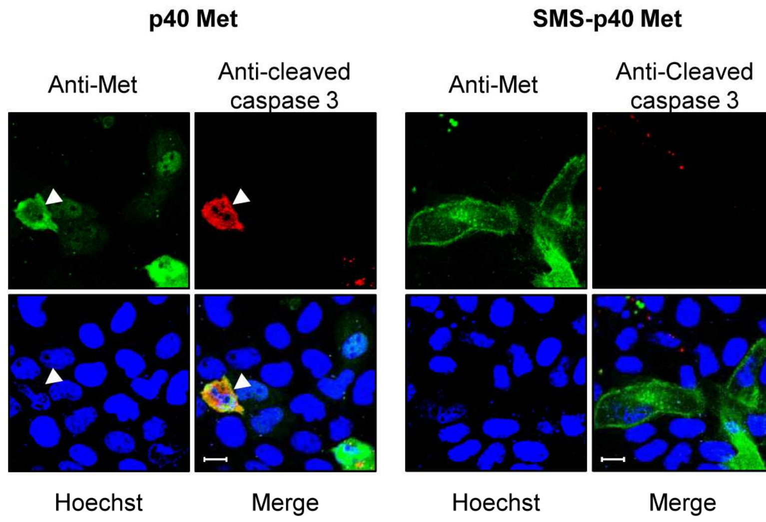


Figure 3



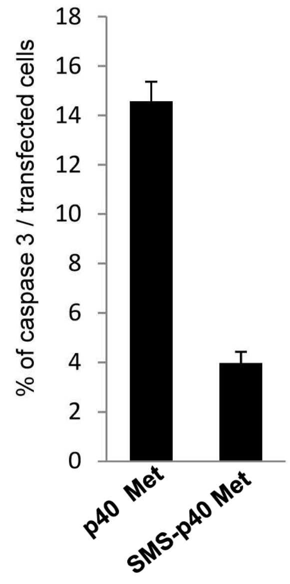


Figure 4

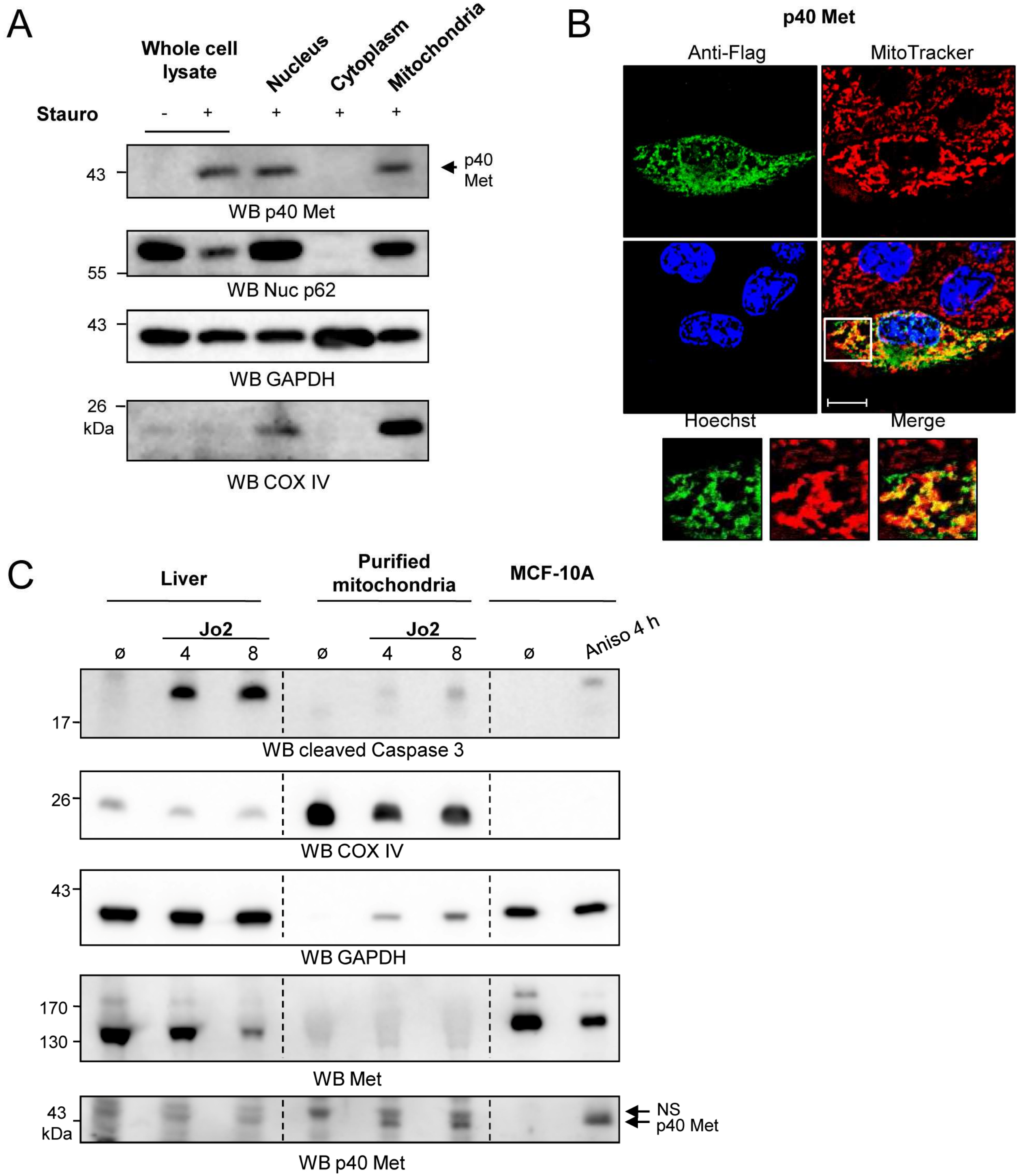
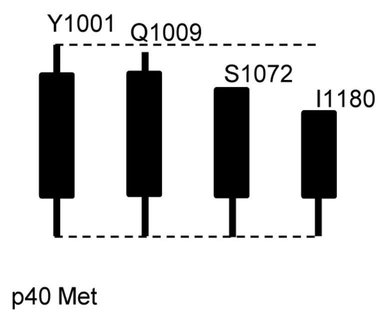


Figure 5

A



p40 Met

B

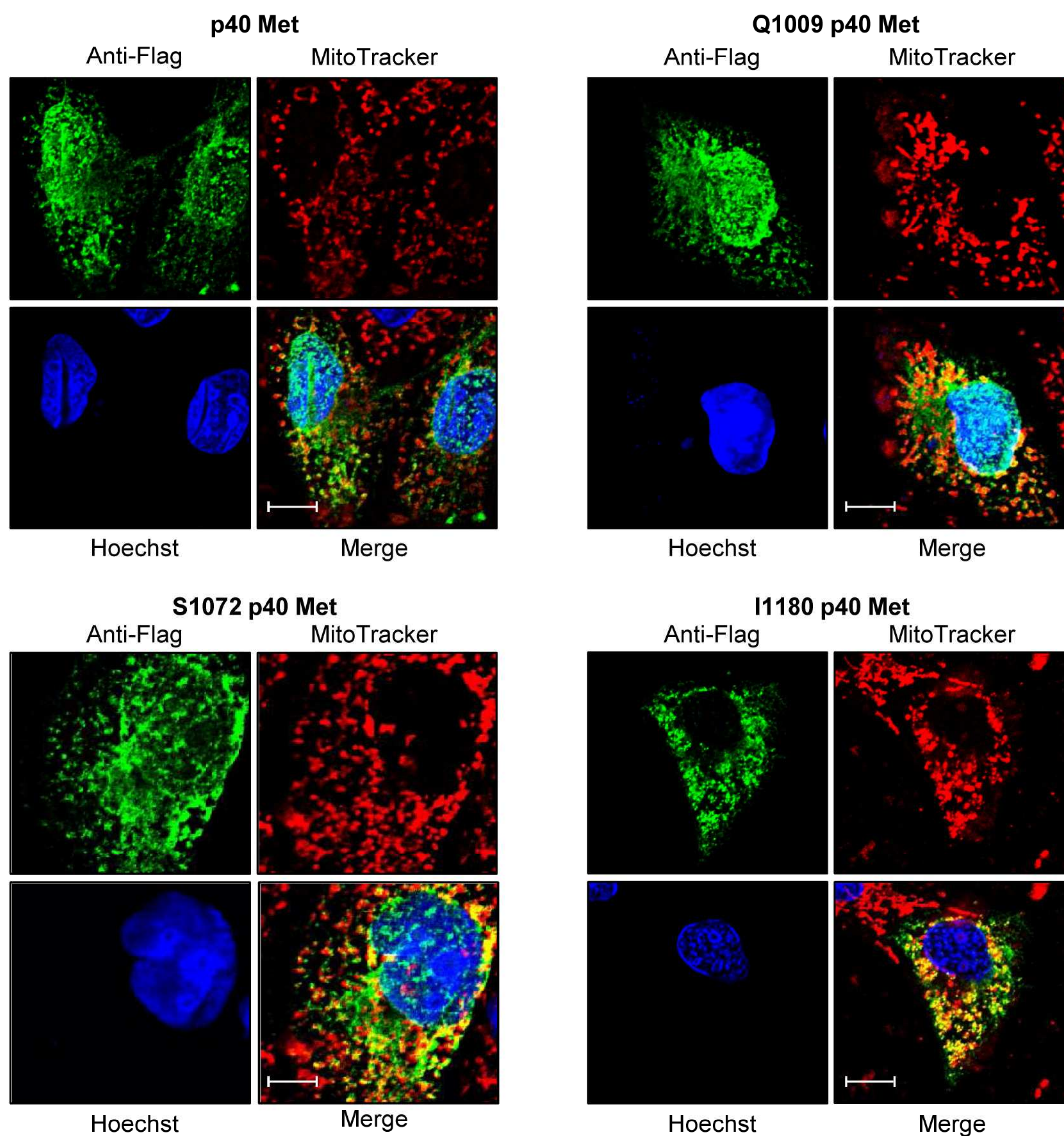
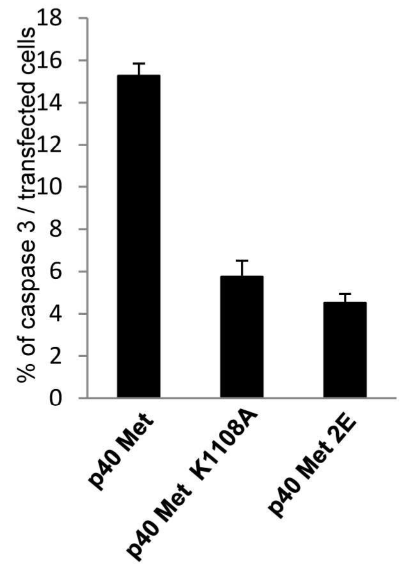
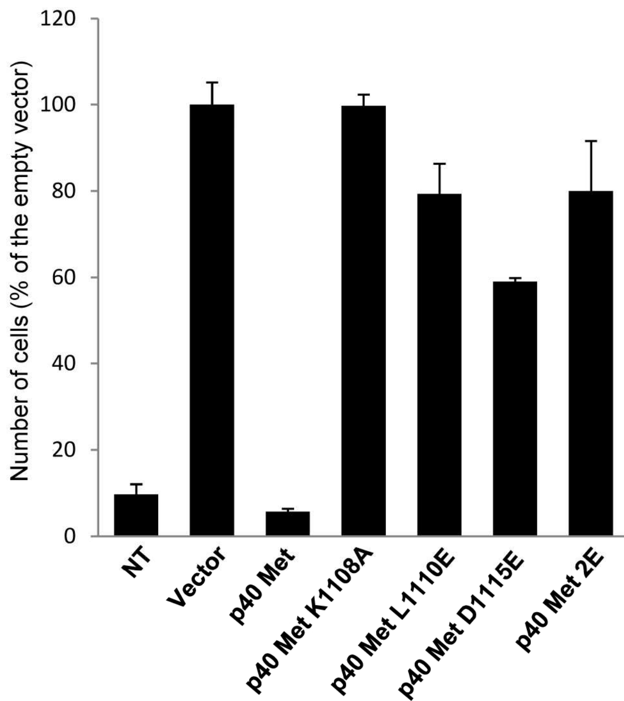


Figure 6





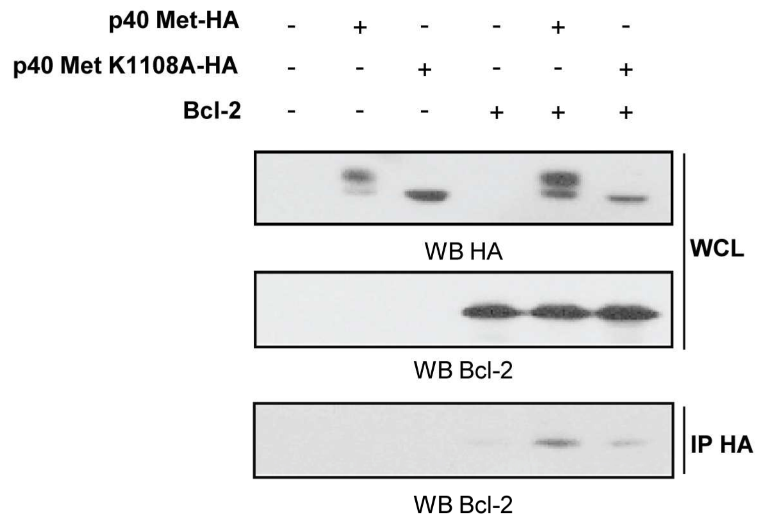


Figure 7

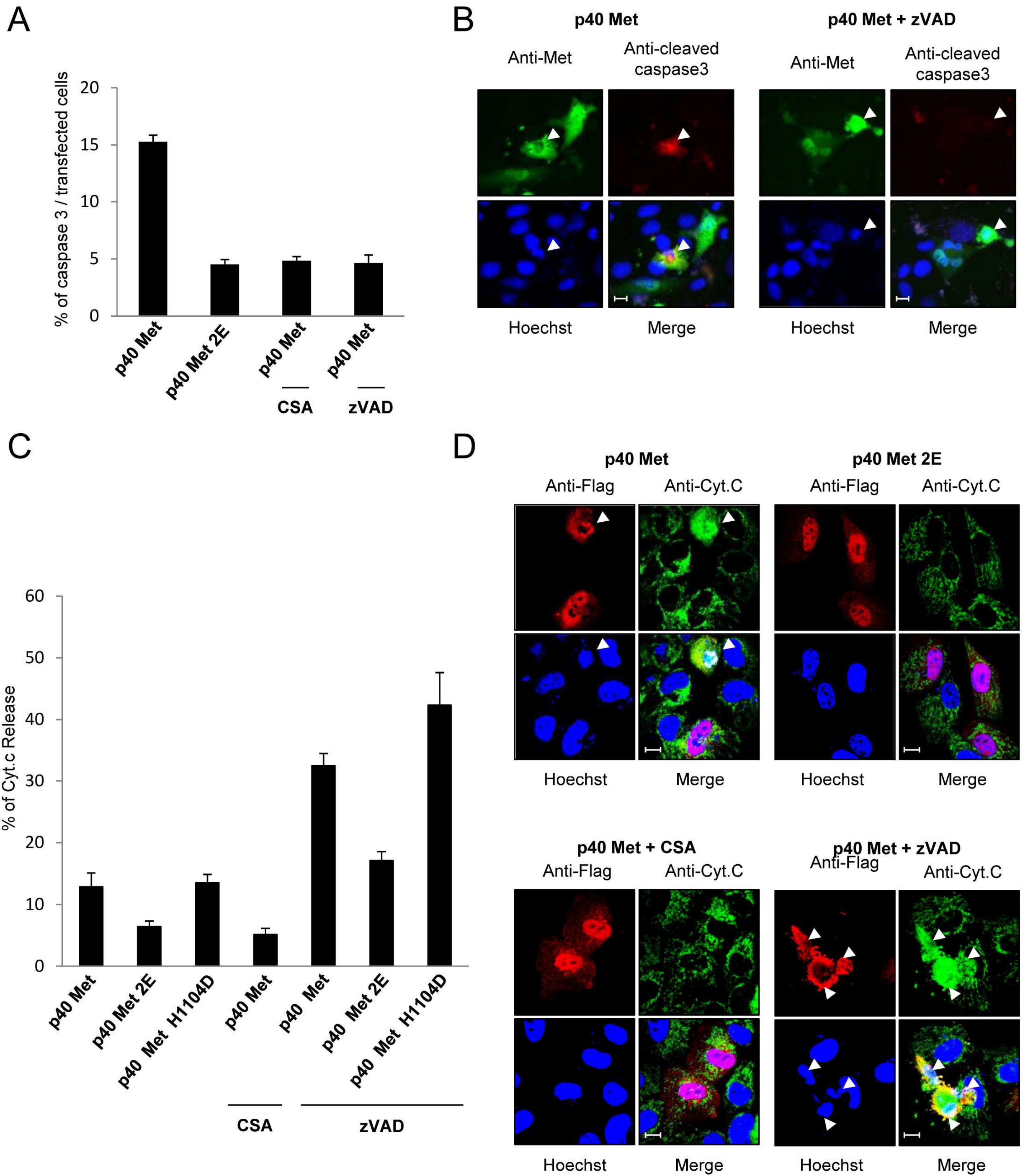


Figure 8

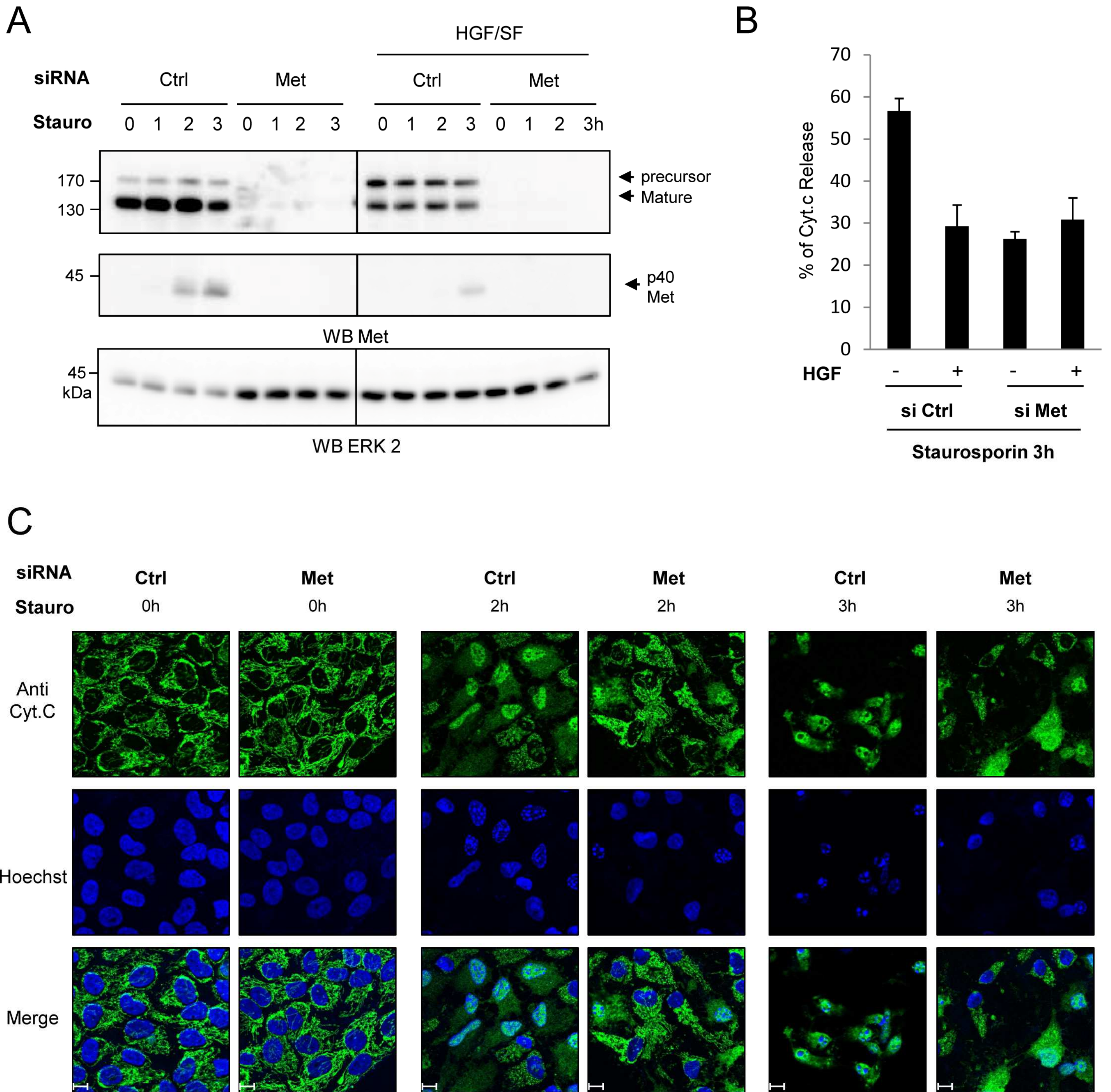


Figure 9

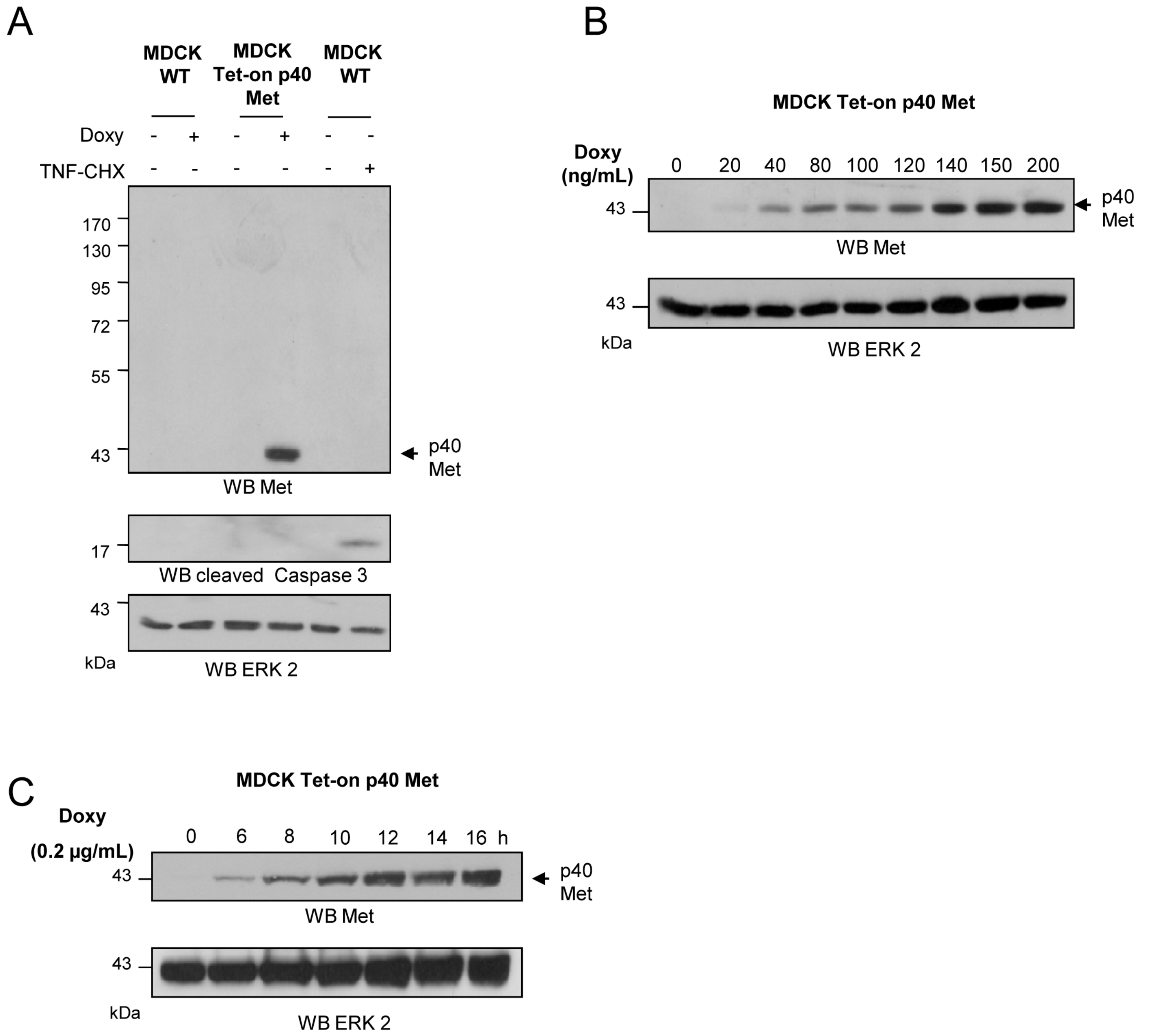
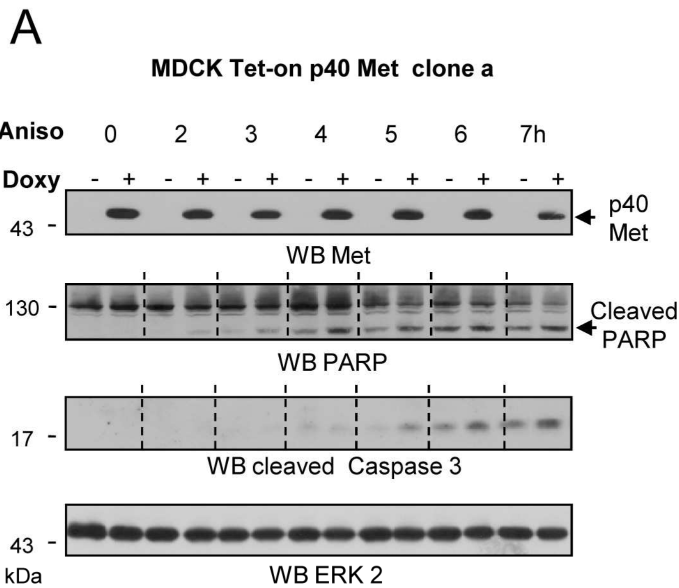
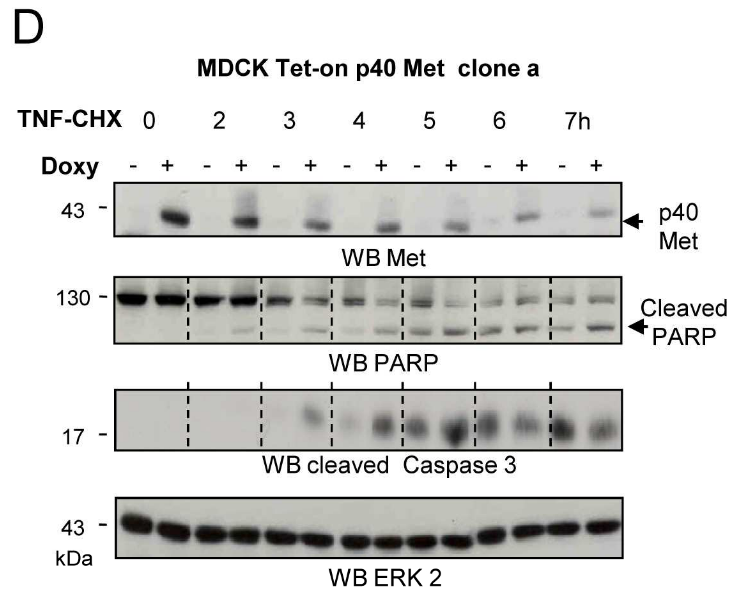
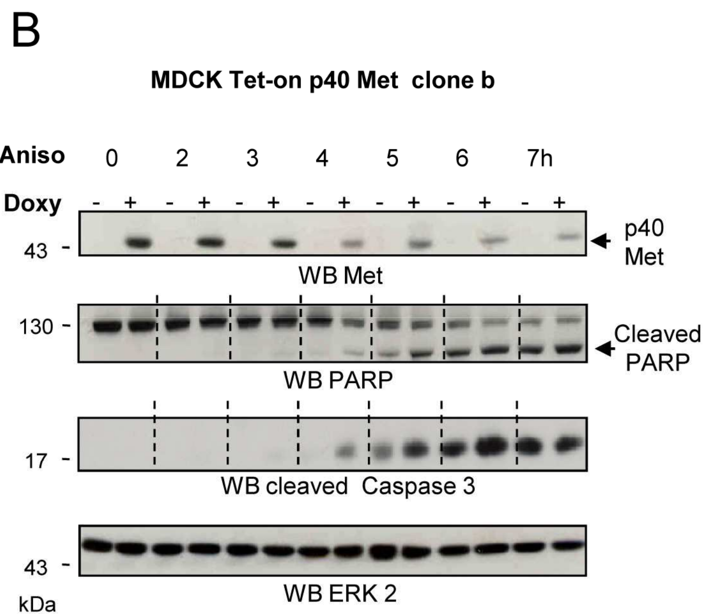
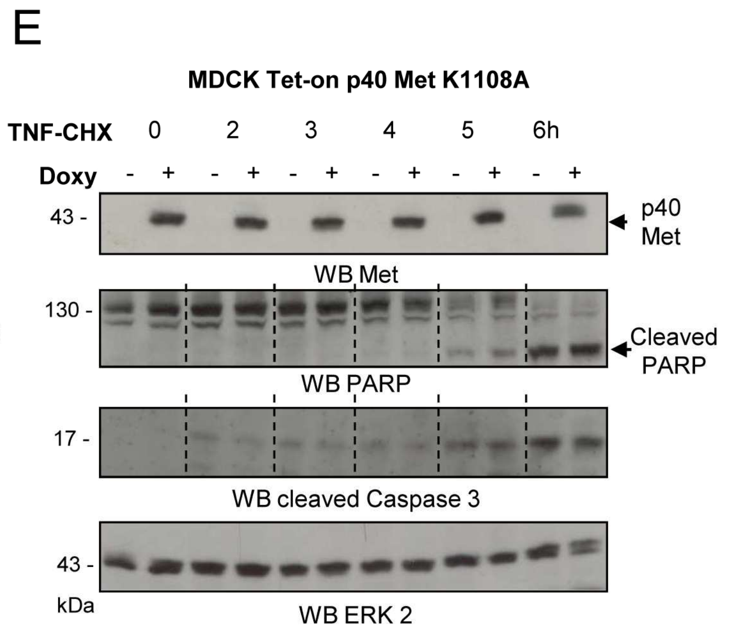


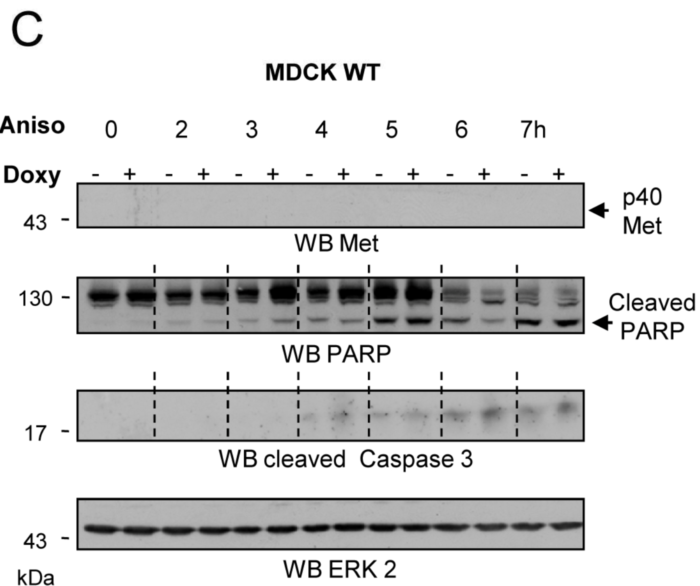
Figure S1











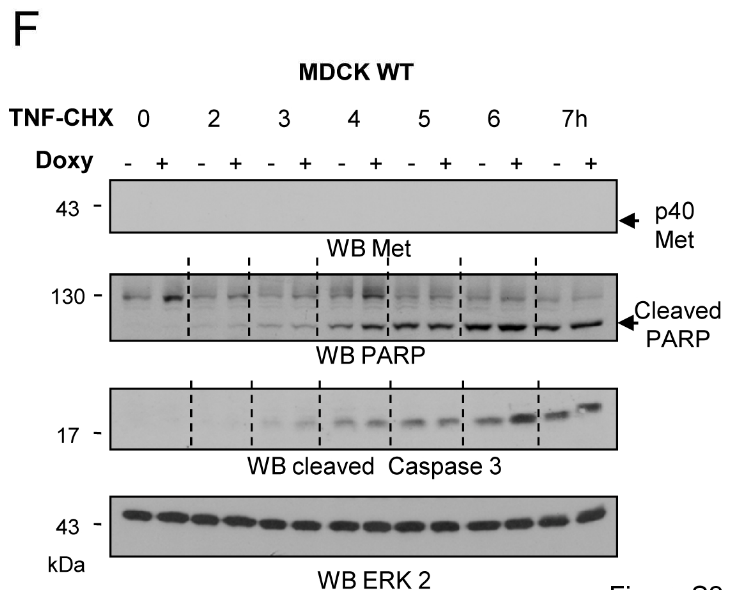


Figure S2

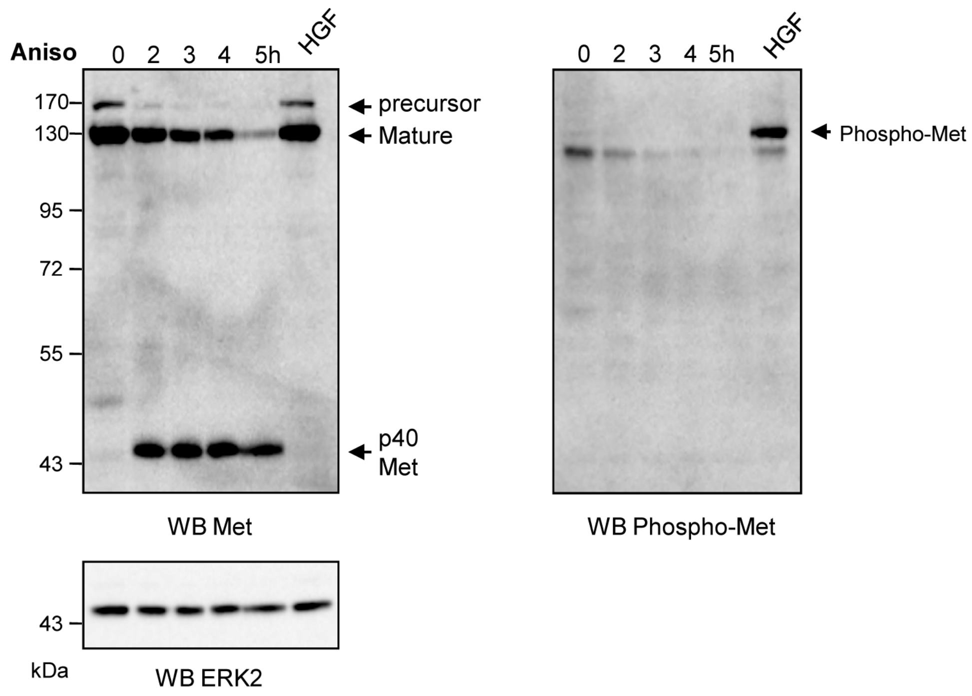


Figure S3

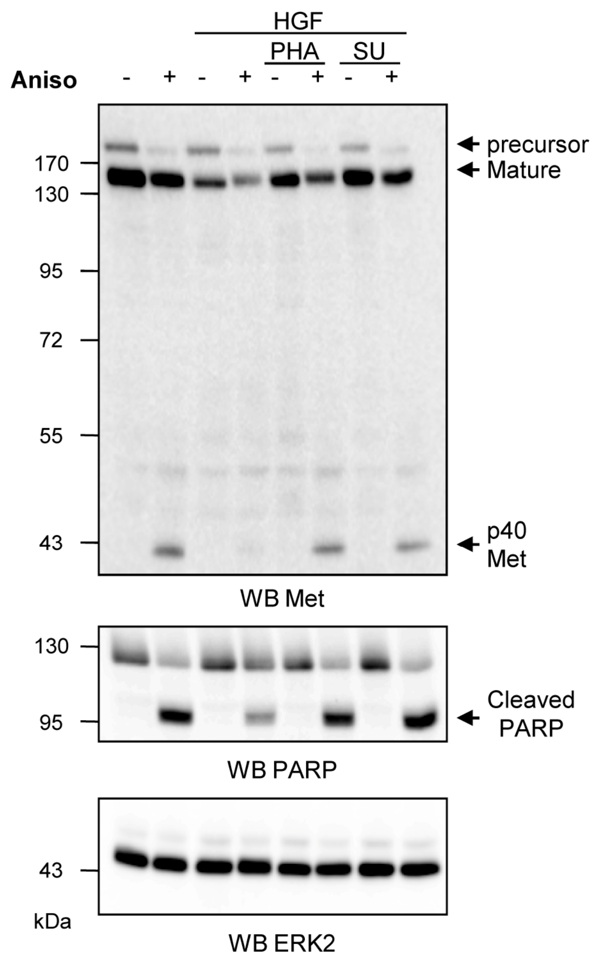
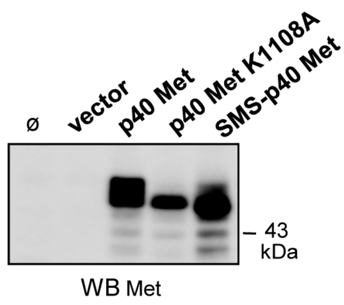
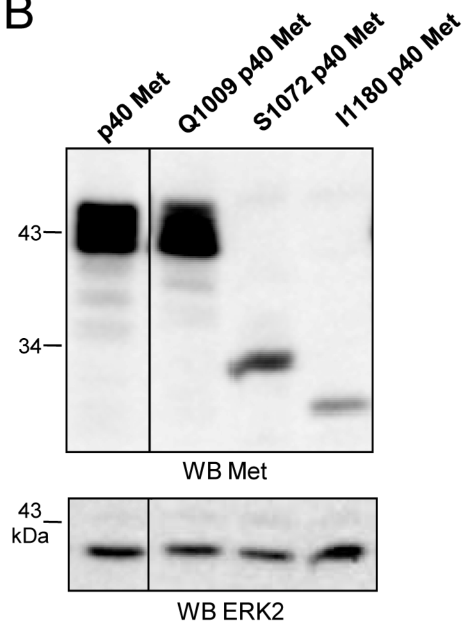


Figure S4





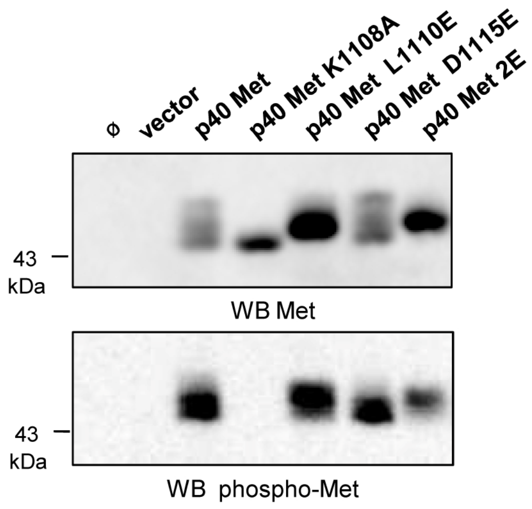


Figure S5

II) A la recherche de nouveaux partenaires

De façon à identifier les événements initiaux impliqués dans l'amplification de l'apoptose contrôlée par p40 Met, nous avons fait réaliser un criblage double hybride utilisant le fragment p40 Met comme appât afin d'identifier ses partenaires.

Cette stratégie s'est révélée efficace dans le cadre de l'étude des propriétés apoptotiques du récepteur à dépendance Patched (Ptc) En effet, le criblage double hybride à permis d'identifier qu'en absence de son ligand (Sonic hedgehog), Patched recrute un complexe multiprotéique formé de la protéine adaptatrice DRAL (downregulated in rhabdomyosarcoma LIM-domain protein) et de la caspase 9 (recrutée par l'intermédiaire du domaine CARD de DRAL). Cette étude montre également que l'activation des capacités apoptotiques de Patched dépend de la formation de ce complexe (Mille, Frederic 2009).

Dans notre cas, la totalité des partenaires identifiés sont des protéines à domaines SH2 capable de reconnaitre les tyrosines phosphorylées d'un récepteur activé. Ce résultat s'expliquerait par la dimérisation forcée de p40 Met induite par sa fusion avec le domaine de liaison à l'ADN et de dimérisation Gal-4 utilisé pour le crible. Ainsi ce crible, ne nous a pas permis d'identifier des protéines se liant à un fragment de Met non-phosphorylé.

La plupart des partenaires identifiés sont des protéines de signalisation déjà bien connues (Gab-1, PI3-K) validant l'activation de l'appât. De manière intéressante, certaines de ces protéines à domaines SH2 ne sont pas des partenaires connus de Met. L'un de ces partenaires est désoramais étudié par Ghaffar Muharram un ancien post-doctorant de l'équipe qui effectue depuis un stage post-doctorat au sein de l'équipe de Johanna Ivaska. Dans cette équipe, spécialisée dans les mécanismes moléculaires régulant la migration cellulaire (VTT Medical Biotechnology), il étudie l'implication et les fonctions de la tensine, une des protéines candidates issues de notre criblage double hybride. Cette protéine pourrait, par son action sur le cytosquelette, intervenir dans la migration cellulaire induite par l'HGF/SF suite à son interaction avec Met.

Liste des protéines candidates:

TNS (Tensin) isoforme 3 et 4, VAV isoforme 1 et 3, YES 1, ARIH2 (anti-Ariadne Homolog 2), SOCS (Suppressor of cytokine signaling) isoforme 1 et 2

III) Implications des gangliosides dans l'activation de Met indépendante du ligand

Nous l'avons vu dans l'introduction, le récepteur Met possède de nombreux partenaires protéiques membranaires. Ces partenaires modulent directement l'activité de Met et participent donc à la signalisation. Les travaux menés en collaboration avec l'équipe de Philippe Delannoy et plus particulièrement Aurélie Cazet qui a soutenu sa thèse nous ont permis de dévoiler un mécanisme de régulation de l'activité de Met par les gangliosides.

Les gangliosides sont des structures constituées d'un céramide et d'une chaîne de sucres hydrophiles. Leurs chaînes saccharidiques est plus ou moins complexe et est le résultat de l'addition séquentielle par un ensemble de glycosyltransférases spécifiques de différents monosaccharides tel que l'acide sialique. Au niveau cellulaire, les gangliosides sont retrouvés au niveau de la bicouche lipidique. Leurs parties saccharidiques est alors exposées du côté extracellulaire. Ils sont également retrouvés dans des vésicules d'endocytose ou d'exocysose, mais aussi dans des compartiments intracellulaires tels que le réticulum ou la mitochondrie. Ils sont exprimés dans les tissus des Mammifères selon un profil spécifique de l'espèce, du tissu ou de la cellule, mais aussi en fonction du stade de développement de l'organe ou de différenciation cellulaire. Depuis plusieurs années, les gangliosides GD3 et le GD2 sont décrits comme des marqueurs onco-fœtaux de mauvais pronostic dans les cancers d'origine neuro-ectodermique et du cancer du poumon à petites cellules (Thampoe *et al.*, 1989; Ruan and Lloyd, 1992; Oblinger *et al.*, 2006). Plus récemment, des études ont montré que l'expression des enzymes impliquées dans la biosynthèse des gangliosides peut également être altérée dans les tumeurs de sein. Ainsi, deux études cliniques ont montré que l'expression du gène *st8sia1*, codant l'enzyme GD3synthase impliquée dans la biosynthèse des gangliosides complexes, est augmentée dans les tumeurs mammaires hormonoindépendantes (Estrogen Receptor) et corrélée à un grade histopronostique élevé (Ruckhaberle *et al.*, 2008; Ruckhaberle *et al.*, 2009).

Afin de mieux définir le rôle de l'expression de *st8sia1*, l'équipe du Professeur Delannoy a surexprimé ce gène dans les cellules tumorales mammaires hormonoindépendantes MDA-MB-231. La surexpression de ce gène codant la GD3 synthase perturbe le profil gangliosidique de la cellule au profit d'un ganglioside majoritaire le GD2. Les résultats de l'étude phénotypique montre que cette surexpression renforce les capacités prolifératives et migratoires des cellules. De plus, l'analyse du profil d'activation de 42 RTK

a permis de détecter une activation constitutive du récepteur Met en condition de sevrage au sérum tandis que la majorité des autres récepteurs demeure inactive. Ces travaux ont donc permis de mettre en lumière un nouveau mécanisme d'activation de Met en absence d'HGF/SF contrôlée par les gangliosides. Ces études ont fait l'objet de deux publications qui sont présentées en annexe où je signe respectivement deuxième et cinquième auteur (Cazet *et al.*) Steenackers, 2011

CONCLUSION

1) La double facette des Récepteurs Tyrosine Kinase

Les études sur le fonctionnement des RTK ont abouti à un dogme mécanistique selon lequel la fonction de ces récepteurs est associée à la fixation du ligand tandis qu'en son absence, le RTK est considéré comme inactif. L'émergence du concept de récepteur à dépendance a cependant bouleversé ce dogme. Ainsi, en absence de leur ligand, les RTK de la famille des récepteurs à dépendance peuvent être clivés par des caspases et les fragments générés, sont capables d'induire la mort cellulaire. En revanche, leur mode de fonctionnement reste méconnu.

Les études des récepteurs à dépendance DCC et UNC5H2 montrent qu'ils sont capables de recruter et d'activer les caspases en absence de leur ligand, la nétrine-1. Ce fonctionnement s'apparente à celui des récepteurs de mort qui activent la voie extrinsèque de l'apoptose en recrutant et en activant les caspases initiatrices. Les études menées sur le fragment p40 Met montrent qu'il est capable d'amplifier l'apoptose via la perméabilisation mitochondriale (Figure 25). Le récepteur à dépendance Met, par l'intermédiaire de son émissaire p40 Met, est donc capable de participer de manière originale au contrôle de la voie intrinsèque de l'apoptose.

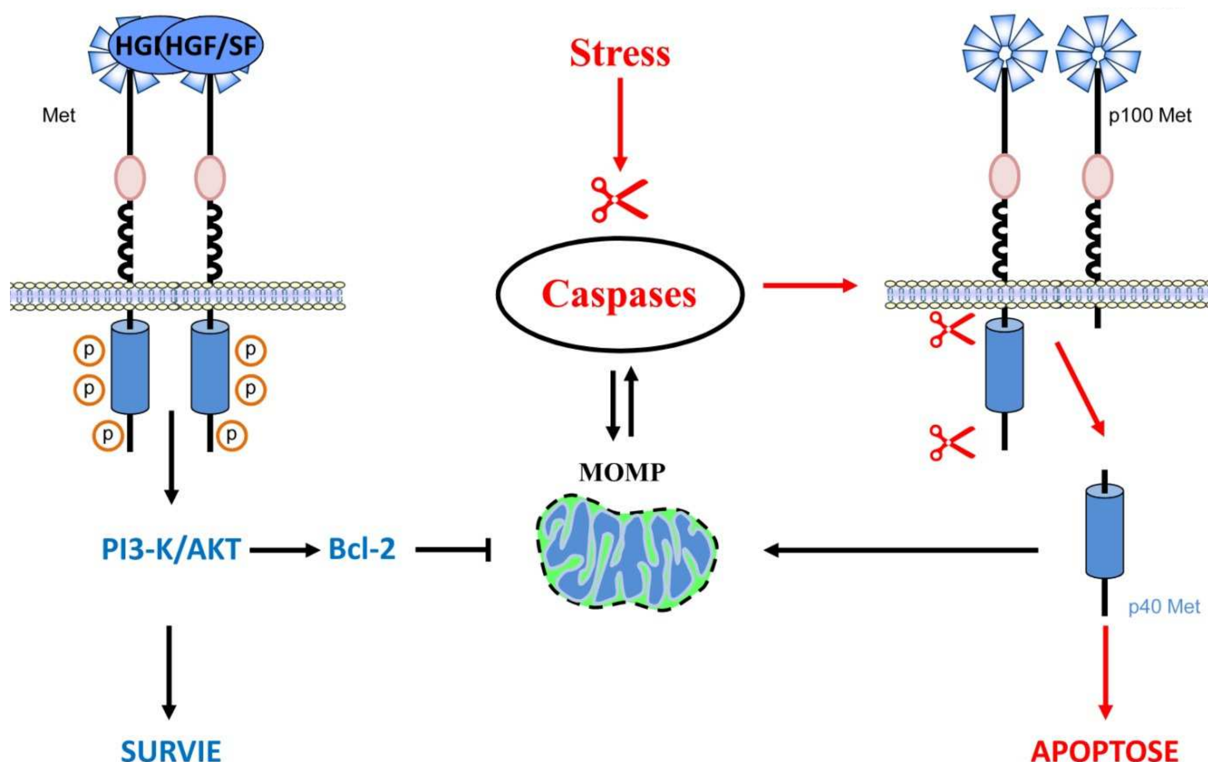


Figure 25: Contrôle de l'apoptose mitochondriale par le récepteur à dépendance Met

Afin de déterminer si la perte des clivages caspases de Met induit un phénotype particulier, le laboratoire a engagé une collaboration avec la clinique de la souris dans le but d'établir une souris KI exprimant une version de Met non clivable par les caspases.

Dans des contextes physiologiques comme la neurogenèse où l'apoptose est très marquée, on peut envisager que des souris exprimant ces versions de Met présentent des défauts dans l'élimination des neurones surnuméraires. De la même façon, l'inhibition des capacités apoptotiques de Met pourrait être liée à l'acquisition par les cellules de caractères transformants.

2) p40 Met et Cancer

La perte d'expression des récepteurs à dépendance DCC, UNC5H2 ou Patched est retrouvée dans de nombreux cancers (Bredesen *et al.*, 2004). Leur réexpression dans des lignées transformées indépendamment de leur ligand peut conduire à l'apoptose mais aussi inhiber la croissance sans ancrage et la transformation cellulaire (Meimei *et al.*, ; Forcet *et al.*, 2001; Thiebault *et al.*, 2003; Oliver *et al.*, 2005). Ces récepteurs sont donc considérés comme des suppresseurs de tumeurs et la perte de leur expression dans les cancers est considérée comme un avantage sélectif permettant à la tumeur de ne plus être dépendante de la présence du ligand pour sa survie. Contrairement à ces récepteurs, l'implication de Met dans les cancers est plutôt associée à la dérégulation de son activité kinase qui fait suite à sa surexpression (ou celle de son ligand) ou à des mutations activatrices. Contrairement à DCC ou UNCH5H2, la dépendance contrôlée par Met serait associée à une perte de ses capacités apoptotiques et non à sa perte d'expression. L'activation de Met est liée à sa phosphorylation sur le résidu Y1003. Celle-ci pourrait constituer, en masquant le site de clivage caspase juxtamembranaire, un mécanisme d'échappement à la dépendance en défavorisant la création du fragment p40 Met.

Dans le cadre des cancers liés à Met, on observe le plus souvent une dérégulation de l'activité kinase du récepteur qui fait suite à une surexpression du récepteur. Les capacités apoptotiques de Met nous permettent de proposer une stratégie thérapeutique alliant les inhibiteurs de l'activité kinase et les traitements chimiothérapeutiques, connus pour induire habituellement une mort cellulaire de type apoptotique. Le traitement chimiothérapeutique activerait l'apoptose tandis que les inhibiteurs pourraient créer un pool de récepteurs déphosphorylés. Incapables d'induire la survie, ils pourraient dans le même temps être la cible des caspases et produire le fragment pro-apoptotique p40 Met. Dans ce contexte, les

traitements chimiothérapeutiques associés aux inhibiteurs de l'activité kinase du récepteur pourraient alors constituer des outils thérapeutiques beaucoup plus efficaces qu'en combinaison par exemple avec des anticorps qui activent la dégradation du récepteur Met par le mécanisme du PS-RIP (le DN-30). La détection du fragment p40 Met dans des biopsies de tumeurs pourrait constituer un facteur prédictif de l'efficacité de la réponse au traitement. A l'inverse, en cas d'échappement thérapeutique, les tumeurs traitées par la chimiothérapie ne produiraient pas de fragment.

3) Effets des mutations du domaine kinase sur l'activité de p40 Met

Certaines mutations situées dans le domaine kinase de Met ont été répertoriées dans des tumeurs variées et les conséquences sur l'activité de Met ont, pour certaines, pu être élucidées. Le site Sanger (http://www.sanger.ac.uk) a référencé dans un cas de cancer de l'ovaire une mutation au niveau du site C-terminal de clivage par les caspases (D1380N) qui pourrait inhiber ou limiter la génération de p40 Met. De manière intéressante, des mutations situées dans le domaine BH3 (A1108S, G1119A/V) ont également été recensées. Dans ce contexte, nous avons donc testé les conséquences de la mutation A1108S sur les activités apoptotiques de p40 Met. Ce variant ne présente plus d'activités apoptotiques contrairement à celui portant la mutation H1106D (retrouvée dans des cancers du rein) située en dehors de ce domaine. Ces résultats préliminaires sont encourageants, mais les mécanismes d'action de ces mutations restent à approfondir pour comprendre leur implication dans les cancers.

BIBLIOGRAPHIE

Abella, J.V., Peschard, P., Naujokas, M.A., Lin, T., Saucier, C., Urbe, S., and Park, M. (2005). Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. Mol Cell Biol 25, 9632-9645.

Abounader, R., Lal, B., Luddy, C., Koe, G., Davidson, B., Rosen, E.M., and Laterra, J. (2002). In vivo targeting of SF/HGF and c-met expression via U1snRNA/ribozymes inhibits glioma growth and angiogenesis and promotes apoptosis. Faseb J 16, 108-110.

Aebersold, D.M., Landt, O., Berthou, S., Gruber, G., Beer, K.T., Greiner, R.H., and Zimmer, Y. (2003). Prevalence and clinical impact of Met Y1253D-activating point mutation in radiotherapy-treated squamous cell cancer of the oropharynx. Oncogene 22, 8519-8523.

Algeciras-Schimnich, A., Shen, L., Barnhart, B.C., Murmann, A.E., Burkhardt, J.K., and Peter, M.E. (2002). Molecular ordering of the initial signaling events of CD95. Mol Cell Biol 22, 207-220.

Anand, R., Kim, A.Y., Brent, M., and Marmorstein, R. (2008). Biochemical analysis of MST1 kinase: elucidation of a C-terminal regulatory region. Biochemistry 47, 6719-6726.

Andermarcher, E., Surani, M.A., and Gherardi, E. (1996). Co-expression of the HGF/SF and c-met genes during early mouse embryogenesis precedes reciprocal expression in adjacent tissues during organogenesis. Dev Genet 18, 254-266.

Arakaki, N., Kazi, J.A., Kazihara, T., Ohnishi, T., and Daikuhara, Y. (1998). Hepatocyte growth factor/scatter factor activates the apoptosis signaling pathway by increasing caspase-3 activity in sarcoma 180 cells. Biochem Biophys Res Commun 245, 211-215.

Arrieta, O., Garcia, E., Guevara, P., Garcia-Navarrete, R., Ondarza, R., Rembao, D., and Sotelo, J. (2002). Hepatocyte growth factor is associated with poor prognosis of malignant gliomas and is a predictor for recurrence of meningioma. Cancer 94, 3210-3218.

Artigiani, S., Conrotto, P., Fazzari, P., Gilestro, G.F., Barberis, D., Giordano, S., Comoglio, P.M., and Tamagnone, L. (2004). Plexin-B3 is a functional receptor for semaphorin 5A. EMBO Rep 5, 710-714.

Asaoka, Y., Tada, M., Ikenoue, T., Seto, M., Imai, M., Miyabayashi, K., Yamamoto, K., Yamamoto, S., Kudo, Y., Mohri, D., Isomura, Y., Ijichi, H., Tateishi, K., Kanai, F., Ogawa, S., Omata, M., and Koike, K. Gastric cancer cell line Hs746T harbors a splice site mutation of c-Met causing juxtamembrane domain deletion. Biochem Biophys Res Commun 394, 1042-1046.

Ashkenazi, A., and Dixit, V.M. (1998). Death receptors: signaling and modulation. Science 281, 1305-1308.

Bachleitner-Hofmann, T., Sun, M.Y., Chen, C.T., Tang, L., Song, L., Zeng, Z., Shah, M., Christensen, J.G., Rosen, N., Solit, D.B., and Weiser, M.R. (2008). HER kinase activation confers resistance to MET tyrosine kinase inhibition in MET oncogene-addicted gastric cancer cells. Mol Cancer Ther 7, 3499-3508.

Bagai, R., Fan, W., and Ma, P.C. ARQ-197, an oral small-molecule inhibitor of c-Met for the treatment of solid tumors. IDrugs 13, 404-414.

Bardelli, A., Longati, P., Gramaglia, D., Stella, M.C., and Comoglio, P.M. (1997). Gab1 coupling to the HGF/Met receptor multifunctional docking site requires binding of Grb2 and correlates with the transforming potential. Oncogene 15, 3103-3111.

Basanez, G., Zhang, J., Chau, B.N., Maksaev, G.I., Frolov, V.A., Brandt, T.A., Burch, J., Hardwick, J.M., and Zimmerberg, J. (2001). Pro-apoptotic cleavage products of Bcl-xL form cytochrome c-conducting pores in pure lipid membranes. J Biol Chem 276, 31083-31091.

Basilico, C., Arnesano, A., Galluzzo, M., Comoglio, P.M., and Michieli, P. (2008). A high affinity hepatocyte growth factor-binding site in the immunoglobulin-like region of Met. J Biol Chem 283, 21267-21277.

Batandier, C., Leverve, X., and Fontaine, E. (2004). Opening of the mitochondrial permeability transition pore induces reactive oxygen species production at the level of the respiratory chain complex I. J Biol Chem 279, 17197-17204.

Bennett, K.L., Modrell, B., Greenfield, B., Bartolazzi, A., Stamenkovic, I., Peach, R., Jackson, D.G., Spring, F., and Aruffo, A. (1995). Regulation of CD44 binding to hyaluronan by glycosylation of variably spliced exons. J Cell Biol 131, 1623-1633.

Benvenuti, S., and Comoglio, P.M. (2007). The MET receptor tyrosine kinase in invasion and metastasis. J Cell Physiol 213, 316-325.

Bernardi, P. (1999). Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol Rev 79, 1127-1155.

Bernet, A., and Mehlen, P. (2005). [Netrin-1 and its dependence receptors: role in colorectal cancers]. Pathol Biol (Paris) 53, 328-333.

Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G.F. (2003). Met, metastasis, motility and more. Nat Rev Mol Cell Biol 4, 915-925.

Birchmeier, C., and Gherardi, E. (1998). Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends Cell Biol 8, 404-410.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature 376, 768-771.

Boccaccio, C., Ando, M., Tamagnone, L., Bardelli, A., Michieli, P., Battistini, C., and Comoglio, P.M. (1998). Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. Nature 391, 285-288.

Bordeaux, M.C., Forcet, C., Granger, L., Corset, V., Bidaud, C., Billaud, M., Bredesen, D.E., Edery, P., and Mehlen, P. (2000). The RET proto-oncogene induces apoptosis: a novel mechanism for Hirschsprung disease. Embo J 19, 4056-4063.

Borowiak, M., Garratt, A.N., Wustefeld, T., Strehle, M., Trautwein, C., and Birchmeier, C. (2004). Met provides essential signals for liver regeneration. Proc Natl Acad Sci U S A 101, 10608-10613.

Bottaro, D.P., Rubin, J.S., Faletto, D.L., Chan, A.M., Kmiecik, T.E., Vande Woude, G.F., and Aaronson, S.A. (1991). Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science 251, 802-804.

Bredesen, D.E., Mehlen, P., and Rabizadeh, S. (2004). Apoptosis and dependence receptors: a molecular basis for cellular addiction. Physiol Rev 84, 411-430.

Brenner, C., and Grimm, S. (2006). The permeability transition pore complex in cancer cell death. Oncogene 25, 4744-4756.

Brinkmann, V., Foroutan, H., Sachs, M., Weidner, K.M., and Birchmeier, W. (1995). Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells. J Cell Biol 131, 1573-1586.

Caulin, C., Salvesen, G.S., and Oshima, R.G. (1997). Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. J Cell Biol 138, 1379-1394.

Cazet, A., Lefebvre, J., Adriaenssens, E., Julien, S., Bobowski, M., Grigoriadis, A., Tutt, A., Tulasne, D., Le Bourhis, X., and Delannoy, P. GD synthase expression enhances proliferation and tumor growth of MDA-MB-231 breast cancer cells through c-Met activation. Mol Cancer Res 8, 1526-1535.

Cecchi, F., Rabe, D.C., and Bottaro, D.P. Targeting the HGF/Met signalling pathway in cancer. Eur J Cancer 46, 1260-1270.

Chan, A., Rubin, J., Bottaro, D., Hirschfield, D., Chedid, M., and Aaronson, S.A. (1993). Isoforms of human HGF and their biological activities. Exs 65, 67-79.

Chiara, F., Michieli, P., Pugliese, L., and Comoglio, P.M. (2003). Mutations in the met oncogene unveil a "dual switch" mechanism controlling tyrosine kinase activity. J Biol Chem 278, 29352-29358.

Chmielowiec, J., Borowiak, M., Morkel, M., Stradal, T., Munz, B., Werner, S., Wehland, J., Birchmeier, C., and Birchmeier, W. (2007). c-Met is essential for wound healing in the skin. J Cell Biol 177, 151-162.

Christensen, J.G., Burrows, J., and Salgia, R. (2005). c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. Cancer Lett 225, 1-26.

Clarke, P.G. (1990). Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol (Berl) 181, 195-213.

Clem, R.J., Sheu, T.T., Richter, B.W., He, W.W., Thornberry, N.A., Duckett, C.S., and Hardwick, J.M. (2001). c-IAP1 is cleaved by caspases to produce a proapoptotic C-terminal fragment. J Biol Chem 276, 7602-7608.

Comito, G., Calvani, M., Giannoni, E., Bianchini, F., Calorini, L., Torre, E., Migliore, C., Giordano, S., and Chiarugi, P. HIF-1alpha stabilization by mitochondrial ROS promotes Met-dependent invasive growth and vasculogenic mimicry in melanoma cells. Free Radic Biol Med 51, 893-904.

Conrotto, P., Corso, S., Gamberini, S., Comoglio, P.M., and Giordano, S. (2004). Interplay between scatter factor receptors and B plexins controls invasive growth. Oncogene 23, 5131-5137.

Cooper, C.S., Blair, D.G., Oskarsson, M.K., Tainsky, M.A., Eader, L.A., and Vande Woude, G.F. (1984). Characterization of human transforming genes from chemically transformed, teratocarcinoma, and pancreatic carcinoma cell lines. Cancer Res 44, 1-10.

Cory, S., Huang, D.C., and Adams, J.M. (2003). The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene 22, 8590-8607.

Cristiani, C., Rusconi, L., Perego, R., Schiering, N., Kalisz, H.M., Knapp, S., and Isacchi, A. (2005). Regulation of the wild-type and Y1235D mutant Met kinase activation. Biochemistry 44, 14110-14119.

Croft, D.R., Coleman, M.L., Li, S., Robertson, D., Sullivan, T., Stewart, C.L., and Olson, M.F. (2005). Actin-myosin-based contraction is responsible for apoptotic nuclear disintegration. J Cell Biol 168, 245-255.

Crompton, M. (1999). The mitochondrial permeability transition pore and its role in cell death. Biochem J 341 (Pt 2), 233-249.

Crompton, M., Ellinger, H., and Costi, A. (1988). Inhibition by cyclosporin A of a Ca2+-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. Biochem J 255, 357-360.

Davidson, A.M., and Halestrap, A.P. (1990). Partial inhibition by cyclosporin A of the swelling of liver mitochondria in vivo and in vitro induced by sub-micromolar [Ca2+], but not by butyrate. Evidence for two distinct swelling mechanisms. Biochem J 268, 147-152.

de la Torre, J.R., Hopker, V.H., Ming, G.L., Poo, M.M., Tessier-Lavigne, M., Hemmati-Brivanlou, A., and Holt, C.E. (1997). Turning of retinal growth cones in a netrin-1 gradient mediated by the netrin receptor DCC. Neuron 19, 1211-1224.

Degterev, A., and Yuan, J. (2008). Expansion and evolution of cell death programmes. Nat Rev Mol Cell Biol 9, 378-390.

Deheuninck, J., Foveau, B., Goormachtigh, G., Leroy, C., Ji, Z., Tulasne, D., and Fafeur, V. (2008). Caspase cleavage of the MET receptor generates an HGF interfering fragment. Biochem Biophys Res Commun 367, 573-577.

Deheuninck, J., Goormachtigh, G., Foveau, B., Ji, Z., Leroy, C., Ancot, F., Villeret, V., Tulasne, D., and Fafeur, V. (2009). Phosphorylation of the MET receptor on juxtamembrane tyrosine residue 1001 inhibits its caspase-dependent cleavage. Cell Signal 21, 1455-1463.

Ebens, A., Brose, K., Leonardo, E.D., Hanson, M.G., Jr., Bladt, F., Birchmeier, C., Barres, B.A., and Tessier-Lavigne, M. (1996). Hepatocyte growth factor/scatter factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. Neuron 17, 1157-1172.

Edison, N., Zuri, D., Maniv, I., Bornstein, B., Lev, T., Gottfried, Y., Kemeny, S., Garcia-Fernandez, M., Kagan, J., and Larisch, S. The IAP-antagonist ARTS initiates caspase activation upstream of cytochrome C and SMAC/Diablo. Cell Death Differ.

Engelman, J.A., Zejnullahu, K., Mitsudomi, T., Song, Y., Hyland, C., Park, J.O., Lindeman, N., Gale, C.M., Zhao, X., Christensen, J., Kosaka, T., Holmes, A.J., Rogers, A.M., Cappuzzo, F., Mok, T., Lee, C., Johnson, B.E., Cantley, L.C., and Janne, P.A. (2007). MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 316, 1039-1043.

Epstein, J.A., Shapiro, D.N., Cheng, J., Lam, P.Y., and Maas, R.L. (1996). Pax3 modulates expression of the c-Met receptor during limb muscle development. Proc Natl Acad Sci U S A 93, 4213-4218.

Fafeur, V., Tulasne, D., Queva, C., Vercamer, C., Dimster, V., Mattot, V., Stehelin, D., Desbiens, X., and Vandenbunder, B. (1997). The ETS1 transcription factor is expressed during epithelial-

mesenchymal transitions in the chick embryo and is activated in scatter factor-stimulated MDCK epithelial cells. Cell Growth Differ 8, 655-665.

Fazeli, A., Dickinson, S.L., Hermiston, M.L., Tighe, R.V., Steen, R.G., Small, C.G., Stoeckli, E.T., Keino-Masu, K., Masu, M., Rayburn, H., Simons, J., Bronson, R.T., Gordon, J.I., Tessier-Lavigne, M., and Weinberg, R.A. (1997). Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. Nature 386, 796-804.

Fearon, E.R., and Pierceall, W.E. (1995). The deleted in colorectal cancer (DCC) gene: a candidate tumour suppressor gene encoding a cell surface protein with similarity to neural cell adhesion molecules. Cancer Surv 24, 3-17.

Ferracini, R., Longati, P., Naldini, L., Vigna, E., and Comoglio, P.M. (1991). Identification of the major autophosphorylation site of the Met/hepatocyte growth factor receptor tyrosine kinase. J Biol Chem 266, 19558-19564.

Finegold, D.N., Schacht, V., Kimak, M.A., Lawrence, E.C., Foeldi, E., Karlsson, J.M., Baty, C.J., and Ferrell, R.E. (2008). HGF and MET mutations in primary and secondary lymphedema. Lymphat Res Biol 6, 65-68.

Fischer, U., Janicke, R.U., and Schulze-Osthoff, K. (2003). Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ 10, 76-100.

Fixman, E.D., Holgado-Madruga, M., Nguyen, L., Kamikura, D.M., Fournier, T.M., Wong, A.J., and Park, M. (1997). Efficient cellular transformation by the Met oncoprotein requires a functional Grb2 binding site and correlates with phosphorylation of the Grb2-associated proteins, Cbl and Gab1. J Biol Chem 272, 20167-20172.

Follenzi, A., Bakovic, S., Gual, P., Stella, M.C., Longati, P., and Comoglio, P.M. (2000). Cross-talk between the proto-oncogenes Met and Ron. Oncogene 19, 3041-3049.

Forcet, C., Ye, X., Granger, L., Corset, V., Shin, H., Bredesen, D.E., and Mehlen, P. (2001). The dependence receptor DCC (deleted in colorectal cancer) defines an alternative mechanism for caspase activation. Proc Natl Acad Sci U S A 98, 3416-3421.

Foveau, B., Ancot, F., Leroy, C., Petrelli, A., Reiss, K., Vingtdeux, V., Giordano, S., Fafeur, V., and Tulasne, D. (2009). Down-regulation of the met receptor tyrosine kinase by presenilin-dependent regulated intramembrane proteolysis. Mol Biol Cell 20, 2495-2507.

Foveau, B., Leroy, C., Ancot, F., Deheuninck, J., Ji, Z., Fafeur, V., and Tulasne, D. (2007). Amplification of apoptosis through sequential caspase cleavage of the MET tyrosine kinase receptor. Cell Death Differ 14, 752-764.

Fresno Vara, J.A., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C., and Gonzalez-Baron, M. (2004). PI3K/Akt signalling pathway and cancer. Cancer Treat Rev 30, 193-204.

Fuentes-Prior, P., and Salvesen, G.S. (2004). The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J 384, 201-232.

Fujita, N., Nagahashi, A., Nagashima, K., Rokudai, S., and Tsuruo, T. (1998). Acceleration of apoptotic cell death after the cleavage of Bcl-XL protein by caspase-3-like proteases. Oncogene 17, 1295-1304.

Furge, K.A., Zhang, Y.W., and Vande Woude, G.F. (2000). Met receptor tyrosine kinase: enhanced signaling through adapter proteins. Oncogene 19, 5582-5589.

Galluzzi, L., Vitale, I., Abrams, J.M., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., Dawson, T.M., Dawson, V.L., El-Deiry, W.S., Fulda, S., Gottlieb, E., Green, D.R., Hengartner, M.O., Kepp, O., Knight, R.A., Kumar, S., Lipton, S.A., Lu, X., Madeo, F., Malorni, W., Mehlen, P., Nunez, G., Peter, M.E., Piacentini, M., Rubinsztein, D.C., Shi, Y., Simon, H.U., Vandenabeele, P., White, E., Yuan, J., Zhivotovsky, B., Melino, G., and Kroemer, G. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ.

Galvani, A.P., Cristiani, C., Carpinelli, P., Landonio, A., and Bertolero, F. (1995). Suramin modulates cellular levels of hepatocyte growth factor receptor by inducing shedding of a soluble form. Biochem Pharmacol 50, 959-966.

Gambarotta, G., Boccaccio, C., Giordano, S., Ando, M., Stella, M.C., and Comoglio, P.M. (1996). Ets up-regulates MET transcription. Oncogene 13, 1911-1917.

Gherardi, E., Sandin, S., Petoukhov, M.V., Finch, J., Youles, M.E., Ofverstedt, L.G., Miguel, R.N., Blundell, T.L., Vande Woude, G.F., Skoglund, U., and Svergun, D.I. (2006). Structural basis of hepatocyte growth factor/scatter factor and MET signalling. Proc Natl Acad Sci U S A 103, 4046-4051.

Giordano, S., Corso, S., Conrotto, P., Artigiani, S., Gilestro, G., Barberis, D., Tamagnone, L., and Comoglio, P.M. (2002). The semaphorin 4D receptor controls invasive growth by coupling with Met. Nat Cell Biol 4, 720-724.

Goldschneider, D., and Mehlen, P. Dependence receptors: a new paradigm in cell signaling and cancer therapy. Oncogene 29, 1865-1882.

Graveel, C., Su, Y., Koeman, J., Wang, L.M., Tessarollo, L., Fiscella, M., Birchmeier, C., Swiatek, P., Bronson, R., and Vande Woude, G. (2004). Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. Proc Natl Acad Sci U S A 101, 17198-17203.

Gual, P., Giordano, S., Williams, T.A., Rocchi, S., Van Obberghen, E., and Comoglio, P.M. (2000). Sustained recruitment of phospholipase C-gamma to Gab1 is required for HGF-induced branching tubulogenesis. Oncogene 19, 1509-1518.

Guenebeaud, C., Goldschneider, D., Castets, M., Guix, C., Chazot, G., Delloye-Bourgeois, C., Eisenberg-Lerner, A., Shohat, G., Zhang, M., Laudet, V., Kimchi, A., Bernet, A., and Mehlen, P. The dependence receptor UNC5H2/B triggers apoptosis via PP2A-mediated dephosphorylation of DAP kinase. Mol Cell 40, 863-876.

Guo, A., Villen, J., Kornhauser, J., Lee, K.A., Stokes, M.P., Rikova, K., Possemato, A., Nardone, J., Innocenti, G., Wetzel, R., Wang, Y., MacNeill, J., Mitchell, J., Gygi, S.P., Rush, J., Polakiewicz, R.D., and Comb, M.J. (2008). Signaling networks assembled by oncogenic EGFR and c-Met. Proc Natl Acad Sci U S A 105, 692-697.

Guzy, R.D., Hoyos, B., Robin, E., Chen, H., Liu, L., Mansfield, K.D., Simon, M.C., Hammerling, U., and Schumacker, P.T. (2005). Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. Cell Metab 1, 401-408.

Halestrap, A.P., and Davidson, A.M. (1990). Inhibition of Ca2(+)-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem J 268, 153-160.

Hamanoue, M., Takemoto, N., Matsumoto, K., Nakamura, T., Nakajima, K., and Kohsaka, S. (1996). Neurotrophic effect of hepatocyte growth factor on central nervous system neurons in vitro. J Neurosci Res 43, 554-564.

Hammond, D.E., Carter, S., and Clague, M.J. (2004). Met receptor dynamics and signalling. Curr Top Microbiol Immunol 286, 21-44.

Hanahan, D., and Weinberg, R.A. Hallmarks of cancer: the next generation. Cell 144, 646-674.

Hanna, J.A., Bordeaux, J., Rimm, D.L., and Agarwal, S. (2009). The function, proteolytic processing, and histopathology of Met in cancer. Adv Cancer Res 103, 1-23.

Hanzawa, M., Shindoh, M., Higashino, F., Yasuda, M., Inoue, N., Hida, K., Ono, M., Kohgo, T., Nakamura, M., Notani, K., Fukuda, H., Totsuka, Y., Yoshida, K., and Fujinaga, K. (2000). Hepatocyte growth factor upregulates E1AF that induces oral squamous cell carcinoma cell invasion by activating matrix metalloproteinase genes. Carcinogenesis 21, 1079-1085.

Hegde, R., Srinivasula, S.M., Zhang, Z., Wassell, R., Mukattash, R., Cilenti, L., DuBois, G., Lazebnik, Y., Zervos, A.S., Fernandes-Alnemri, T., and Alnemri, E.S. (2002). Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. J Biol Chem 277, 432-438.

Heideman, D.A., Snijders, P.J., Bloemena, E., Meijer, C.J., Offerhaus, G.J., Meuwissen, S.G., Gerritsen, W.R., and Craanen, M.E. (2001). Absence of tpr-met and expression of c-met in human gastric mucosa and carcinoma. J Pathol 194, 428-435.

Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F., and Chao, M.V. (1991). High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature 350, 678-683.

Herrick, J., Michalet, X., Conti, C., Schurra, C., and Bensimon, A. (2000). Quantifying single gene copy number by measuring fluorescent probe lengths on combed genomic DNA. Proc Natl Acad Sci U S A 97, 222-227.

Higashio, K., Shima, N., Goto, M., Itagaki, Y., Nagao, M., Yasuda, H., and Morinaga, T. (1990). Identity of a tumor cytotoxic factor from human fibroblasts and hepatocyte growth factor. Biochem Biophys Res Commun 170, 397-404.

Higuchi, O., Mizuno, K., Vande Woude, G.F., and Nakamura, T. (1992). Expression of c-met proto-oncogene in COS cells induces the signal transducing high-affinity receptor for hepatocyte growth factor. FEBS Lett 301, 282-286.

Hofmann, K., Bucher, P., and Tschopp, J. (1997). The CARD domain: a new apoptotic signalling motif. Trends Biochem Sci 22, 155-156.

Horvitz, H.R. (1999). Genetic control of programmed cell death in the nematode Caenorhabditis elegans. Cancer Res 59, 1701s-1706s.

Huh, C.G., Factor, V.M., Sanchez, A., Uchida, K., Conner, E.A., and Thorgeirsson, S.S. (2004). Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. Proc Natl Acad Sci U S A 101, 4477-4482.

Hunter, D.R., Haworth, R.A., and Southard, J.H. (1976). Relationship between configuration, function, and permeability in calcium-treated mitochondria. J Biol Chem 251, 5069-5077.

Irvine, R.A., Adachi, N., Shibata, D.K., Cassell, G.D., Yu, K., Karanjawala, Z.E., Hsieh, C.L., and Lieber, M.R. (2005). Generation and characterization of endonuclease G null mice. Mol Cell Biol 25, 294-302.

Ishibe, S., Karihaloo, A., Ma, H., Zhang, J., Marlier, A., Mitobe, M., Togawa, A., Schmitt, R., Czyczk, J., Kashgarian, M., Geller, D.S., Thorgeirsson, S.S., and Cantley, L.G. (2009). Met and the epidermal growth factor receptor act cooperatively to regulate final nephron number and maintain collecting duct morphology. Development 136, 337-345.

Ivan, M., Bond, J.A., Prat, M., Comoglio, P.M., and Wynford-Thomas, D. (1997). Activated ras and ret oncogenes induce over-expression of c-met (hepatocyte growth factor receptor) in human thyroid epithelial cells. Oncogene 14, 2417-2423.

Jackson, D.G., Bell, J.I., Dickinson, R., Timans, J., Shields, J., and Whittle, N. (1995). Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon. J Cell Biol 128, 673-685.

Jagadeeswaran, R., Ma, P.C., Seiwert, T.Y., Jagadeeswaran, S., Zumba, O., Nallasura, V., Ahmed, S., Filiberti, R., Paganuzzi, M., Puntoni, R., Kratzke, R.A., Gordon, G.J., Sugarbaker, D.J., Bueno, R., Janamanchi, V., Bindokas, V.P., Kindler, H.L., and Salgia, R. (2006). Functional analysis of c-Met/hepatocyte growth factor pathway in malignant pleural mesothelioma. Cancer Res 66, 352-361.

Jahani-Asl, A., Basak, A., and Tsang, B.K. (2007). Caspase-3-mediated cleavage of Akt: involvement of non-consensus sites and influence of phosphorylation. FEBS Lett 581, 2883-2888.

Jakobi, R. (2004). Subcellular targeting regulates the function of caspase-activated protein kinases in apoptosis. Drug Resist Updat 7, 11-17.

Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C.P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G.F. (1997a). Activating mutations for the met tyrosine kinase receptor in human cancer. Proc Natl Acad Sci U S A 94, 11445-11450.

Jeffers, M., Taylor, G.A., Weidner, K.M., Omura, S., and Vande Woude, G.F. (1997b). Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. Mol Cell Biol 17, 799-808.

Jeffers, M.F. (1999). Activating mutations in the Met receptor overcome the requirement for autophosphorylation of tyrosines crucial for wild type signaling. Oncogene 18, 5120-5125.

Jiang, W.G., Hiscox, S.E., Parr, C., Martin, T.A., Matsumoto, K., Nakamura, T., and Mansel, R.E. (1999). Antagonistic effect of NK4, a novel hepatocyte growth factor variant, on in vitro angiogenesis of human vascular endothelial cells. Clin Cancer Res 5, 3695-3703.

Jin, H., Yang, R., Li, W., Ogasawara, A.K., Schwall, R., Eberhard, D.A., Zheng, Z., Kahn, D., and Paoni, N.F. (2003). Early treatment with hepatocyte growth factor improves cardiac function in experimental heart failure induced by myocardial infarction. J Pharmacol Exp Ther 304, 654-660.

Jin, H., Yang, R., Zheng, Z., Romero, M., Ross, J., Bou-Reslan, H., Carano, R.A., Kasman, I., Mai, E., Young, J., Zha, J., Zhang, Z., Ross, S., Schwall, R., Colbern, G., and Merchant, M. (2008). MetMAb, the one-armed 5D5 anti-c-Met antibody, inhibits orthotopic pancreatic tumor growth and improves survival. Cancer Res 68, 4360-4368.

Jo, M., Stolz, D.B., Esplen, J.E., Dorko, K., Michalopoulos, G.K., and Strom, S.C. (2000). Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. J Biol Chem 275, 8806-8811.

Jung, W., Castren, E., Odenthal, M., Vande Woude, G.F., Ishii, T., Dienes, H.P., Lindholm, D., and Schirmacher, P. (1994). Expression and functional interaction of hepatocyte growth factor-scatter factor and its receptor c-met in mammalian brain. J Cell Biol 126, 485-494.

Kaufmann, S.H., and Earnshaw, W.C. (2000). Induction of apoptosis by cancer chemotherapy. Exp Cell Res 256, 42-49.

Kaufmann, T., Strasser, A., and Jost, P.J. Fas death receptor signalling: roles of Bid and XIAP. Cell Death Differ.

Kawaida, K., Matsumoto, K., Shimazu, H., and Nakamura, T. (1994). Hepatocyte growth factor prevents acute renal failure and accelerates renal regeneration in mice. Proc Natl Acad Sci U S A 91, 4357-4361.

Kermorgant, S., and Parker, P.J. (2008). Receptor trafficking controls weak signal delivery: a strategy used by c-Met for STAT3 nuclear accumulation. J Cell Biol 182, 855-863.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26, 239-257.

Kim, H., Tu, H.C., Ren, D., Takeuchi, O., Jeffers, J.R., Zambetti, G.P., Hsieh, J.J., and Cheng, E.H. (2009). Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. Mol Cell 36, 487-499.

Kirchhofer, D., Yao, X., Peek, M., Eigenbrot, C., Lipari, M.T., Billeci, K.L., Maun, H.R., Moran, P., Santell, L., Wiesmann, C., and Lazarus, R.A. (2004). Structural and functional basis of the serine protease-like hepatocyte growth factor beta-chain in Met binding and signaling. J Biol Chem 279, 39915-39924.

Kirsch, D.G., Doseff, A., Chau, B.N., Lim, D.S., de Souza-Pinto, N.C., Hansford, R., Kastan, M.B., Lazebnik, Y.A., and Hardwick, J.M. (1999). Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. J Biol Chem 274, 21155-21161.

Kong-Beltran, M., Seshagiri, S., Zha, J., Zhu, W., Bhawe, K., Mendoza, N., Holcomb, T., Pujara, K., Stinson, J., Fu, L., Severin, C., Rangell, L., Schwall, R., Amler, L., Wickramasinghe, D., and Yauch, R. (2006). Somatic mutations lead to an oncogenic deletion of met in lung cancer. Cancer Res 66, 283-289.

Kong-Beltran, M., Stamos, J., and Wickramasinghe, D. (2004). The Sema domain of Met is necessary for receptor dimerization and activation. Cancer Cell 6, 75-84.

Kosai, K., Matsumoto, K., Nagata, S., Tsujimoto, Y., and Nakamura, T. (1998). Abrogation of Fas-induced fulminant hepatic failure in mice by hepatocyte growth factor. Biochem Biophys Res Commun 244, 683-690.

Krammer, P.H. (2000). CD95's deadly mission in the immune system. Nature 407, 789-795.

Kroemer, G. (2003). Mitochondrial control of apoptosis: an introduction. Biochem Biophys Res Commun 304, 433-435.

Kruger, R.P., Aurandt, J., and Guan, K.L. (2005). Semaphorins command cells to move. Nat Rev Mol Cell Biol 6, 789-800.

Lamballe, F., Genestine, M., Caruso, N., Arce, V., Richelme, S., Helmbacher, F., and Maina, F. Pool-specific regulation of motor neuron survival by neurotrophic support. J Neurosci 31, 11144-11158.

Lee, J.H., Han, S.U., Cho, H., Jennings, B., Gerrard, B., Dean, M., Schmidt, L., Zbar, B., and Vande Woude, G.F. (2000). A novel germ line juxtamembrane Met mutation in human gastric cancer. Oncogene 19, 4947-4953.

Lehninger, A.L. (1964). The Transfer of Energy in Oxidative Phosphorylation. Bull Soc Chim Biol (Paris) 46, 1555-1575.

Levy, D.E., and Darnell, J.E., Jr. (2002). Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 3, 651-662.

Li, L.Y., Luo, X., and Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412, 95-99.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993). Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature 363, 85-88.

Liang, C.C., and Chen, H.C. (2001). Sustained activation of extracellular signal-regulated kinase stimulated by hepatocyte growth factor leads to integrin alpha 2 expression that is involved in cell scattering. J Biol Chem 276, 21146-21152.

Liang, T.J., Reid, A.E., Xavier, R., Cardiff, R.D., and Wang, T.C. (1996). Transgenic expression of tpr-met oncogene leads to development of mammary hyperplasia and tumors. J Clin Invest 97, 2872-2877.

Lin, J.C., Naujokas, M., Zhu, H., Nolet, S., and Park, M. (1998). Intron-exon structure of the MET gene and cloning of an alternatively-spliced Met isoform reveals frequent exon-skipping of a single large internal exon. Oncogene 16, 833-842.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86, 147-157.

Liu, Y., Chattopadhyay, N., Qin, S., Szekeres, C., Vasylyeva, T., Mahoney, Z.X., Taglienti, M., Bates, C.M., Chapman, H.A., Miner, J.H., and Kreidberg, J.A. (2009). Coordinate integrin and c-Met signaling regulate Wnt gene expression during epithelial morphogenesis. Development 136, 843-853.

Lock, L.S., Frigault, M.M., Saucier, C., and Park, M. (2003). Grb2-independent recruitment of Gab1 requires the C-terminal lobe and structural integrity of the Met receptor kinase domain. J Biol Chem 278, 30083-30090.

Lock, L.S., Royal, I., Naujokas, M.A., and Park, M. (2000). Identification of an atypical Grb2 carboxyl-terminal SH3 domain binding site in Gab docking proteins reveals Grb2-dependent and -independent recruitment of Gab1 to receptor tyrosine kinases. J Biol Chem 275, 31536-31545.

Lokker, N.A., Mark, M.R., Luis, E.A., Bennett, G.L., Robbins, K.A., Baker, J.B., and Godowski, P.J. (1992). Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding. Embo J 11, 2503-2510.

Longati, P., Bardelli, A., Ponzetto, C., Naldini, L., and Comoglio, P.M. (1994). Tyrosines1234-1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). Oncogene 9, 49-57.

Loud, A.V. (1968). A quantitative stereological description of the ultrastructure of normal rat liver parenchymal cells. J Cell Biol 37, 27-46.

Luthi, A.U., and Martin, S.J. (2007). The CASBAH: a searchable database of caspase substrates. Cell Death Differ 14, 641-650.

Ma, P.C., Jagadeeswaran, R., Jagadeesh, S., Tretiakova, M.S., Nallasura, V., Fox, E.A., Hansen, M., Schaefer, E., Naoki, K., Lader, A., Richards, W., Sugarbaker, D., Husain, A.N., Christensen, J.G., and Salgia, R. (2005). Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. Cancer Res 65, 1479-1488.

Ma, P.C., Kijima, T., Maulik, G., Fox, E.A., Sattler, M., Griffin, J.D., Johnson, B.E., and Salgia, R. (2003). c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res 63, 6272-6281.

Ma, P.C., Tretiakova, M.S., MacKinnon, A.C., Ramnath, N., Johnson, C., Dietrich, S., Seiwert, T., Christensen, J.G., Jagadeeswaran, R., Krausz, T., Vokes, E.E., Husain, A.N., and Salgia, R. (2008). Expression and mutational analysis of MET in human solid cancers. Genes Chromosomes Cancer 47, 1025-1037.

MacFarlane, M., and Williams, A.C. (2004). Apoptosis and disease: a life or death decision. EMBO Rep 5, 674-678.

Maina, F., Casagranda, F., Audero, E., Simeone, A., Comoglio, P.M., Klein, R., and Ponzetto, C. (1996). Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development. Cell 87, 531-542.

Maina, F., Hilton, M.C., Andres, R., Wyatt, S., Klein, R., and Davies, A.M. (1998). Multiple roles for hepatocyte growth factor in sympathetic neuron development. Neuron 20, 835-846.

Maina, F., Hilton, M.C., Ponzetto, C., Davies, A.M., and Klein, R. (1997). Met receptor signaling is required for sensory nerve development and HGF promotes axonal growth and survival of sensory neurons. Genes Dev 11, 3341-3350.

Maroun, C.R., Holgado-Madruga, M., Royal, I., Naujokas, M.A., Fournier, T.M., Wong, A.J., and Park, M. (1999a). The Gab1 PH domain is required for localization of Gab1 at sites of cell-cell contact and epithelial morphogenesis downstream from the met receptor tyrosine kinase. Mol Cell Biol 19, 1784-1799.

Maroun, C.R., Moscatello, D.K., Naujokas, M.A., Holgado-Madruga, M., Wong, A.J., and Park, M. (1999b). A conserved inositol phospholipid binding site within the pleckstrin homology domain of the Gab1 docking protein is required for epithelial morphogenesis. J Biol Chem 274, 31719-31726.

Martens, T., Schmidt, N.O., Eckerich, C., Fillbrandt, R., Merchant, M., Schwall, R., Westphal, M., and Lamszus, K. (2006). A novel one-armed anti-c-Met antibody inhibits glioblastoma growth in vivo. Clin Cancer Res 12, 6144-6152.

Martins, G.J., Plachez, C., and Powell, E.M. (2007). Loss of embryonic MET signaling alters profiles of hippocampal interneurons. Dev Neurosci 29, 143-158.

Massari, S., and Azzone, G.F. (1972). The equivalent pore radius of intact and damaged mitochondria and the mechanism of active shrinkage. Biochim Biophys Acta 283, 23-29.

Matsumoto, K., Date, K., Shimura, H., and Nakamura, T. (1996). Acquisition of invasive phenotype in gallbladder cancer cells via mutual interaction of stromal fibroblasts and cancer cells as mediated by hepatocyte growth factor. Jpn J Cancer Res 87, 702-710.

Matsumoto, K., Kataoka, H., Date, K., and Nakamura, T. (1998). Cooperative interaction between alpha- and beta-chains of hepatocyte growth factor on c-Met receptor confers ligand-induced receptor tyrosine phosphorylation and multiple biological responses. J Biol Chem 273, 22913-22920.

Matsumoto, K., and Nakamura, T. (2003). NK4 (HGF-antagonist/angiogenesis inhibitor) in cancer biology and therapeutics. Cancer Sci 94, 321-327.

Matsumoto, K., and Nakamura, T. (2006). Hepatocyte growth factor and the Met system as a mediator of tumor-stromal interactions. Int J Cancer 119, 477-483.

Matsumoto, K., and Nakamura, T. (2008). NK4 gene therapy targeting HGF-Met and angiogenesis. Front Biosci 13, 1943-1951.

Maulik, G., Shrikhande, A., Kijima, T., Ma, P.C., Morrison, P.T., and Salgia, R. (2002). Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. Cytokine Growth Factor Rev 13, 41-59.

Mazelin, L., Bernet, A., Bonod-Bidaud, C., Pays, L., Arnaud, S., Gespach, C., Bredesen, D.E., Scoazec, J.Y., and Mehlen, P. (2004). Netrin-1 controls colorectal tumorigenesis by regulating apoptosis. Nature 431, 80-84.

Mazzone, M., Basilico, C., Cavassa, S., Pennacchietti, S., Risio, M., Naldini, L., Comoglio, P.M., and Michieli, P. (2004). An uncleavable form of pro-scatter factor suppresses tumor growth and dissemination in mice. J Clin Invest 114, 1418-1432.

Mehlen, P., Rabizadeh, S., Snipas, S.J., Assa-Munt, N., Salvesen, G.S., and Bredesen, D.E. (1998). The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis. Nature 395, 801-804.

Meier, P., Finch, A., and Evan, G. (2000). Apoptosis in development. Nature 407, 796-801.

Meimei, L., Peiling, L., Baoxin, L., Changmin, L., Rujin, Z., and Chunjie, H. Lost expression of DCC gene in ovarian cancer and its inhibition in ovarian cancer cells. Med Oncol 28, 282-289.

Michieli, P., Mazzone, M., Basilico, C., Cavassa, S., Sottile, A., Naldini, L., and Comoglio, P.M. (2004). Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. Cancer Cell 6, 61-73.

Mitra, A., Chakrabarti, J., and Chatterjee, A. (2003). Binding of alpha5 monoclonal antibody to cell surface alpha5beta1 integrin modulates MMP-2 and MMP-7 activity in B16F10 melanoma cells. J Environ Pathol Toxicol Oncol 22, 167-178.

Miura, M., Zhu, H., Rotello, R., Hartwieg, E.A., and Yuan, J. (1993). Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell 75, 653-660.

Montesano, R., Schaller, G., and Orci, L. (1991). Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. Cell 66, 697-711.

Montesano, R., Soriano, J.V., Malinda, K.M., Ponce, M.L., Bafico, A., Kleinman, H.K., Bottaro, D.P., and Aaronson, S.A. (1998). Differential effects of hepatocyte growth factor isoforms on epithelial and endothelial tubulogenesis. Cell Growth Differ 9, 355-365.

Moumen, A., Patane, S., Porras, A., Dono, R., and Maina, F. (2007). Met acts on Mdm2 via mTOR to signal cell survival during development. Development 134, 1443-1451.

Mourali, J., Benard, A., Lourenco, F.C., Monnet, C., Greenland, C., Moog-Lutz, C., Racaud-Sultan, C., Gonzalez-Dunia, D., Vigny, M., Mehlen, P., Delsol, G., and Allouche, M. (2006). Anaplastic lymphoma kinase is a dependence receptor whose proapoptotic functions are activated by caspase cleavage. Mol Cell Biol 26, 6209-6222.

Mueller, K.L., Hunter, L.A., Ethier, S.P., and Boerner, J.L. (2008). Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells. Cancer Res 68, 3314-3322.

Munshi, N., Jeay, S., Li, Y., Chen, C.R., France, D.S., Ashwell, M.A., Hill, J., Moussa, M.M., Leggett, D.S., and Li, C.J. ARQ 197, a novel and selective inhibitor of the human c-Met receptor tyrosine kinase with antitumor activity. Mol Cancer Ther 9, 1544-1553.

Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S., and Dixit, V.M. (1998). An induced proximity model for caspase-8 activation. J Biol Chem 273, 2926-2930.

Nakamura, T., Nawa, K., and Ichihara, A. (1984). Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem Biophys Res Commun 122, 1450-1459.

Naldini, L., Weidner, K.M., Vigna, E., Gaudino, G., Bardelli, A., Ponzetto, C., Narsimhan, R.P., Hartmann, G., Zarnegar, R., Michalopoulos, G.K., and et al. (1991). Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. Embo J 10, 2867-2878.

Naumann, T., Casademunt, E., Hollerbach, E., Hofmann, J., Dechant, G., Frotscher, M., and Barde, Y.A. (2002). Complete deletion of the neurotrophin receptor p75NTR leads to long-lasting increases in the number of basal forebrain cholinergic neurons. J Neurosci 22, 2409-2418.

Navab, R., Liu, J., Seiden-Long, I., Shih, W., Li, M., Bandarchi, B., Chen, Y., Lau, D., Zu, Y.F., Cescon, D., Zhu, C.Q., Organ, S., Ibrahimov, E., Ohanessian, D., and Tsao, M.S. (2009). Co-overexpression of Met and hepatocyte growth factor promotes systemic metastasis in NCI-H460 non-small cell lung carcinoma cells. Neoplasia 11, 1292-1300.

Nicholls, D.G. (2005). Mitochondria and calcium signaling. Cell Calcium 38, 311-317.

Nicoleau, C., Benzakour, O., Agasse, F., Thiriet, N., Petit, J., Prestoz, L., Roger, M., Jaber, M., and Coronas, V. (2009). Endogenous hepatocyte growth factor is a niche signal for subventricular zone neural stem cell amplification and self-renewal. Stem Cells 27, 408-419.

Niki, M., Isozaki, H., Toyoda, M., Ishibashi, T., Fujii, K., Nomura, E., Mabuchi, H., Nakamura, M., Nishiguchi, K., and Tanigawa, N. (1999). Serum human hepatocyte growth factor (hHGF) is elevated in patients with metastatic gastric carcinoma. Hepatogastroenterology 46, 568-573.

Oblinger, J.L., Pearl, D.K., Boardman, C.L., Saqr, H., Prior, T.W., Scheithauer, B.W., Jenkins, R.B., Burger, P.C., and Yates, A.J. (2006). Diagnostic and prognostic value of glycosyltransferase mRNA in glioblastoma multiforme patients. Neuropathol Appl Neurobiol 32, 410-418.

Ohmichi, H., Matsumoto, K., and Nakamura, T. (1996). In vivo mitogenic action of HGF on lung epithelial cells: pulmotrophic role in lung regeneration. Am J Physiol 270, L1031-1039.

Okamoto, K., and Shaw, J.M. (2005). Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. Annu Rev Genet 39, 503-536.

Olaku, V., Matzke, A., Mitchell, C., Hasenauer, S., Sakkaravarthi, A., Pace, G., Ponta, H., and Orian-Rousseau, V. c-Met recruits ICAM-1 as a coreceptor to compensate for the loss of CD44 in Cd44 null mice. Mol Biol Cell 22, 2777-2786.

Oliver, T.G., Read, T.A., Kessler, J.D., Mehmeti, A., Wells, J.F., Huynh, T.T., Lin, S.M., and Wechsler-Reya, R.J. (2005). Loss of patched and disruption of granule cell development in a pre-neoplastic stage of medulloblastoma. Development 132, 2425-2439.

Onozato, R., Kosaka, T., Kuwano, H., Sekido, Y., Yatabe, Y., and Mitsudomi, T. (2009). Activation of MET by gene amplification or by splice mutations deleting the juxtamembrane domain in primary resected lung cancers. J Thorac Oncol 4, 5-11.

Orian-Rousseau, V., Chen, L., Sleeman, J.P., Herrlich, P., and Ponta, H. (2002). CD44 is required for two consecutive steps in HGF/c-Met signaling. Genes Dev 16, 3074-3086.

Orian-Rousseau, V., Morrison, H., Matzke, A., Kastilan, T., Pace, G., Herrlich, P., and Ponta, H. (2007). Hepatocyte growth factor-induced Ras activation requires ERM proteins linked to both CD44v6 and F-actin. Mol Biol Cell 18, 76-83.

Ow, Y.P., Green, D.R., Hao, Z., and Mak, T.W. (2008). Cytochrome c: functions beyond respiration. Nat Rev Mol Cell Biol 9, 532-542.

Owen, K.A., Qiu, D., Alves, J., Schumacher, A.M., Kilpatrick, L.M., Li, J., Harris, J.L., and Ellis, V. Pericellular activation of hepatocyte growth factor by the transmembrane serine proteases matriptase and hepsin, but not by the membrane-associated protease uPA. Biochem J 426, 219-228.

Park, M., Dean, M., Kaul, K., Braun, M.J., Gonda, M.A., and Vande Woude, G. (1987). Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc Natl Acad Sci U S A 84, 6379-6383.

Parrish, J., Li, L., Klotz, K., Ledwich, D., Wang, X., and Xue, D. (2001). Mitochondrial endonuclease G is important for apoptosis in C. elegans. Nature 412, 90-94.

Pelicci, G., Giordano, S., Zhen, Z., Salcini, A.E., Lanfrancone, L., Bardelli, A., Panayotou, G., Waterfield, M.D., Ponzetto, C., Pelicci, P.G., and et al. (1995). The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein. Oncogene 10, 1631-1638.

Pepper, M.S., Soriano, J.V., Menoud, P.A., Sappino, A.P., Orci, L., and Montesano, R. (1995). Modulation of hepatocyte growth factor and c-met in the rat mammary gland during pregnancy, lactation, and involution. Exp Cell Res 219, 204-210.

Perkins, G., Renken, C., Martone, M.E., Young, S.J., Ellisman, M., and Frey, T. (1997). Electron tomography of neuronal mitochondria: three-dimensional structure and organization of cristae and membrane contacts. J Struct Biol 119, 260-272.

Peschard, P., Fournier, T.M., Lamorte, L., Naujokas, M.A., Band, H., Langdon, W.Y., and Park, M. (2001). Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. Mol Cell 8, 995-1004.

Peschard, P., and Park, M. (2007). From Tpr-Met to Met, tumorigenesis and tubes. Oncogene 26, 1276-1285.

Petrelli, A., Circosta, P., Granziero, L., Mazzone, M., Pisacane, A., Fenoglio, S., Comoglio, P.M., and Giordano, S. (2006). Ab-induced ectodomain shedding mediates hepatocyte growth factor receptor down-regulation and hampers biological activity. Proc Natl Acad Sci U S A 103, 5090-5095.

Petronilli, V., Szabo, I., and Zoratti, M. (1989). The inner mitochondrial membrane contains ion-conducting channels similar to those found in bacteria. FEBS Lett 259, 137-143.

Phaneuf, D., Moscioni, A.D., LeClair, C., Raper, S.E., and Wilson, J.M. (2004). Generation of a mouse expressing a conditional knockout of the hepatocyte growth factor gene: demonstration of impaired liver regeneration. DNA Cell Biol 23, 592-603.

Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., and Comoglio, P.M. (1994). A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell 77, 261-271.

Ponzetto, C., Giordano, S., Peverali, F., Della Valle, G., Abate, M.L., Vaula, G., and Comoglio, P.M. (1991). c-met is amplified but not mutated in a cell line with an activated met tyrosine kinase. Oncogene 6, 553-559.

Ponzetto, C., Zhen, Z., Audero, E., Maina, F., Bardelli, A., Basile, M.L., Giordano, S., Narsimhan, R., and Comoglio, P. (1996). Specific uncoupling of GRB2 from the Met receptor. Differential effects on transformation and motility. J Biol Chem 271, 14119-14123.

Popsueva, A., Poteryaev, D., Arighi, E., Meng, X., Angers-Loustau, A., Kaplan, D., Saarma, M., and Sariola, H. (2003). GDNF promotes tubulogenesis of GFRalpha1-expressing MDCK cells by Src-mediated phosphorylation of Met receptor tyrosine kinase. J Cell Biol 161, 119-129.

Prat, M., Crepaldi, T., Gandino, L., Giordano, S., Longati, P., and Comoglio, P. (1991). C-terminal truncated forms of Met, the hepatocyte growth factor receptor. Mol Cell Biol 11, 5954-5962.

Puthalakath, H., and Strasser, A. (2002). Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ 9, 505-512.

Rahimi, N., Hung, W., Tremblay, E., Saulnier, R., and Elliott, B. (1998). c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. J Biol Chem 273, 33714-33721.

Rao, L., Perez, D., and White, E. (1996). Lamin proteolysis facilitates nuclear events during apoptosis. J Cell Biol 135, 1441-1455.

Rhim, J.S., Kim, C.M., Arnstein, P., Huebner, R.J., Weisburger, E.K., and Nelson Ress, W.A. (1975). Transformation of human osteosarcoma cells by a chemical carcinogen. J Natl Cancer Inst 55, 1291-1294.

Ridley, A.J., Comoglio, P.M., and Hall, A. (1995). Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells. Mol Cell Biol 15, 1110-1122.

Riedl, S.J., and Salvesen, G.S. (2007). The apoptosome: signalling platform of cell death. Nat Rev Mol Cell Biol 8, 405-413.

Rodrigues, G.A., Falasca, M., Zhang, Z., Ong, S.H., and Schlessinger, J. (2000). A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling. Mol Cell Biol 20, 1448-1459.

Rodrigues, G.A., and Park, M. (1993). Dimerization mediated through a leucine zipper activates the oncogenic potential of the met receptor tyrosine kinase. Mol Cell Biol 13, 6711-6722.

Rong, S., Segal, S., Anver, M., Resau, J.H., and Vande Woude, G.F. (1994). Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. Proc Natl Acad Sci U S A 91, 4731-4735.

Rotonda, J., Nicholson, D.W., Fazil, K.M., Gallant, M., Gareau, Y., Labelle, M., Peterson, E.P., Rasper, D.M., Ruel, R., Vaillancourt, J.P., Thornberry, N.A., and Becker, J.W. (1996). The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. Nat Struct Biol 3, 619-625.

Ruan, S., and Lloyd, K.O. (1992). Glycosylation pathways in the biosynthesis of gangliosides in melanoma and neuroblastoma cells: relative glycosyltransferase levels determine ganglioside patterns. Cancer Res 52, 5725-5731.

Ruchaud, S., Korfali, N., Villa, P., Kottke, T.J., Dingwall, C., Kaufmann, S.H., and Earnshaw, W.C. (2002). Caspase-6 gene disruption reveals a requirement for lamin A cleavage in apoptotic chromatin condensation. Embo J 21, 1967-1977.

Ruckhaberle, E., Karn, T., Rody, A., Hanker, L., Gatje, R., Metzler, D., Holtrich, U., and Kaufmann, M. (2009). Gene expression of ceramide kinase, galactosyl ceramide synthase and ganglioside GD3 synthase is associated with prognosis in breast cancer. J Cancer Res Clin Oncol 135, 1005-1013.

Ruckhaberle, E., Rody, A., Engels, K., Gaetje, R., von Minckwitz, G., Schiffmann, S., Grosch, S., Geisslinger, G., Holtrich, U., Karn, T., and Kaufmann, M. (2008). Microarray analysis of altered sphingolipid metabolism reveals prognostic significance of sphingosine kinase 1 in breast cancer. Breast Cancer Res Treat 112, 41-52.

Sadanandam, A., Rosenbaugh, E.G., Singh, S., Varney, M., and Singh, R.K. Semaphorin 5A promotes angiogenesis by increasing endothelial cell proliferation, migration, and decreasing apoptosis. Microvasc Res 79, 1-9.

Saucier, C., Khoury, H., Lai, K.M., Peschard, P., Dankort, D., Naujokas, M.A., Holash, J., Yancopoulos, G.D., Muller, W.J., Pawson, T., and Park, M. (2004). The Shc adaptor protein is critical for VEGF induction by Met/HGF and ErbB2 receptors and for early onset of tumor angiogenesis. Proc Natl Acad Sci U S A 101, 2345-2350.

Scarpino, S., Cancellario d'Alena, F., Di Napoli, A., Pasquini, A., Marzullo, A., and Ruco, L.P. (2004). Increased expression of Met protein is associated with up-regulation of hypoxia inducible factor-1 (HIF-1) in tumour cells in papillary carcinoma of the thyroid. J Pathol 202, 352-358.

Schaeper, U., Gehring, N.H., Fuchs, K.P., Sachs, M., Kempkes, B., and Birchmeier, W. (2000). Coupling of Gab1 to c-Met, Grb2, and Shp2 mediates biological responses. J Cell Biol 149, 1419-1432.

Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. (1995). Scatter factor/hepatocyte growth factor is essential for liver development. Nature 373, 699-702.

Schmidt, L., Duh, F.M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S.W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U.R., Feltis, J.T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C.J., Walther, M.M., Tsui, L.C., Geil, L., Orcutt, M.L., Stackhouse, T., Lipan, J., Slife, L., Brauch, H., Decker, J., Niehans, G., Hughson, M.D., Moch, H., Storkel, S., Lerman, M.I., Linehan, W.M., and Zbar, B. (1997). Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet 16, 68-73.

Schmidt, M.H., and Dikic, I. (2005). The Cbl interactome and its functions. Nat Rev Mol Cell Biol 6, 907-918.

Schutte, B., Henfling, M., Kolgen, W., Bouman, M., Meex, S., Leers, M.P., Nap, M., Bjorklund, V., Bjorklund, P., Bjorklund, B., Lane, E.B., Omary, M.B., Jornvall, H., and Ramaekers, F.C. (2004). Keratin 8/18 breakdown and reorganization during apoptosis. Exp Cell Res 297, 11-26.

Sebbagh, M., Renvoize, C., Hamelin, J., Riche, N., Bertoglio, J., and Breard, J. (2001). Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. Nat Cell Biol 3, 346-352.

Seki, T., Hagiya, M., Shimonishi, M., Nakamura, T., and Shimizu, S. (1991). Organization of the human hepatocyte growth factor-encoding gene. Gene 102, 213-219.

Shima, N., Itagaki, Y., Nagao, M., Yasuda, H., Morinaga, T., and Higashio, K. (1991a). A fibroblast-derived tumor cytotoxic factor/F-TCF (hepatocyte growth factor/HGF) has multiple functions in vitro. Cell Biol Int Rep 15, 397-408.

Shima, N., Nagao, M., Ogaki, F., Tsuda, E., Murakami, A., and Higashio, K. (1991b). Tumor cytotoxic factor/hepatocyte growth factor from human fibroblasts: cloning of its cDNA, purification and characterization of recombinant protein. Biochem Biophys Res Commun 180, 1151-1158.

Shimamura, M., Sato, N., Sata, M., Wakayama, K., Ogihara, T., and Morishita, R. (2007). Expression of hepatocyte growth factor and c-Met after spinal cord injury in rats. Brain Res 1151, 188-194.

Siegfried, J.M., Weissfeld, L.A., Singh-Kaw, P., Weyant, R.J., Testa, J.R., and Landreneau, R.J. (1997). Association of immunoreactive hepatocyte growth factor with poor survival in resectable non-small cell lung cancer. Cancer Res 57, 433-439.

Smolen, G.A., Sordella, R., Muir, B., Mohapatra, G., Barmettler, A., Archibald, H., Kim, W.J., Okimoto, R.A., Bell, D.W., Sgroi, D.C., Christensen, J.G., Settleman, J., and Haber, D.A. (2006). Amplification of MET may identify a subset of cancers with extreme sensitivity to the selective tyrosine kinase inhibitor PHA-665752. Proc Natl Acad Sci U S A 103, 2316-2321.

Smyth, L.A., and Brady, H.J. (2005). cMet and Fas receptor interaction inhibits death-inducing signaling complex formation in endothelial cells. Hypertension 46, 100-106.

Soman, N.R., Correa, P., Ruiz, B.A., and Wogan, G.N. (1991). The TPR-MET oncogenic rearrangement is present and expressed in human gastric carcinoma and precursor lesions. Proc Natl Acad Sci U S A 88, 4892-4896.

Sonnenberg, E., Meyer, D., Weidner, K.M., and Birchmeier, C. (1993). Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. J Cell Biol 123, 223-235.

Soriano, J.V., Pepper, M.S., Orci, L., and Montesano, R. (1998). Roles of hepatocyte growth factor/scatter factor and transforming growth factor-beta1 in mammary gland ductal morphogenesis. J Mammary Gland Biol Neoplasia 3, 133-150.

Spix, J.K., Chay, E.Y., Block, E.R., and Klarlund, J.K. (2007). Hepatocyte growth factor induces epithelial cell motility through transactivation of the epidermal growth factor receptor. Exp Cell Res 313, 3319-3325.

Stamos, J., Lazarus, R.A., Yao, X., Kirchhofer, D., and Wiesmann, C. (2004). Crystal structure of the HGF beta-chain in complex with the Sema domain of the Met receptor. Embo J 23, 2325-2335.

Stevens, L., McClelland, L., Fricke, A., Williamson, M., Kuo, I., and Scott, G. Plexin B1 suppresses c-Met in melanoma: a role for plexin B1 as a tumor-suppressor protein through regulation of c-Met. J Invest Dermatol 130, 1636-1645.

Stoker, M., Gherardi, E., Perryman, M., and Gray, J. (1987). Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature 327, 239-242.

Strasser, A., Puthalakath, H., Bouillet, P., Huang, D.C., O'Connor, L., O'Reilly, L.A., Cullen, L., Cory, S., and Adams, J.M. (2000). The role of bim, a proapoptotic BH3-only member of the Bcl-2 family in cell-death control. Ann N Y Acad Sci 917, 541-548.

Susin, S.A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K.F., Irinopoulou, T., Prevost, M.C., Brothers, G., Mak, T.W., Penninger, J., Earnshaw, W.C., and Kroemer, G. (2000). Two distinct pathways leading to nuclear apoptosis. J Exp Med 192, 571-580.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M., and Kroemer, G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397, 441-446.

Susin, S.A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., and Kroemer, G. (1996). Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J Exp Med 184, 1331-1341.

Tait, S.W., and Green, D.R. Cell survival in tough times: The mitochondrial recovery plan. Cell Cycle 9, 4254-4255.

Tajima, H., Matsumoto, K., and Nakamura, T. (1991). Hepatocyte growth factor has potent anti-proliferative activity in various tumor cell lines. FEBS Lett 291, 229-232.

Takada, Y., Ye, X., and Simon, S. (2007). The integrins. Genome Biol 8, 215.

Takayama, H., LaRochelle, W.J., Sharp, R., Otsuka, T., Kriebel, P., Anver, M., Aaronson, S.A., and Merlino, G. (1997). Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. Proc Natl Acad Sci U S A 94, 701-706.

Tauszig-Delamasure, S., Yu, L.Y., Cabrera, J.R., Bouzas-Rodriguez, J., Mermet-Bouvier, C., Guix, C., Bordeaux, M.C., Arumae, U., and Mehlen, P. (2007). The TrkC receptor induces apoptosis when the dependence receptor notion meets the neurotrophin paradigm. Proc Natl Acad Sci U S A 104, 13361-13366.

Tempest, P.R., Stratton, M.R., and Cooper, C.S. (1988). Structure of the met protein and variation of met protein kinase activity among human tumour cell lines. Br J Cancer 58, 3-7.

Thampoe, I.J., Furukawa, K., Vellve, E., and Lloyd, K.O. (1989). Sialyltransferase levels and ganglioside expression in melanoma and other cultured human cancer cells. Cancer Res 49, 6258-6264.

Thiebault, K., Mazelin, L., Pays, L., Llambi, F., Joly, M.O., Scoazec, J.Y., Saurin, J.C., Romeo, G., and Mehlen, P. (2003). The netrin-1 receptors UNC5H are putative tumor suppressors controlling cell death commitment. Proc Natl Acad Sci U S A 100, 4173-4178.

Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T., and Nicholson, D.W. (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem 272, 17907-17911.

Toi, M., Taniguchi, T., Ueno, T., Asano, M., Funata, N., Sekiguchi, K., Iwanari, H., and Tominaga, T. (1998). Significance of circulating hepatocyte growth factor level as a prognostic indicator in primary breast cancer. Clin Cancer Res 4, 659-664.

Toiyama, Y., Miki, C., Inoue, Y., Okugawa, Y., Tanaka, K., and Kusunoki, M. (2009). Serum hepatocyte growth factor as a prognostic marker for stage II or III colorectal cancer patients. Int J Cancer 125, 1657-1662.

Toole, B.P. (1990). Hyaluronan and its binding proteins, the hyaladherins. Curr Opin Cell Biol 2, 839-844.

Toschi, L., and Cappuzzo, F. Clinical implications of MET gene copy number in lung cancer. Future Oncol 6, 239-247.

Trusolino, L., Bertotti, A., and Comoglio, P.M. (2001). A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth. Cell 107, 643-654.

Tulasne, D., Deheuninck, J., Lourenco, F.C., Lamballe, F., Ji, Z., Leroy, C., Puchois, E., Moumen, A., Maina, F., Mehlen, P., and Fafeur, V. (2004). Proapoptotic function of the MET tyrosine kinase receptor through caspase cleavage. Mol Cell Biol 24, 10328-10339.

Tulasne, D., Paumelle, R., Leroy, C., Reveneau, S., Vandenbunder, B., and Fafeur, V. (2002). Involvement of RAS-ERK signaling in multiple biological responses to HGF/SF. Ann N Y Acad Sci 973, 105-108.

Tulasne, D., Paumelle, R., Weidner, K.M., Vandenbunder, B., and Fafeur, V. (1999). The multisubstrate docking site of the MET receptor is dispensable for MET-mediated RAS signaling and cell scattering. Mol Biol Cell 10, 551-565.

Tyner, J.W., Fletcher, L.B., Wang, E.Q., Yang, W.F., Rutenberg-Schoenberg, M.L., Beadling, C., Mori, M., Heinrich, M.C., Deininger, M.W., Druker, B.J., and Loriaux, M.M. MET receptor sequence variants R970C and T992I lack transforming capacity. Cancer Res 70, 6233-6237.

Uehara, Y., Minowa, O., Mori, C., Shiota, K., Kuno, J., Noda, T., and Kitamura, N. (1995). Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature 373, 702-705.

Underiner, T.L., Herbertz, T., and Miknyoczki, S.J. Discovery of small molecule c-Met inhibitors: Evolution and profiles of clinical candidates. Anticancer Agents Med Chem 10, 7-27.

van der Klei, I.J., Veenhuis, M., and Neupert, W. (1994). A morphological view on mitochondrial protein targeting. Microsc Res Tech 27, 284-293.

van der Neut, R., Krimpenfort, P., Calafat, J., Niessen, C.M., and Sonnenberg, A. (1996). Epithelial detachment due to absence of hemidesmosomes in integrin beta 4 null mice. Nat Genet 13, 366-369.

van der Voort, R., Taher, T.E., Wielenga, V.J., Spaargaren, M., Prevo, R., Smit, L., David, G., Hartmann, G., Gherardi, E., and Pals, S.T. (1999). Heparan sulfate-modified CD44 promotes hepatocyte

growth factor/scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met. J Biol Chem 274, 6499-6506.

van Loo, G., Schotte, P., van Gurp, M., Demol, H., Hoorelbeke, B., Gevaert, K., Rodriguez, I., Ruiz-Carrillo, A., Vandekerckhove, J., Declercq, W., Beyaert, R., and Vandenabeele, P. (2001). Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. Cell Death Differ 8, 1136-1142.

Vande Walle, L., Lamkanfi, M., and Vandenabeele, P. (2008). The mitochondrial serine protease HtrA2/Omi: an overview. Cell Death Differ 15, 453-460.

Vanhaesebroeck, B., Ali, K., Bilancio, A., Geering, B., and Foukas, L.C. (2005). Signalling by PI3K isoforms: insights from gene-targeted mice. Trends Biochem Sci 30, 194-204.

Vaux, D.L., and Silke, J. (2003). Mammalian mitochondrial IAP binding proteins. Biochem Biophys Res Commun 304, 499-504.

Velcich, A., Corner, G., Palumbo, L., and Augenlicht, L. (1999). Altered phenotype of HT29 colonic adenocarcinoma cells following expression of the DCC gene. Oncogene 18, 2599-2606.

Vendelin, M., Beraud, N., Guerrero, K., Andrienko, T., Kuznetsov, A.V., Olivares, J., Kay, L., and Saks, V.A. (2005). Mitochondrial regular arrangement in muscle cells: a "crystal-like" pattern. Am J Physiol Cell Physiol 288, C757-767.

Wajih, N., Walter, J., and Sane, D.C. (2002). Vascular origin of a soluble truncated form of the hepatocyte growth factor receptor (c-met). Circ Res 90, 46-52.

Wang, W., Marimuthu, A., Tsai, J., Kumar, A., Krupka, H.I., Zhang, C., Powell, B., Suzuki, Y., Nguyen, H., Tabrizizad, M., Luu, C., and West, B.L. (2006). Structural characterization of autoinhibited c-Met kinase produced by coexpression in bacteria with phosphatase. Proc Natl Acad Sci U S A 103, 3563-3568.

Wang, X., DeFrances, M.C., Dai, Y., Pediaditakis, P., Johnson, C., Bell, A., Michalopoulos, G.K., and Zarnegar, R. (2002). A mechanism of cell survival: sequestration of Fas by the HGF receptor Met. Mol Cell 9, 411-421.

Weidner, K.M., Sachs, M., Riethmacher, D., and Birchmeier, W. (1995). Mutation of juxtamembrane tyrosine residue 1001 suppresses loss-of-function mutations of the met receptor in epithelial cells. Proc Natl Acad Sci U S A 92, 2597-2601.

Widmann, C., Gibson, S., and Johnson, G.L. (1998). Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals. J Biol Chem 273, 7141-7147.

Wilhelmsen, K., Litjens, S.H., and Sonnenberg, A. (2006). Multiple functions of the integrin alpha6beta4 in epidermal homeostasis and tumorigenesis. Mol Cell Biol 26, 2877-2886.

Willis, S.N., Fletcher, J.I., Kaufmann, T., van Delft, M.F., Chen, L., Czabotar, P.E., Ierino, H., Lee, E.F., Fairlie, W.D., Bouillet, P., Strasser, A., Kluck, R.M., Adams, J.M., and Huang, D.C. (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science 315, 856-859.

Winberg, M.L., Noordermeer, J.N., Tamagnone, L., Comoglio, P.M., Spriggs, M.K., Tessier-Lavigne, M., and Goodman, C.S. (1998). Plexin A is a neuronal semaphorin receptor that controls axon guidance. Cell 95, 903-916.

Wood, W., Turmaine, M., Weber, R., Camp, V., Maki, R.A., McKercher, S.R., and Martin, P. (2000). Mesenchymal cells engulf and clear apoptotic footplate cells in macrophageless PU.1 null mouse embryos. Development 127, 5245-5252.

Wyllie, A.H., Kerr, J.F., and Currie, A.R. (1980). Cell death: the significance of apoptosis. Int Rev Cytol 68, 251-306.

Yamamoto, K., Morishita, R., Hayashi, S., Matsushita, H., Nakagami, H., Moriguchi, A., Matsumoto, K., Nakamura, T., Kaneda, Y., and Ogihara, T. (2001). Contribution of Bcl-2, but not Bcl-xL and Bax, to antiapoptotic actions of hepatocyte growth factor in hypoxia-conditioned human endothelial cells. Hypertension 37, 1341-1348.

Yamamoto, Y., Livet, J., Pollock, R.A., Garces, A., Arce, V., deLapeyriere, O., and Henderson, C.E. (1997). Hepatocyte growth factor (HGF/SF) is a muscle-derived survival factor for a subpopulation of embryonic motoneurons. Development 124, 2903-2913.

Yamashita, J., Ogawa, M., Yamashita, S., Nomura, K., Kuramoto, M., Saishoji, T., and Shin, S. (1994). Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. Cancer Res 54, 1630-1633.

Yao, Y., and Marassi, F.M. (2009). BAX and BAK caught in the act. Mol Cell 36, 353-354.

Yeo, T.T., Chua-Couzens, J., Butcher, L.L., Bredesen, D.E., Cooper, J.D., Valletta, J.S., Mobley, W.C., and Longo, F.M. (1997). Absence of p75NTR causes increased basal forebrain cholinergic neuron size, choline acetyltransferase activity, and target innervation. J Neurosci 17, 7594-7605.

Yo, Y., Morishita, R., Nakamura, S., Tomita, N., Yamamoto, K., Moriguchi, A., Matsumoto, K., Nakamura, T., Higaki, J., and Ogihara, T. (1998). Potential role of hepatocyte growth factor in the maintenance of renal structure: anti-apoptotic action of HGF on epithelial cells. Kidney Int 54, 1128-1138.

Youle, R.J., and Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 9, 47-59.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M., and Horvitz, H.R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell 75, 641-652.

Zaffaroni, D., Spinola, M., Galvan, A., Falvella, F.S., Pazzaglia, S., Saran, A., Mancuso, M.T., Galbiati, F., Pignatiello, C., Cabrera, W., Ibanez, O., Manenti, G., and Dragani, T.A. (2005). Met proto-oncogene juxtamembrane rare variations in mouse and humans: differential effects of Arg and Cys alleles on mouse lung tumorigenesis. Oncogene 24, 1084-1090.

Zeng, Z.S., Weiser, M.R., Kuntz, E., Chen, C.T., Khan, S.A., Forslund, A., Nash, G.M., Gimbel, M., Yamaguchi, Y., Culliford, A.T.t., D'Alessio, M., Barany, F., and Paty, P.B. (2008). c-Met gene amplification is associated with advanced stage colorectal cancer and liver metastases. Cancer Lett 265, 258-269.

Zhang, J., Dong, M., Li, L., Fan, Y., Pathre, P., Dong, J., Lou, D., Wells, J.M., Olivares-Villagomez, D., Van Kaer, L., Wang, X., and Xu, M. (2003). Endonuclease G is required for early embryogenesis and normal apoptosis in mice. Proc Natl Acad Sci U S A 100, 15782-15787.

Zhang, Y., Guessous, F., Kofman, A., Schiff, D., and Abounader, R. XL-184, a MET, VEGFR-2 and RET kinase inhibitor for the treatment of thyroid cancer, glioblastoma multiforme and NSCLC. IDrugs 13, 112-121.

Zhang, Y.W., Wang, L.M., Jove, R., and Vande Woude, G.F. (2002). Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis. Oncogene 21, 217-226.

ANNEXES

Oncogene (2009) 28, 2185–2195
© 2009 Macmillan Publishers Limited All rights reserved 0950-9232/09 $32.00
www.nature.com/onc

REVIEW

Proteolytic cleavages give receptor tyrosine kinases the gift of ubiquity

F Ancot, B Foveau, J Lefebvre, C Leroy and D Tulasne

CNRS UMR 8161, Institut de Biologie de Lille-Institut Pasteur de Lille-Université de Lille 1-Université de Lille 2, Lille, France

Receptor tyrosine kinases (RTK) constitute a large family of membrane receptors which, in response to their respective ligand, transmit information into cells. RTK regulate multiple biological responses, and their deregulation is often associated with tumourigenesis. The intracellular signalling pathways initiated by full-length membrane RTK are studied extensively, but many RTK fragments showing unexpected cellular localization have been observed. These fragments are generated by proteolytic cleavages, catalyzed notably by caspases, membrane metalloproteases or γ-secretase. Interestingly, these cleavages, in addition to regulating membrane receptor levels, generate active fragments that can regulate biological processes, such as transcription or the survival/apoptosis balance. Thus, proteolytic cleavages release RTK from the membrane and extend their functions. Furthermore, the RTK proteolysis are involved in regulating cell transformation, which highlights their potential as attractive targets for therapeutic strategies.
Oncogene (2009) **28,** 2185–2195; doi:10.1038/onc.2009.88; published online 4 May 2009

Keywords: receptor tyrosine kinase; cleavages; caspase; metalloprotease; γ-secretase; signalling

RTK as a membrane mediator between the extracellular and intracellular compartments

In multicellular organisms, the dialogue between cells is crucial to harmonious development and, in adults, to tissue homeostasis. This dialogue operates notably through soluble cytokines and their cognate membrane receptors. Cytokines secreted by cells into the extracellular environment can bind with high affinity to the extracellular domains of their respective specific membrane receptors expressed on the surface of target cells. Ligand binding triggers activation of the receptor, which transmits the information from the extracellular to the intracellular compartment. Receptor tyrosine kinases (RTK) constitute a large family of information-transmitting membrane receptors. The variability of their extracellular regions results in a wide specificity range as regards cytokine ligands. Inversely, their intracellular regions possess a conserved tyrosine kinase domain, involved in message transmission (Hubbard and Till, 2000). Ligand-activated RTK can initiate multiple biological responses, including proliferation, cell motility, survival and differentiation.

Receptor tyrosine kinases are activated through dimerization and trans-autophosphorylation of tyrosine residues. The intracellular region of an RTK then becomes a signalling platform that can recruit numerous cytoplasmic proteins through phosphorylated tyrosine residues. In turn, these signalling proteins activate signalling pathways commonly organized in a cascade, which will propagate the information inside the cell (Schlessinger, 2000). The signalling cascades are often interconnected and are able to activate or downregulate each other (Amit *et al*., 2007). Therefore, the entire intracellular signalling mechanism is viewed as a network rather than as a superposition of multiple linear pathways.

In keeping with the involvement of RTK in controlling a wide range of biological responses, their deregulation is associated with numerous types of cancer. Uncontrolled RTK activation involves several mechanisms, including tyrosine kinase activation by mutations, generation of constitutive fusion through chromosomal translocation, and in most cases, aberrant overexpression of the RTK and/or its ligand, leading to an autocrine or paracrine activation loop (Kolibaba and Druker, 1997).

RTK are more than membrane receptors

The signalling pathways initiated by ligand-activated membrane-anchored RTK are studied extensively, the challenge being to unravel the relationship between activated signalling pathways and integrated biological responses. Yet RTK do not exist only at the cell surface; they have been observed in the extracellular environment, the cytoplasm and even in the nucleus. These unexpected locations are mostly the consequence of proteolytic cleavages. Recently, the molecular mechanisms underlying these RTK cleavages have been decrypted. In this review, we describe these recent findings, involving caspases, membrane metalloproteases and the γ-secretase complex as proteases targeting RTK. Interestingly, in addition to inactivating RTK, the proteolytic cleavages generate active fragments acting in other cell compartments. Thus, in parallel with the classical signalling cascades initiated by full-length membrane RTK, novel signalling mechanisms have emerged, in which proteolytic fragments, like emissaries, transmit information directly.

Correspondence: Dr D Tulasne, CNRS UMR 8161, Institut de Biologie de Lille, Institut Pasteur de Lille, B.P.447, 59021 Lille, France.
E-mail: david.tulasne@ibl.fr
Received 10 June 2008; revised 23 March 2009; accepted 26 March 2009; published online 4 May 2009

RTK are reaped by caspases

Apoptotic cell death is triggered by a variety of physiological and pathological stimuli. Its characteristic morphological features include cell rounding, membrane blebbing, cytoplasmic condensation and DNA fragmentation. During apoptosis, caspases forming a family of cystein-dependent aspartate-directed proteases, can cleave a wide range of substrates contributing to the characteristic morphological modifications of apoptosis (Luthi and Martin, 2007). Caspases can also contribute to promoting cell death by inactivating survival mechanisms. They can notably cleave several RTK, which are the major mediators of the survival response.

The Epidermal Growth Factor Receptor (EGFR), a member of the EGFR family, mediates critical survival signals, but in response to a variety of apoptotic stimuli, it is downregulated by activated caspases (Bae *et al*., 2001; He *et al*., 2003; Zhuang *et al*., 2003; He *et al*., 2006) that cleave the receptor within its C-terminal tail (Figure 1a). Interestingly, cells expressing uncleavable EGFR show slightly delayed cell death. This suggests that caspase cleavages suppress potential survival signalling through the downregulation of EGFR, identifying caspases as modulators of cell signalling (He *et al*., 2006).

RTK are on the way to dependence

The caspase cleavages of EGFR proteins fail to generate detectable stable fragments. In contrast, caspase cleavages of the RTK RET, Met, TrkC, ALK, ErbB2 and EphA4 generate fragments acting directly on cell fate (Bordeaux *et al*., 2000; Tikhomirov *et al*., 2005; Benoit *et al*., 2004; Tulasne *et al*., 2004; Mourali *et al*., 2006; Foveau *et al*., 2007; Tauszig-Delamasure *et al*., 2007; Strohecker *et al*., 2008; Furne *et al*., 2009). These fragments, generated by one or several cleavages, isolate the entire kinase domain of Met and RET, create a truncated kinase domain from TrkC, ALK and EphA4 or from the C-terminal truncation in ErbB2 (Figure 1a). Thus, these cleavages dissociate the extracellular ligand-binding domain from the kinase domain or the C-terminal binding site for signalling proteins, thereby potentially abolishing the receptor function. Interestingly, the cytoplasmic fragments generated from RET, Met, TrkC and ErbB2, as well as from the remaining membrane-anchored fragment of ALK and EphA4 can all promote apoptosis. Therefore, in addition to inactivating these survival receptors, the caspase cleavages convert them to proapoptotic factors (Figure 1a). In contrast, except in the case of the orphan receptor, ErbB2, ligand stimulation inhibits the caspase cleavage of these receptors, probably through inhibition of caspase activity after induction of survival responses. Thus, the proteolytic RTK fragments generated by the caspase cleavage can promote apoptosis, a response opposite to the survival signalling induced by the ligand-activated full-length receptor (Figure 1b). It is worth noting that although ErbB2 is an orphan receptor, it is able to contribute to cell survival through its association with other EGFRs or through its overexpression in tumour cells.

The proapoptotic function mediated by these RTK in the absence of their respective ligands, contrasting with their anti-apoptotic function upon ligand stimulation, is the hallmark of the emerging family of dependence receptors. Although a receptor is usually considered inactive until bound by its ligand, these receptors have been shown to induce cell death in the absence of the ligand. Thus, expression of these receptors leads to dependence on the ligand for cell survival (Bredesen *et al*., 2005). Very little is known about the physiological involvement of the proapoptotic fragments released from RTK. However, it has been shown that ligand-activated TrkC induces neuron survival, whereas ligand withdrawal induces apoptosis. Interestingly, caspase-uncleavable TrkC impairs apoptosis induced by ligand withdrawal, showing that this neuronal cell death is not caused only by the loss of a survival response, but also to a proapoptotic activity induced by cleaved TrkC (Tauszig-Delamasure *et al*., 2007). EphA4 is also involved in regulating the survival/apoptosis balance in the nervous system. Indeed, EphA4-deficient mice show excessive numbers of neuroblasts in the sub-ventricular zone, whereas the absence of its ligand, the EphrinB3, results in increased cell death. Furthermore, treatment with soluble EphrinB3 into the lateral ventricle reduced cell death. Altogether, these observations support the involvement of EphA4 in neurogenesis through a dependence mechanism (Furne *et al*., 2009). In the case of RET, cleavage by caspases has been linked to Hirschsprung's disease (an intestinal malformation characterized by the absence of parasympathetic neurons in the hindgut), as Hirschsprung's-associated RET mutations impair the ligand downregulation of RET proapoptotic activity (Bordeaux *et al*., 2000). Thus, caspase cleavages of these three RTK are associated with the regulation of the neuronal survival/apoptosis balance, a tightly regulated process. Met and ALK are also expressed in the developing nervous system, and ligand-activated Met induces neuron survival (Allouche, 2007; Runeberg-Roos and Saarma, 2007; Maina and Klein, 1999). This raises the possibility that the proapoptotic properties showed by these receptors in the absence of the ligand might contribute to neuronal cell death during development. The future challenge will be to unravel the physiological relevance of caspase-dependent cleavage of RTK notably during development. With this aim, several laboratories are developing genetically modified mice expressing an uncleavable receptor mutated at the caspase site.

Both survival receptors and messengers of death

Although the survival signalling pathways activated upon RTK stimulation are well described, the down-

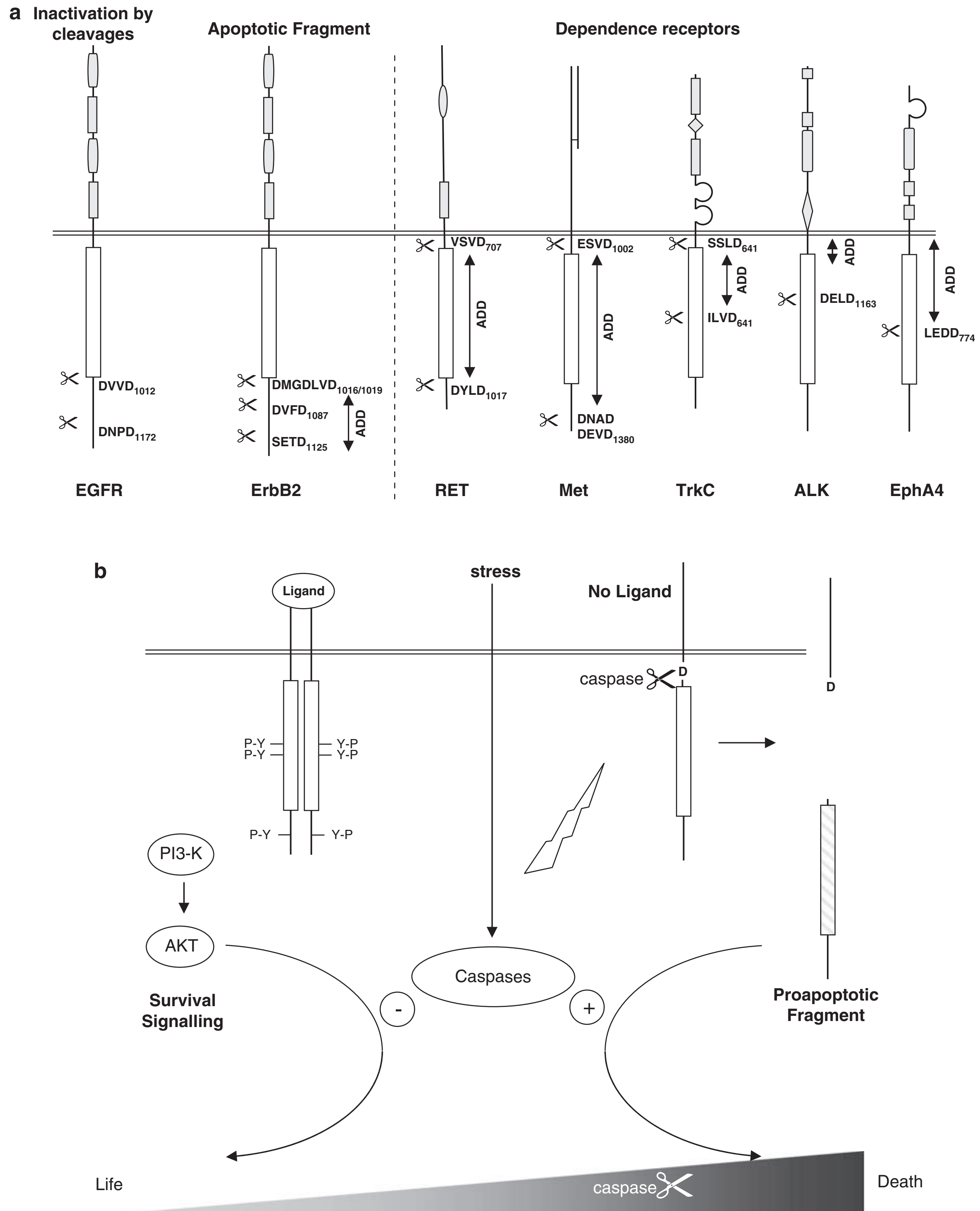


Figure 1 Receptor tyrosine kinases are targets of caspases. (**a**) The extracellular domains, the intracellular kinase domain (white box) (Hubbard and Till, 2000) and the identified caspase sites of receptor tyrosine kinase (RTK) are represented. On the left are represented the epidermal growth factor receptor (EGFR), inactivated by caspase cleavages, and the ErbB2 receptor, in which caspase cleavages generate apoptotic fragments. On the right are represented the RTK classified as dependence receptor, able to induce survival in response to their ligand and apoptosis through the generation of proapoptotic fragments. The identified addiction dependence domains (ADD) involved in the proapoptotic response are indicated. (**b**) Representation of the dependence mechanism induced by RTK. In the presence of ligand (on the left), activated RTK initiates survival signalling pathways, including activation of PI3-K–AKT signalling, which counteract apoptosis notably through the inhibition of caspase activity. In contrast, in the absence of ligand (on the right), some RTK are cleaved by caspases generating proapoptotic fragment able to regulate caspase activity and to amplify cell death.

stream responses induced by RTK fragments remain poorly understood. The proapoptotic domain exposed upon caspase cleavage (named ADD for Addiction Dependence Domain in the dependence receptor family) differs among these RTK, extending through the entire kinase domain in RET and Met, being located in the N-terminal part of the kinase domain in TrkC, the N-terminal region of ErbB2 and within the juxtamembrane domain in ALK (Figure 1a). These distinct locations suggest that these RTK might not share the same cell-death-promoting mechanism.

It has been shown in pituitary cells that the proapoptotic property of RET is associated with the formation of a complex between RET, caspase-3 and PKCδ. This promotes RET cleavage, leading finally to p53 expression and apoptosis (Canibano *et al*., 2007). In the case of ErbB2, it has been shown that the fragment generated through the caspase-dependent cleavage contains a BH3-like domain involved in its mitochondrial localization and its association with the anti-apoptotic protein, Bcl-x_L. These properties are necessary for mitochondrial cytochrome *c* release and apoptosis induced by the ErbB2 fragment (Strohecker *et al*., 2008). Thus, these receptors are able to promote cell death through the generation of fragments acting directly on the apoptotic machinery. In the light of these signalling mechanisms, we can propose that proapoptotic fragments of RTK act directly on the apoptotic process. This contrasts with the survival response, mediated by ligand-activated membrane receptors and based on the activation of signalling cascades (Figure 1b).

Shedding releases RTK from the membrane

Cell-membrane-bound proteins can be cleaved proteolytically in the juxtamembrane region through a process called ectodomain shedding, releasing soluble extracellular domains. Ectodomain shedding is a widespread form of proteolytic processing affecting many proteins, including growth factors, cytokines, adhesion molecules, receptors and proteins of the immune system (Huovila *et al*., 2005). Several extracellular RTK fragments have been observed in the extracellular medium. This unexpected localization is the consequence of ectodomain shedding, evidenced for at least 15 RTK (Table 1).

The shedding activity is attributed to metalloproteases such as matrix metalloproteases (MMP) and A Disintegrins And Metalloproteases (ADAM) or to aspartic proteases (BACE). Among them, the ADAM proteins seem to be responsible for the majority of RTK shedding. Notable in this respect is ADAM17, initially identified as TNF-α-converting enzyme. It has been shown that ADAM17 cleaves CSFR, VEGFR2, ErbB4 and c-Kit, whereas EphB2 is processed by ADAM10 (Table 1). In addition, it has been shown that multiple sheddases can cleave the same substrate. Accordingly, we have shown that silencing of ADAM17 inhibits only partial shedding from Met, suggesting that other metalloproteases could process Met (Foveau *et al*., 2009). Moreover, it has recently been shown in NIH3T3 cells that shedding from Met involves ADAM10 (Kopitz *et al*., 2007). Thus, shedding from RTK could involve several metalloproteases, which could be engaged differently according to the cell type or the stimulus. However, for several RTK the proteases responsible for shedding are not yet known.

Shedding involving ADAM proteins can occur constitutively or in response to different stimuli, such as treatment with phorbol esters (PMA, phorbol myristate acetate), PKC activation, growth factor or cytokine stimulation and calcium influx (Blobel, 2005) (Table 1). In addition, shedding can be activated upon ligand stimulation, as evidenced for EGFR, CSFR, TrkA, and VEGFR (Table 1). Different stimuli can activate shedding of the same RTK. For instance, Met shedding is reported to be induced by PMA (Crepaldi *et al*., 1994; Jeffers *et al*., 1997), suramin (Galvani *et al*., 1995), EGF and LPA (lysophosphatidic acid), HGF/SF (Wajih *et al*., 2002), or by an antibody directed against the extracellular domain of Met (DN30) (Petrelli *et al*., 2006). Although numerous stimuli are known to be involved in the modulation of shedding, the underlying mechanisms are poorly understood. However, it has been recently shown that mitogen-activated protein kinase (MAPK) pathways are involved in activating ADAM-mediated ectodomain shedding, as VEGF enhances ADAM17-mediated VEGFR2 shedding through the involvement of MAPK Erk and p38 and PKC, thereby also triggering shedding from other ADAM17 substrates (Swendeman *et al*., 2008).

This proteolysis occurs within the extracellular juxtamembrane domain of the receptor, releasing the extracellular ligand-binding domain into the medium and generating cell-bound fragments. ADAM-mediated ectodomain shedding from cell adhesion molecules has been implicated in cell–cell interactions and in cell migration (Tousseyn *et al*., 2006). For growth factors or cytokines, the main consequence of ectodomain shedding is to allow a membrane-anchored growth factor or cytokine (such as TNF-α or an EGFR ligand) to participate in paracrine signalling (Blobel, 2005).

Although the biological involvement of RTK ectodomain shedding is less understood, this cleavage may play several roles in the regulation of RTK signalling. First, ectodomain shedding is involved in the downmodulation of RTK, as PMA- or ligand-stimulation-induced shedding from several RTK, such as CSF-1R (Downing *et al*., 1989), c-Kit (Yee *et al*., 1994) and TrkA (Diaz-Rodriguez *et al*., 1999) leads to their downregulation through a decrease in cell surface receptor. This downregulation might have functional consequences on RTK signalling, as PMA-induced shedding from TrkA prevents its ligand-induced autophosphorylation (Cabrera *et al*., 1996; Diaz-Rodriguez *et al*., 1999).

In addition, the released extracellular domains might act as decoys associating with the ligand, which would then be unavailable for the full-length membrane-bound receptor (Figure 2). This potential decoy function is supported by the ability of some extracellular fragments

Table 1 Shedding and γ-secretase cleavages of RTK

	Shedding	*References*	*γ-Secretase cleavage*	*References*
CSFR	Shed by ADAM17 Enhanced by CSF, PMA, IL2-4 Downregulation of CSFR in response to PMA	(Rovida *et al*., 2001; Wilhelmsen and van der Geer, 2004)	Downregulation of CSFR Nuclear localization of CSFR-ICD	(Kasuga *et al*., 2007)
c-Met	Involvement of ADAM17 Enhanced by PMA, EGF, LPA, suramin, DN30 antibody, HGF MET-NTF potential decoy	(Prat *et al*., 1991; Galvani *et al*., 1995; Jeffers *et al*., 1997; Nath *et al*., 2001; Wajih *et al*., 2002; Athauda *et al*., 2006; Petrelli *et al*., 2006)	Ligand-independent down-regulation Nuclear localization of MET-ICD	(Foveau *et al*., 2009)
Tie1 receptor	Enhanced by PMA, VEGF, TNF-α, shear stress, inflammatory cytokines Modulates ligand responsiveness of associated receptor Tie2	(Yabkowitz *et al*., 1997, 1999; McCarthy *et al*., 1999; Chen-Konak *et al*., 2003; Marron *et al*., 2007)	Prevent accumulation of deleterious phosphorylated Tie1-ICD	(Marron *et al*., 2007)
IGF-IR	Induced by PKC	(McElroy *et al*., 2007)	Labile IGF-IR-ICD	(McElroy *et al*., 2007)
EphB2	Shed by ADAM10 in response to ligand Enhanced by EphrinB, calcium influx, *N*-methyl-D-aspartic acid	(Litterst *et al*., 2007)	Ligand-dependent downregulation	(Litterst *et al*., 2007)
ErbB4	Shed by ADAM17 Enhanced by neuregulins, PMA, pervanadate	(Vecchi *et al*., 1996, 1998; Vecchi and Carpenter, 1997; Rio *et al*., 2000)	ErbB4-ICD regulates transcriptional responses; induced apoptosis	(Linggi and Carpenter, 2006; Ni *et al*., 2001; Sardi *et al*., 2006; Williams *et al*., 2004)
Ryk			Nuclear localization of Ryk-ICD, enhanced by Wnt3 involved in neuronal differentiation	(Lyu *et al*., 2008)
TrkA	Enhanced by PMA, NGF TrkA-CTF active fragment, induces neurite outgrowth	(Cabrera *et al*., 1996; Diaz-Rodriguez *et al*., 1999)		
Non catalytic TrkCNC2	Enhanced by PMA, NT3	(Mateos *et al*., 2003)		
c-Kit	Shed by ADAM17 Enhanced by PMA Kit-CTF potential decoy	(Lev *et al*., 1992; Yee *et al*., 1993; Turner *et al*., 1995; Dahlen *et al*., 2001; Cruz *et al*., 2004)		
ErbB2	Shed by ADAM10 Enhanced by PMA, pervanadate, toremifene ErbB2-NTF correlates with malignancy Inhibited by TIMP1	(Warri *et al*., 1996; Codony-Servat *et al*., 1999; Molina *et al*., 2001; Nagy *et al*., 2005; Saez *et al*., 2006)		
DDR1	Induced by collagen	(Slack *et al*., 2006)		
Tie 2 receptor	Enhanced by VEGF, PMA	(Findley *et al*., 2007)		
VEGFR2	Shed by ADAM17 Enhanced by PMA, VEGF-A, calcium ionophore ionomycin	(Swendeman *et al*., 2008)		
EGFR	Enhanced by PMA, heat-inactivated fetal bovine serum, pervanadate and EGFR ligands (EGF and TGF-α)	(Perez-Torres *et al*., 2008) (Sanderson *et al*., 2008)		

Abbreviations: ADAM, A Disintegrins And Metalloproteases; EGFR, epidermal Growth factor receptor; ICD, intracellular domain; LPA, lysophosphatidic acid; NTF, N-terminal fragment; RTK, receptor tyrosine kinase.
Approximately 15 RTK are shed by metalloproteases. These cleavages involve mainly the ADAM17 and are induced by various stimuli. The extracellular NTF generated could act as a decoy fragment as evidenced for some of them. For about half of the shed RTK (upper part of the table), metalloprotease cleavage is followed by γ-secretase cleavage. This further cleavage is involved in the downregulation of RTK and in the generation of active fragment able to regulate biological functions, such as transcriptional response.

to bind their ligand efficiently, as evidenced for the extracellular fragments of c-Kit and Met (Turner *et al*., 1995; Wajih *et al*., 2002). Furthermore, conditioned medium containing shed Met fragments can inhibit intracellular signalling induced by ligand-activated full-length receptor (Petrelli *et al*., 2006; Wajih *et al*., 2002).

Alternatively, ADAM-mediated shedding from RTK can initiate signalling pathways through generation of the membrane-bound fragment. The membrane-anchored fragments generated by shedding from TrkA and ErbB2 show increased autokinase activity (Diaz-Rodriguez *et al*., 1999; Xia *et al*., 2004). In addition, the membrane-bound fragment of TrkA can associate with signalling proteins, such as p85 PI3-K and PLCγ, suggesting that the truncated fragment is signalling-competent (Diaz-Rodriguez *et al*., 1999). In breast cancer cells, it has been shown that heregulin stimulates phosphorylation of the membrane-anchored fragment ($p95^{ErbB2}$) generated by the metalloprotease-dependent cleavage of ErbB2, when heterodimerized with ErbB3; this results in the activation of downstream signalling mediators, such as Akt (Xia *et al*., 2004).

Downregulation of RTK by presenilin-dependent regulated intramembrane proteolysis

The role of shedding, notably in degradation, can be understood as part of a more complex process. Ectodomain shedding from several RTK by metalloprotease-dependent cleavage is also the checkpoint of another protease, the γ-secretase complex (Table 1). The γ-secretase complex cannot efficiently cleave full-length type-1 proteins, such as RTK, and initial ectodomain removal is a prerequisite to intramembrane cleavage of the remaining stub (Brown *et al*., 2000). Detailed genetic and biochemical studies have shown that this second cleavage involves the γ-secretase complex, including APH-1, Nicastrin, Pen-2 and presenilin 1–2 as catalytic cores (De Strooper, 2003; Edbauer *et al*., 2003; Fraering *et al*., 2004; Parks and Curtis, 2007). This proteolytic process in two sequential steps has been named Presenilin-dependent Regulated Intramembrane Proteolysis (PS-RIP). This mechanism plays a crucial role in Alzheimer's disease, as PS-RIP of amyloid precursor protein leads to the generation of the Aβ fragments found in the brains of patients (Wilquet and De Strooper, 2004). Three fragments are potentially generated by PS-RIP, namely an extracellular and soluble N-terminal fragment (NTF), an intermediate membrane-anchored C-terminal fragment (CTF) and an intracellular domain (ICD) generated by further cleavage by the γ-secretase complex.

A common feature of the ICD generated from RTK is their high instability. As evidenced, for instance, for CSF-R, IGF-IR, Tie1, EphB2 and Met the ICD is rapidly degraded through a proteasome-dependent mechanism (Wilhelmsen and van der Geer, 2004; Litterst *et al*., 2007; Marron *et al*., 2007; McElroy *et al*., 2007; Foveau *et al*., 2009) (Figure 2). Moreover,

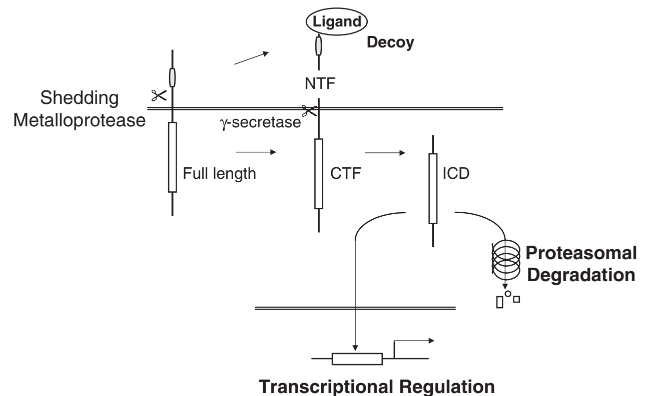


Figure 2 Presenilin-dependent regulated intramembrane proteolysis of receptor tyrosine kinase (RTK). Presenilin-dependent regulated intramembrane proteolysis (PS-RIP) of RTK is initiated by extracellular shedding within the juxtamembrane region by membrane metalloproteases. This cleavage leads to the generation of an extracellular N-terminal fragment (NTF) and a membrane-anchored C-terminal fragment (CTF). The extracellular NTF can potentially act as a decoy fragment through binding to the ligand. The CTF is cleaved in turn by the γ-secretase complex, leading to the generation of a cytoplasmic fragment (or ICD for intracellular domain). The ICD fragments are extremely labile through their proteasomal degradation, which contributes to the downregulation of RTK. In addition, some ICD show nuclear localization and regulate gene transcription.

PS-RIP shares common features with the ligand-dependent degradation described for RTK, including endocytosis and ubiquitination. For instance, processing of the EphB2 receptor is greatly stimulated by the ephrinB ligand, which stimulates ubiquitination and rapid endosomal degradation of the intracellular fragment (Litterst *et al*., 2007). Downregulation of RTK through PS-RIP also affects the associated signalling pathways. Indeed, as recently shown in the case of the RTK, Tie1, its proteolyses by PS-RIP leads to an amplified ligand activation of the associated receptor, Tie2 (Marron *et al*., 2007; McCarthy *et al*., 1999; Yabkowitz *et al*., 1999). The authors propose that release of the extracellular domain of Tie1 permits ligand access to Tie2, presumably by preventing spatial hindrance.

On the whole, PS-RIP can be considered an efficient mechanism for clearing out the membrane-anchored domains of many type-1 proteins, a function that has earned this complex the name of proteasome of the membrane (Kopan and Ilagan, 2004). Interestingly, the two steps of RTK PS-RIP, generating both a labile intracellular fragment and an extracellular soluble fragment, could participate together in downregulating receptor signalling, through both degradation of the intracellular tyrosine kinase domain and generation of an extracellular decoy inhibiting ligand activation of the remaining membrane-anchored receptor.

Both membrane receptors and transcriptional regulators

Although most PS-RIP-dependent fragments are extremely labile, some ICD are active fragments showing

physiological functions. For instance, the EGFR-family member, ErbB4, is cleaved sequentially by ADAM17 and γ-secretase complex in response to neuregulin. Unexpectedly, this proteolysis generates an ICD fragment (ErbB4-ICD) showing nuclear localization (Cheng *et al*., 2003; Ni *et al*., 2001). Furthermore, ErbB4-ICD is involved in regulating several biological responses triggered by ligand-activated ErbB4, according to the complex formed with different transcriptional regulators. In the lactating breast epithelium, ErbB4-ICD localizes to the nucleus with an intrinsic nuclear localization signal, promotes nuclear translocation of the transcription factor, STAT5A (signal transducer and activator of transcription 5A), and thereby activates the promoter of the β-casein-encoding gene (Williams *et al*., 2004; Vidal *et al*., 2005). In contrast, ErbB4-ICD acts in astrocytes as a transcriptional repressor, through the formation of a complex with the adaptor, TAB2, and the co-repressor, N-CoR, which translocates into the nucleus to repress promoters of astrocytic genes (Sardi *et al*., 2006). Importantly, the physiological involvement of this transcriptional repression has been evidenced during embryonic development; ErbB4 knockout mice show precocious astrogenesis, a phenotype rescued by the ectopic expression of PS-RIP-sensitive ErbB4 and ErbB4-ICD in electroporated embryos. PS-RIP of ErbB4 probably contributes to the regulation of additional physiological or pathological responses, as ErbB4-ICD interacts also with Mdm2, a regulator of p53, and ETO2, a transcriptional repressor (Arasada and Carpenter, 2005; Linggi and Carpenter, 2006).

Nuclear signalling mediated by a membrane receptor fragment was initially evidenced for Notch. In response to the Notch ligand, Jagged, the generated ICD of Notch translocates into the nucleus, in which it binds transcription factors. This results in the displacement of negative co-repressors and in the transcription of Notch-responsive genes (Bray, 2006). This mechanism is the canonical signalling pathway propagating Notch-dependent responses. In contrast, RTK were believed until recently to regulate transcription solely through the activation of protein kinase cascades that ultimately phosphorylate transcription factors, regulating their activity (Schlessinger, 2000). Thus, PS-RIP might represent an alternative or parallel signalling pathway, in which generated proteolytic fragments themselves transmit information and act directly on the key regulators of transcription (Schlessinger and Lemmon, 2006). Interestingly, the PS-RIP-dependent signalling could represent the major mechanism of signal transduction for a particular RTK. The Ryk RTK is a receptor of Wnt proteins required for the differentiation of neuronal progenitor cells during cortical development. However, unlike other RTK, Ryk lacks tyrosine kinase activity leaving unresolved its mechanism to transduce signal from the cell surface to the nucleus. It has been recently shown that Ryk is processed by the PS-RIP leading to the generation of a Ryk-ICD fragment that is able to translocate into the nucleus in response to the Wnt3 stimulation. The generation of Ryk-ICD is required for Wnt3-dependent differentiation of the primary culture of neuronal progenitor cells, showing that PS-RIP of Ryk represents an efficient mechanism of signalling required for neuronal determination (Lyu *et al*., 2008). Other PS-RIP-dependent fragments of RTK have been observed in the nucleus, suggesting that the proteolytic cleavage could give these receptors the gift of ubiquity (Kasuga *et al*., 2007; Wilhelmsen and van der Geer, 2004).

Proteolytic cleavages of RTK as gatekeepers of cell transformation

Deregulation of RTK signalling is one of the major molecular defects associated with tumourigenesis. In most cases, aberrant overexpression of an RTK and/or its ligand leads to an autocrine or paracrine activation loop. Although RTK overexpression has been attributed mainly to gene amplification or to increased transcriptional induction, aberrant RTK activation can also result from defective downregulation. For instance, deficient ligand-dependent downregulation of EGFR, Met, PDGFR, and CSFR (involving ubiquitination and lysosomal degradation) is associated with cell transformation (Bache *et al*., 2004).

As both caspase cleavages and PS-RIP downregulate RTK expression and RTK-mediated biological responses, one might speculate that these processes could prevent tumourigenesis promoted by overexpressed RTK. In normal epithelial cells, accordingly, the PS-RIP-uncleavable Met receptor shows an extended half-life and membrane accumulation. Consequently, this receptor triggers ligand-independent biological responses, including cell migration (Foveau *et al*., 2009).

Proteolytic cleavages can also prevent accumulation of deleterious fragments. Indeed, degradation of type I receptor by PS-RIP involves initial ectodomain shedding. However, for numerous RTK, truncation of the extracellular domain leads to the generation of active proteins often showing transforming properties. For IGFR, ErbB4 and Tie1, ectodomain shedding leads to the generation of constitutively phosphorylated membrane-tethered fragments, but their accumulation is prevented by γ-secretase cleavage and subsequent degradation of the fragment (Linggi *et al*., 2006; Marron *et al*., 2007; McElroy *et al*., 2007). Thus, γ-secretase cleavage might be considered as a protective cleavage preventing the generation of transforming fragments.

In contrast, ectodomain shedding from ErbB2 is not followed by γ-secretase cleavage. Consequently, in cancer cells overexpressing ErbB2, ectodomain shedding is associated with the accumulation of membrane-anchored ErbB2-CTF fragment (or $p95^{ErbB2}$) (Christianson *et al*., 1998). This fragment appears to be constitutively phosphorylated (Segatto *et al*., 1988; Molina *et al*., 2001) and is associated with worse outcome among patients overexpressing ErbB2 (Saez *et al*., 2006). This suggests that ErbB2-CTF is a deleterious fragment. Furthermore, expression of the ErbB2-CTF fragment is associated with resistance to Herceptin treatment, Herceptin being a humanized

antagonist antibody directed against ErbB2 and used to treat ErbB2-overexpressing metastatic breast cancers. ErbB2-CTF is indeed potentially insensitive to this antibody targeting the extracellular region of the receptor (Scaltriti *et al*., 2007). In consequence, ADAM inhibition is envisaged as a novel therapeutic approach to optimize the activity of other treatments, such as Herceptin or tyrosine kinase inhibitor in overexpressed ErbB2 cancers (Liu *et al*., 2006; Witters *et al*., 2008).

In addition to degrading RTK, proteolytic cleavages generate active fragments that might play a role in tumourigenesis. For instance, a γ-secretase fragment of ErbB4 (ErbB4-ICD), which can translocate to the nucleus to regulate gene expression, can also promote apoptosis. This conclusion rests on the observation that ErbB4-ICD appears in the cytoplasm, in which it co-localizes with mitochondria. ErbB4-ICD can interact with Bcl-2 to promote Bak-dependent permeabilization of the mitochondria and apoptosis. Interestingly, ErbB4-ICD induces efficient apoptosis in various transformed cell lines, and in human breast tumours, an association between tumour cell apoptosis and cytoplasmic localization of ErbB4-ICD has been observed (Naresh *et al*., 2006; Vidal *et al*., 2007). These observations suggest that PS-RIP of ErbB4 could counteract cell transformation through the generation of a proapoptotic fragment.

Deregulation of the survival/apoptosis balance in favour of survival is often associated with cell transformation. The deregulated activity of RET, TrkC, Met and ALK, four RTK classified as dependence receptors, is associated with various tumours sharing a common feature, namely constitutive tyrosine kinase activation induced by aberrant ligand expression, receptor overexpression or fusion of the kinase domain to dimerization domains (Runeberg-Roos and Saarma, 2007; Chiarle *et al*., 2008; Migliore and Giordano, 2008). It has been established that the constitutive activation of these RTK contributes to cell transformation through the activation of signalling pathways that positively regulate survival, growth or motility. Yet one might speculate that ligand stimulation, which impairs the caspase-dependent cleavage of these receptors and thus, reduces their proapoptotic properties, might also contribute to cell transformation. According to this model, cell transformation favoured by cell survival would result from both activation of RTK-triggered positive signalling and inhibition of RTK proapoptotic properties.

From proteolysis to therapy

Receptor tyrosine kinases and their downstream signalling pathways are frequent targets of efforts to develop anti-cancer strategies (Gschwind *et al*., 2004). An example is the development of many antagonist antibodies, like Herceptin, directed against the RTK extracellular domain. Another attractive strategy might be to reinforce RTK proteolysis, for instance, so as to induce the degradation of an overexpressed receptor in transformed cells. In this line, an anti-Met antibody directed against the extracellular domain has been shown to downregulate expression of the receptor in Met-overexpressing cells and to hamper Met biological activity, including tumour growth in mice (Petrelli *et al*., 2006). Interestingly, we have recently shown that this antibody reinforces Met PS-RIP, leading to Met downregulation (Foveau *et al*., 2009). Thus, a forced induction of PS-RIP of the RTK receptor by antagonist antibodies could be an attractive therapeutic strategy for preventing its uncontrolled activation.

Shedding from RTK generates extracellular fragments acting as decoy receptors owing to their efficient binding to the ligand (Turner *et al*., 1995; Wajih *et al*., 2002). Although the physiological relevance of the RTK decoy function is largely unknown, decoy approaches have been developed as therapeutic strategies. An artificial soluble extracellular domain of Met or c-Kit can efficiently inhibit relevant ligand-induced cell responses (Dahlen *et al*., 2001; Kong-Beltran *et al*., 2004), and a decoy Met receptor has been shown to prevent tumour growth in a mouse model (Michieli *et al*., 2004).

The number of RTK known to be cleaved by caspases or PS-RIP has recently increased, suggesting that this type of regulation could be widespread within the family. Future challenges will be to understand relationships between canonical signalling cascades and these alternative signalling mechanisms, and to unravel the physiological relevance of fragments or truncated receptors in the multiple biological responses induced by RTK.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the CNRS, the Institut Pasteur de Lille, and INSERM, and by grants from the 'Ligue contre le Cancer, comité Nord', the 'Association pour la Recherche sur le Cancer', the 'Fondation Recherche Médicale, Comité Nord-Pas de Calais' and 'Agence Nationale de la Recherche, Young Investigator Program'. BF was supported by a 'Institut Pasteur/Région Nord-Pas de Calais' fellowship and FA and JL by grants from the French Research and Technology Minister.

References

Allouche M. (2007). ALK is a novel dependence receptor: potential implications in development and cancer. *Cell Cycle* **6**: 1533–1538.

Amit I, Wides R, Yarden Y. (2007). Evolvable signaling networks of receptor tyrosine kinases: relevance of robustness to malignancy and to cancer therapy. *Mol Syst Biol* **3**: 151.

Arasada RR, Carpenter G. (2005). Secretase-dependent tyrosine phosphorylation of Mdm2 by the ErbB-4 intracellular domain fragment. *J Biol Chem* **280**: 30783–30787.

Athauda G, Giubellino A, Coleman JA, Horak C, Steeg PS, Lee MJ *et al*. (2006). c-Met ectodomain shedding rate correlates with malignant potential. *Clin Cancer Res* **12**: 4154–4162.

Bache KG, Slagsvold T, Stenmark H. (2004). Defective downregulation of receptor tyrosine kinases in cancer. *EMBO J* **23**: 2707–2712.

Bae SS, Choi JH, Oh YS, Perry DK, Ryu SH, Suh PG. (2001). Proteolytic cleavage of epidermal growth factor receptor by caspases. *FEBS Lett* **491**: 16–20.

Benoit V, Chariot A, Delacroix L, Deregowski V, Jacobs N, Merville MP *et al*. (2004). Caspase-8-dependent HER-2 cleavage in response to tumor necrosis factor alpha stimulation is counteracted by nuclear factor kappaB through c-FLIP-L expression. *Cancer Res* **64**: 2684–2691.

Blobel CP. (2005). ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol* **6**: 32–43.

Bordeaux MC, Forcet C, Granger L, Corset V, Bidaud C, Billaud M *et al*. (2000). The RET proto-oncogene induces apoptosis: a novel mechanism for Hirschsprung disease. *EMBO J* **19**: 4056–4063.

Bray SJ. (2006). Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* **7**: 678–689.

Bredesen DE, Mehlen P, Rabizadeh S. (2005). Receptors that mediate cellular dependence. *Cell Death Differ* **12**: 1031–1043.

Brown MS, Ye J, Rawson RB, Goldstein JL. (2000). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* **100**: 391–398.

Cabrera N, Diaz-Rodriguez E, Becker E, Martin-Zanca D, Pandiella A. (1996). TrkA receptor ectodomain cleavage generates a tyrosine-phosphorylated cell-associated fragment. *J Cell Biol* **132**: 427–436.

Canibano C, Rodriguez NL, Saez C, Tovar S, Garcia-Lavandeira M, Borrello MG *et al*. (2007). The dependence receptor Ret induces apoptosis in somatotrophs through a Pit-1/p53 pathway, preventing tumor growth. *EMBO J* **26**: 2015–2028.

Chen-Konak L, Guetta-Shubin Y, Yahav H, Shay-Salit A, Zilberman M, Binah O *et al*. (2003). Transcriptional and post-translation regulation of the Tie1 receptor by fluid shear stress changes in vascular endothelial cells. *FASEB J* **17**: 2121–2123.

Cheng QC, Tikhomirov O, Zhou W, Carpenter G. (2003). Ectodomain cleavage of ErbB-4: characterization of the cleavage site and m80 fragment. *J Biol Chem* **278**: 38421–38427.

Chiarle R, Voena C, Ambrogio C, Piva R, Inghirami G. (2008). The anaplastic lymphoma kinase in the pathogenesis of cancer. *Nat Rev Cancer* **8**: 11–23.

Christianson TA, Doherty JK, Lin YJ, Ramsey EE, Holmes R, Keenan EJ *et al*. (1998). NH2-terminally truncated HER-2/neu protein: relationship with shedding of the extracellular domain and with prognostic factors in breast cancer. *Cancer Res* **58**: 5123–5129.

Codony-Servat J, Albanell J, Lopez-Talavera JC, Arribas J, Baselga J. (1999). Cleavage of the HER2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases-1 in breast cancer cells. *Cancer Res* **59**: 1196–1201.

Crepaldi T, Prat M, Giordano S, Medico E, Comoglio PM. (1994). Generation of a truncated hepatocyte growth factor receptor in the endoplasmic reticulum. *J Biol Chem* **269**: 1750–1755.

Cruz AC, Frank BT, Edwards ST, Dazin PF, Peschon JJ, Fang KC. (2004). Tumor necrosis factor-alpha-converting enzyme controls surface expression of c-Kit and survival of embryonic stem cell-derived mast cells. *J Biol Chem* **279**: 5612–5620.

Dahlen DD, Lin NL, Liu YC, Broudy VC. (2001). Soluble Kit receptor blocks stem cell factor bioactivity in vitro. *Leuk Res* **25**: 413–421.

De Strooper B. (2003). Aph-1, Pen-2, and Nicastrin with Presenilin generate an active gamma-secretase complex. *Neuron* **38**: 9–12.

Diaz-Rodriguez E, Cabrera N, Esparis-Ogando A, Montero JC, Pandiella A. (1999). Cleavage of the TrkA neurotrophin receptor by multiple metalloproteases generates signalling-competent truncated forms. *Eur J Neurosci* **11**: 1421–1430.

Downing JR, Roussel MF, Sherr CJ. (1989). Ligand and protein kinase C downmodulate the colony-stimulating factor 1 receptor by independent mechanisms. *Mol Cell Biol* **9**: 2890–2896.

Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, Haass C. (2003). Reconstitution of gamma-secretase activity. *Nat Cell Biol* **5**: 486–488.

Findley CM, Cudmore MJ, Ahmed A, Kontos CD. (2007). VEGF induces Tie2 shedding via a phosphoinositide 3-kinase/Akt dependent pathway to modulate Tie2 signaling. *Arterioscler Thromb Vasc Biol* **27**: 2619–2626.

Foveau B, Ancot F, Leroy C, Petrelli A, Reiss K, Vingtdeux V *et al*. (2009). Downregulation of the Met receptor tyrosine kinase by presenilin-dependent regulated intramembrane proteolysis. *Mol Biol Cell* **20**: 2494–2506.

Foveau B, Leroy C, Ancot F, Deheuninck J, Ji Z, Fafeur V *et al*. (2007). Amplification of apoptosis through sequential caspase cleavage of the MET tyrosine kinase receptor. *Cell Death Differ* **14**: 752–764.

Fraering PC, Ye W, Strub JM, Dolios G, LaVoie MJ, Ostaszewski BL *et al*. (2004). Purification and characterization of the human gamma-secretase complex. *Biochemistry* **43**: 9774–9789.

Furne C, Ricard J, Cabrera JR, Pays L, Bethea JR, Mehlen P *et al*. (2009). EphrinB3 is an anti-apoptotic ligand that inhibits the dependence receptor functions of EphA4 receptors during adult neurogenesis. *Biochim Biophys Acta* **1793**: 231–238.

Galvani AP, Cristiani C, Carpinelli P, Landonio A, Bertolero F. (1995). Suramin modulates cellular levels of hepatocyte growth factor receptor by inducing shedding of a soluble form. *Biochem Pharmacol* **50**: 959–966.

Gschwind A, Fischer OM, Ullrich A. (2004). The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev Cancer* **4**: 361–370.

He YY, Huang JL, Chignell CF. (2006). Cleavage of epidermal growth factor receptor by caspase during apoptosis is independent of its internalization. *Oncogene* **25**: 1521–1531.

He YY, Huang JL, Gentry JB, Chignell CF. (2003). Epidermal growth factor receptor down-regulation induced by UVA in human keratinocytes does not require the receptor kinase activity. *J Biol Chem* **278**: 42457–42465.

Hubbard SR, Till JH. (2000). Protein tyrosine kinase structure and function. *Annu Rev Biochem* **69**: 373–398.

Huovila AP, Turner AJ, Pelto-Huikko M, Karkkainen I, Ortiz RM. (2005). Shedding light on ADAM metalloproteinases. *Trends Biochem Sci* **30**: 413–422.

Jeffers M, Taylor GA, Weidner KM, Omura S, Vande-Woude GF. (1997). Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. *Mol Cell Biol* **17**: 799–808.

Kasuga K, Kaneko H, Nishizawa M, Onodera O, Ikeuchi T. (2007). Generation of intracellular domain of insulin receptor tyrosine kinase by gamma-secretase. *Biochem Biophys Res Commun* **360**: 90–96.

Kolibaba KS, Druker BJ. (1997). Protein tyrosine kinases and cancer. *Biochim Biophys Acta* **1333**: F217–F248.

Kong-Beltran M, Stamos J, Wickramasinghe D. (2004). The Sema domain of Met is necessary for receptor dimerization and activation. *Cancer Cell* **6**: 75–84.

Kopan R, Ilagan MX. (2004). Gamma-secretase: proteasome of the membrane? *Nat Rev Mol Cell Biol* **5**: 499–504.

Kopitz C, Gerg M, Bandapalli OR, Ister D, Pennington CJ, Hauser S *et al*. (2007). Tissue inhibitor of metalloproteinases-1 promotes liver metastasis by induction of hepatocyte growth factor signaling. *Cancer Res* **67**: 8615–8623.

Lev S, Yarden Y, Givol D. (1992). A recombinant ectodomain of the receptor for the stem cell factor (SCF) retains ligand-induced receptor dimerization and antagonizes SCF-stimulated cellular responses. *J Biol Chem* **267**: 10866–10873.

Linggi B, Carpenter G. (2006). ErbB-4 s80 intracellular domain abrogates ETO2-dependent transcriptional repression. *J Biol Chem* **281**: 25373–25380.

Linggi B, Cheng QC, Rao AR, Carpenter G. (2006). The ErbB-4 s80 intracellular domain is a constitutively active tyrosine kinase. *Oncogene* **25**: 160–163.
Litterst C, Georgakopoulos A, Shioi J, Ghersi E, Wisniewski T, Wang R *et al*. (2007). Ligand binding and calcium influx induce distinct ectodomain/gamma-secretase-processing pathways of EphB2 receptor. *J Biol Chem* **282**: 16155–16163.
Liu X, Fridman JS, Wang Q, Caulder E, Yang G, Covington M *et al*. (2006). Selective inhibition of ADAM metalloproteases blocks HER-2 extracellular domain (ECD) cleavage and potentiates the anti-tumor effects of trastuzumab. *Cancer Biol Ther* **5**: 648–656.
Luthi AU, Martin SJ. (2007). The CASBAH: a searchable database of caspase substrates. *Cell Death Differ* **14**: 641–650.
Lyu J, Yamamoto V, Lu W. (2008). Cleavage of the Wnt receptor Ryk regulates neuronal differentiation during cortical neurogenesis. *Dev Cell* **15**: 773–780.
Maina F, Klein R. (1999). Hepatocyte growth factor, a versatile signal for developing neurons. *Nat Neurosci* **2**: 213–217.
Marron MB, Singh H, Tahir TA, Kavumkal J, Kim HZ, Koh GY *et al*. (2007). Regulated proteolytic processing of Tie1 modulates ligand responsiveness of the receptor-tyrosine kinase Tie2. *J Biol Chem* **282**: 30509–30517.
Mateos S, Calothy G, Lamballe F. (2003). The noncatalytic TrkCNC2 receptor is cleaved by metalloproteases upon neurotrophin-3 stimulation. *Oncogene* **22**: 740–745.
McCarthy MJ, Burrows R, Bell SC, Christie G, Bell PR, Brindle NP. (1999). Potential roles of metalloprotease mediated ectodomain cleavage in signaling by the endothelial receptor tyrosine kinase Tie-1. *Lab Invest* **79**: 889–895.
McElroy B, Powell JC, McCarthy JV. (2007). The insulin-like growth factor 1 (IGF-1) receptor is a substrate for gamma-secretase-mediated intramembrane proteolysis. *Biochem Biophys Res Commun* **358**: 1136–1141.
Michieli P, Mazzone M, Basilico C, Cavassa S, Sottile A, Naldini L *et al*. (2004). Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. *Cancer Cell* **6**: 61–73.
Migliore C, Giordano S. (2008). Molecular cancer therapy: can our expectation be MET? *Eur J Cancer* **44**: 641–651.
Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J. (2001). Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res* **61**: 4744–4749.
Mourali J, Benard A, Lourenco FC, Monnet C, Greenland C, Moog-Lutz C *et al*. (2006). Anaplastic lymphoma kinase is a dependence receptor whose proapoptotic functions are activated by caspase cleavage. *Mol Cell Biol* **26**: 6209–6222.
Nagy P, Friedlander E, Tanner M, Kapanen AI, Carraway KL, Isola J *et al*. (2005). Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. *Cancer Res* **65**: 473–482.
Naresh A, Long W, Vidal GA, Wimley WC, Marrero L, Sartor CI *et al*. (2006). The ERBB4/HER4 intracellular domain 4ICD is a BH3-only protein promoting apoptosis of breast cancer cells. *Cancer Res* **66**: 6412–6420.
Nath D, Williamson NJ, Jarvis R, Murphy G. (2001). Shedding of c-Met is regulated by crosstalk between a G-protein coupled receptor and the EGF receptor and is mediated by a TIMP-3 sensitive metalloproteinase. *J Cell Sci* **114**: 1213–1220.
Ni CY, Murphy MP, Golde TE, Carpenter G. (2001). Gamma-secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science* **294**: 2179–2181.
Parks AL, Curtis D. (2007). Presenilin diversifies its portfolio. *Trends Genet* **23**: 140–150.
Perez-Torres M, Valle BL, Maihle NJ, Negron-Vega L, Nieves-Alicea R, Cora EM. (2008). Shedding of epidermal growth factor receptor is a regulated process that occurs with overexpression in malignant cells. *Exp Cell Res* **314**: 2907–2918.
Petrelli A, Circosta P, Granziero L, Mazzone M, Pisacane A, Fenoglio S *et al*. (2006). Ab-induced ectodomain shedding mediates hepatocyte growth factor receptor down-regulation and hampers biological activity. *Proc Natl Acad Sci USA* **103**: 5090–5095.
Prat M, Crepaldi T, Gandino L, Giordano S, Longati P, Comoglio P. (1991). C-terminal truncated forms of Met, the hepatocyte growth factor receptor. *Mol Cell Biol* **11**: 5954–5962.
Rio C, Buxbaum JD, Peschon JJ, Corfas G. (2000). Tumor necrosis factor-alpha-converting enzyme is required for cleavage of erbB4/HER4. *J Biol Chem* **275**: 10379–10387.
Rovida E, Paccagnini A, Del Rosso M, Peschon J, Dello Sbarba P. (2001). TNF-alpha-converting enzyme cleaves the macrophage colony-stimulating factor receptor in macrophages undergoing activation. *J Immunol* **166**: 1583–1589.
Runeberg-Roos P, Saarma M. (2007). Neurotrophic factor receptor RET: structure, cell biology, and inherited diseases. *Ann Med* **39**: 572–580.
Saez R, Molina MA, Ramsey EE, Rojo F, Keenan EJ, Albanell J *et al*. (2006). p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer. *Clin Cancer Res* **12**: 424–431.
Sanderson MP, Keller S, Alonso A, Riedle S, Dempsey PJ, Altevogt P. (2008). Generation of novel, secreted epidermal growth factor receptor (EGFR/ErbB1) isoforms via metalloprotease-dependent ectodomain shedding and exosome secretion. *J Cell Biochem* **103**: 1783–1797.
Sardi SP, Murtie J, Koirala S, Patten BA, Corfas G. (2006). Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. *Cell* **127**: 185–197.
Scaltriti M, Rojo F, Ocana A, Anido J, Guzman M, Cortes J *et al*. (2007). Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. *J Natl Cancer Inst* **99**: 628–638.
Schlessinger J. (2000). Cell signaling by receptor tyrosine kinases. *Cell* **103**: 211–225.
Schlessinger J, Lemmon MA. (2006). Nuclear signaling by receptor tyrosine kinases: the first robin of spring. *Cell* **127**: 45–48.
Segatto O, King CR, Pierce JH, Di Fiore PP, Aaronson SA. (1988). Different structural alterations upregulate in vitro tyrosine kinase activity and transforming potency of the erbB-2 gene. *Mol Cell Biol* **8**: 5570–5574.
Slack BE, Siniaia MS, Blusztajn JK. (2006). Collagen type I selectively activates ectodomain shedding of the discoidin domain receptor 1: involvement of Src tyrosine kinase. *J Cell Biochem* **98**: 672–684.
Strohecker AM, Yehiely F, Chen F, Cryns VL. (2008). Caspase cleavage of HER-2 releases a Bad-like cell death effector. *J Biol Chem* **283**: 18269–18282.
Swendeman S, Mendelson K, Weskamp G, Horiuchi K, Deutsch U, Scherle P *et al*. (2008). VEGF-A stimulates ADAM17-dependent shedding of VEGFR2 and crosstalk between VEGFR2 and ERK signaling. *Circ Res* **103**: 916–918.
Tauszig-Delamasure S, Yu LY, Cabrera JR, Bouzas-Rodriguez J, Mermet-Bouvier C, Guix C *et al*. (2007). The TrkC receptor induces apoptosis when the dependence receptor notion meets the neurotrophin paradigm. *Proc Natl Acad Sci USA* **104**: 13361–13366.
Tikhomirov O, Dikov M, Carpenter G. (2005). Identification of proteolytic fragments from ErbB-2 that induce apoptosis. *Oncogene* **24**: 3906–3913.
Tousseyn T, Jorissen E, Reiss K, Hartmann D. (2006). (Make) stick and cut loose–disintegrin metalloproteases in development and disease. *Birth Defects Res C Embryo Today* **78**: 24–46.
Tulasne D, Deheuninck J, Lourenco FC, Lamballe F, Ji Z, Leroy C *et al*. (2004). Proapoptotic function of the MET tyrosine kinase receptor through caspase cleavage. *Mol Cell Biol* **24**: 10328–10339.
Turner AM, Bennett LG, Lin NL, Wypych J, Bartley TD, Hunt RW *et al*. (1995). Identification and characterization of a soluble c-kit receptor produced by human hematopoietic cell lines. *Blood* **85**: 2052–2058.
Vecchi M, Baulida J, Carpenter G. (1996). Selective cleavage of the heregulin receptor ErbB-4 by protein kinase C activation. *J Biol Chem* **271**: 18989–18995.
Vecchi M, Carpenter G. (1997). Constitutive proteolysis of the ErbB-4 receptor tyrosine kinase by a unique, sequential mechanism. *J Cell Biol* **139**: 995–1003.

Vecchi M, Rudolph-Owen LA, Brown CL, Dempsey PJ, Carpenter G. (1998). Tyrosine phosphorylation and proteolysis. Pervanadate-induced, metalloprotease-dependent cleavage of the ErbB-4 receptor and amphiregulin. *J Biol Chem* **273**: 20589–20595.

Vidal GA, Clark DE, Marrero L, Jones FE. (2007). A constitutively active ERBB4/HER4 allele with enhanced transcriptional coactivation and cell-killing activities. *Oncogene* **26**: 462–466.

Vidal GA, Naresh A, Marrero L, Jones FE. (2005). Presenilin-dependent gamma-secretase processing regulates multiple ERBB4/HER4 activities. *J Biol Chem* **280**: 19777–19783.

Wajih N, Walter J, Sane DC. (2002). Vascular origin of a soluble truncated form of the hepatocyte growth factor receptor (c-met). *Circ Res* **90**: 46–52.

Warri AM, Isola JJ, Harkonen PL. (1996). Anti-oestrogen stimulation of ERBB2 ectodomain shedding from BT-474 human breast cancer cells with ERBB2 gene amplification. *Eur J Cancer* **32A**: 134–140.

Wilhelmsen K, van der Geer P. (2004). Phorbol 12-myristate 13-acetate-induced release of the colony-stimulating factor 1 receptor cytoplasmic domain into the cytosol involves two separate cleavage events. *Mol Cell Biol* **24**: 454–464.

Williams CC, Allison JG, Vidal GA, Burow ME, Beckman BS, Marrero L *et al*. (2004). The ERBB4/HER4 receptor tyrosine kinase regulates gene expression by functioning as a STAT5A nuclear chaperone. *J Cell Biol* **167**: 469–478.

Wilquet V, De Strooper B. (2004). Amyloid-beta precursor protein processing in neurodegeneration. *Curr Opin Neurobiol* **14**: 582–588.

Witters L, Scherle P, Friedman S, Fridman J, Caulder E, Newton R *et al*. (2008). Synergistic inhibition with a dual epidermal growth factor receptor/HER-2/neu tyrosine kinase inhibitor and a disintegrin and metalloprotease inhibitor. *Cancer Res* **68**: 7083–7089.

Xia W, Liu LH, Ho P, Spector NL. (2004). Truncated ErbB2 receptor (p95ErbB2) is regulated by heregulin through heterodimer formation with ErbB3 yet remains sensitive to the dual EGFR/ErbB2 kinase inhibitor GW572016. *Oncogene* **23**: 646–653.

Yabkowitz R, Meyer S, Black T, Elliott G, Merewether LA, Yamane HK. (1999). Inflammatory cytokines and vascular endothelial growth factor stimulate the release of soluble tie receptor from human endothelial cells via metalloprotease activation. *Blood* **93**: 1969–1979.

Yabkowitz R, Meyer S, Yanagihara D, Brankow D, Staley T, Elliott G *et al*. (1997). Regulation of tie receptor expression on human endothelial cells by protein kinase C-mediated release of soluble tie. *Blood* **90**: 706–715.

Yee NS, Hsiau CW, Serve H, Vosseller K, Besmer P. (1994). Mechanism of down-regulation of c-kit receptor. Roles of receptor tyrosine kinase, phosphatidylinositol 3′-kinase, and protein kinase C. *J Biol Chem* **269**: 31991–31998.

Yee NS, Langen H, Besmer P. (1993). Mechanism of kit ligand, phorbol ester, and calcium-induced down-regulation of c-kit receptors in mast cells. *J Biol Chem* **268**: 14189–14201.

Zhuang S, Ouedraogo GD, Kochevar IE. (2003). Downregulation of epidermal growth factor receptor signaling by singlet oxygen through activation of caspase-3 and protein phosphatases. *Oncogene* **22**: 4413–4424.

Published OnlineFirst October 1, 2010; DOI:10.1158/1541-7786.MCR-10-0302

Molecular Cancer Research

G_{D3} Synthase Expression Enhances Proliferation and Tumor Growth of MDA-MB-231 Breast Cancer Cells through c-Met Activation

Aurélie Cazet, Jonathan Lefebvre, Eric Adriaenssens, et al.

Mol Cancer Res 2010;8:1526-1535. Published OnlineFirst October 1, 2010.

Updated Version	Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-10-0302

Cited Articles	This article cites 50 articles, 21 of which you can access for free at: http://mcr.aacrjournals.org/content/8/11/1526.full.html#ref-list-1

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Downloaded from mcr.aacrjournals.org on October 21, 2011
Copyright © 2010 American Association for Cancer Research

Published OnlineFirst October 1, 2010; DOI:10.1158/1541-7786.MCR-10-0302

Molecular Cancer Research

G_{D3} Synthase Expression Enhances Proliferation and Tumor Growth of MDA-MB-231 Breast Cancer Cells through c-Met Activation

Aurélie Cazet[1,2,3], Jonathan Lefebvre[1,4], Eric Adriaenssens[1,4], Sylvain Julien[6], Marie Bobowski[1,2,3], Anita Grigoriadis[7], Andrew Tutt[7], David Tulasne[1,4], Xuefen Le Bourhis[1,5], and Philippe Delannoy[1,2,3]

Abstract

The disialoganglioside G_{D3} is overexpressed in ~50% of invasive ductal breast carcinoma, and the G_{D3} synthase gene (*ST8SIA1*) displays higher expression among estrogen receptor–negative breast cancer tumors, associated with a decreased overall survival of breast cancer patients. However, no relationship between ganglioside expression and breast cancer development and aggressiveness has been reported. We have previously shown that overexpression of G_{D3} synthase induces the accumulation of b- and c-series gangliosides (G_{D3}, G_{D2}, and G_{T3}) at the cell surface of MDA-MB-231 breast cancer cells together with the acquisition of a proliferative phenotype in the absence of serum. Here, we show that phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase pathways are constitutively activated in G_{D3} synthase–expressing cells. Analysis of phosphorylation of tyrosine kinase receptors shows a specific c-Met constitutive activation in G_{D3} synthase–expressing cells, in the absence of its ligand, hepatocyte growth factor/scatter factor. In addition, inhibition of c-Met or downstream signaling pathways reverses the proliferative phenotype. We also show that G_{D3} synthase expression enhances tumor growth in severe combined immunodeficient mice. Finally, a higher expression of *ST8SIA1* and *MET* in the basal subtype of human breast tumors are observed. Altogether, our results show that G_{D3} synthase expression is sufficient to enhance the tumorigenicity of MDA-MB-231 breast cancer cells through a ganglioside-dependent activation of the c-Met receptor. *Mol Cancer Res; 8(11); 1526–35. ©2010 AACR.*

Introduction

Gangliosides, the glycosphingolipids carrying one or more sialic acid residues, are essentially located at the outer leaflet of the plasma membrane in microdomains named "glycosynapse," where tyrosine kinase receptors (RTK) are also located (1). They are often found in growing and developing tissues and complex gangliosides from b- and c-series, initiated by G_{D3} or G_{T3}, respectively; are mainly expressed during embryogenesis; and are restricted to the central nervous system in adults (2). In mammalian cells, the expression of di- and trisialogangliosides increases in pathologic conditions such as cancer. G_{D3}, G_{D2}, and G_{T3} are considered as oncofetal markers in animal and human tumors such as melanoma and neuroblastoma. They play a key role in invasion and metastasis by mediating cell proliferation, migration, adhesion, and angiogenesis (3-5). The restrictive expression in pathologic conditions and the accessibility of gangliosides on the cell surface make them good targets for cancer immunotherapy, as it was well studied in melanoma (6).

Little is known about ganglioside expression in breast cancer. Total ganglioside levels seem to be significantly higher in breast tumors than in normal breast tissues (7). The gangliosides G_{D3}, 9-*O*-acetyl-G_{D3}, and 9-*O*-acetyl-G_{T3} are absent or expressed at a very low levels in normal breast tissues, but are overexpressed in ~50% of invasive ductal breast carcinoma (7). Recently, two clinical studies have shown that the G_{D3} synthase (GD3S) gene (*ST8SIA1*), the key enzyme for the biosynthesis of b- and c-series gangliosides, displayed higher expression among estrogen receptor (ER)–negative breast cancer tumors (8) and had a prognostic impact in breast cancer dependent on ER status: *ST8SIA1* gene expression was associated with poor pathohistologic grading in ER-negative tumors and a reduced

Authors' Affiliations: [1]Univ. Lille Nord de France; [2]Université des Sciences et Technologies de Lille, Unité de Glycobiologie Structurale et Fonctionnelle; [3]Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8576; [4]Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8161, Institut de Biologie de Lille-Institut Pasteur de Lille, Lille, France; [5]Institut National de la Santé et de la Recherche Médicale U908, Villeneuve d'Ascq, France; and [6]Breast Cancer Biology Group and [7]Breakthrough Breast Cancer Research Unit, Guy's Hospital, King's Health Partners Academic Health Sciences Centre, London, United Kingdom

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Corresponding Author: Philippe Delannoy, Structural and Functional Glycobiology Unit, UMR CNRS 8576, University of Sciences and Technologies of Lille, Villeneuve d'Ascq 59655, France. Phone: 33-320-43-69-23; Fax: 33-320-43-65-55. E-mail: philippe.delannoy@univ-lille1.fr

doi: 10.1158/1541-7786.MCR-10-0302

©2010 American Association for Cancer Research.

American Association for Cancer Research

Downloaded from mcr.aacrjournals.org on October 21, 2011
Copyright © 2010 American Association for Cancer Research

Published OnlineFirst October 1, 2010; DOI:10.1158/1541-7786.MCR-10-0302

overall survival of the patients (9). In contrast, a better prognosis for ER-positive samples with high expression of *ST8SIA1* was noticed (9). However, no functional relationship between ganglioside expression and breast cancer development and aggressiveness has been reported. We have recently developed a cellular model derived from MDA-MB-231 breast cancer cells expressing the human GD3S. GD3S-expressing cells accumulate gangliosides of b- and c-series (i.e., G_{D3}, G_{D2}, and G_{T3}) at the cell surface. The change in cell membrane ganglioside composition is associated with morphologic changes, and increased migration and proliferation in the absence of serum or exogenous growth factors. Here, we show that proliferative capacity of MDA-MB-231 GD3S$^+$ clones in serum-free medium is directly correlated to the constitutive activation of c-Met receptor and downstream signaling pathways phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK). Inhibition of either c-Met activity or downstream signaling abrogates the proliferation of GD3S-expressing cells. We also show that GD3S expression induces an increased tumor growth in severe combined immunodeficient (SCID) mice.

Materials and Methods

Antibodies and reagents

Antiphosphorylated extracellular signal-regulated kinase ERK (E-4), anti-ERK (K-23), anti-Akt (H-136), and rabbit polyclonal IgG anti-actin antibodies were purchased from Santa Cruz Biotechnology. Anti-phosphorylated Akt S473, mouse monoclonal antibody directed against the COOH-terminal region of human Met, and rabbit polyclonal antibody against phosphorylated tyrosine 1234 and 1235 of the Met kinase domain were purchased from Cell Signaling Technology. Anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase were purchased from GE Healthcare. Alexa Fluor 488 donkey anti-mouse IgG (H+L) was purchased from Molecular Probes Invitrogen.

U0126, Akt inhibitor VIII, SU11274, and PHA665752 were purchased from Calbiochem Merck Chemicals Ltd. and dissolved in dimethyl sulfoxide (DMSO). Human recombinant hepatocyte growth factor/scatter factor (HGF/SF) was purchased from Peprotech.

Cell culture

Cell culture reagents were purchased from Lonza. The breast cancer cell line MDA-MB-231 was obtained from the American Type Cell Culture Collection. These cells were routinely grown in monolayer cultures and maintained at 37°C in an atmosphere of 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 100 units/mL penicillin-streptomycin. MDA-MB-231 control (empty vector transfected) and MDA-MB-231 GD3S$^+$ clones 4 and 11 were obtained as previously described (10). The transfected cells were cultured in the presence of 1 mg/mL G418 (Invitrogen).

RNA interference assays

Cells were transfected with 20 pmol siRNA (Invitrogen) targeting Met [*Met7* (stealth) 5′-CCAUUUCAACUGAGUUUGCUGUUAA-3′; *Met8* (stealth) 5′-UCCAGAAGAUCAGUUUCCUAAUUCA-3′] or a scramble sequence using 5 μL Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Cells were transfected with the corresponding siRNAs and were collected for cell growth assay or Western blot analysis 24 hours later.

Proliferation assays

Cell growth was analyzed using the MTS reagent (Promega) according to the manufacturer's directions. For pharmacologic inhibition, cells (2×10^3) were seeded in 96-well plates (Thermo Fisher Scientific) and grown in DMEM containing 0.1% FBS. After 12 hours, the medium was replaced with serum-free DMEM containing specific inhibitors: 1 μmol/L U0126, 1 μmol/L Akt inhibitor VIII, 500 nmol/L SU11274, or 100 nmol/L PHA665752. Control cells were treated with DMSO at a 1:1,000 dilution. The medium was changed every 2 days. For inhibition with neutralizing antibody against c-Met, cells were cultured during 5 days in DMEM serum-free medium containing 10 μg/mL of 5D5 Fab or control IgG.

For RNA interference assay, 2×10^3 cells were seeded in 96-well plates in DMEM serum-free medium 1 day after the transfection with siRNA sequences, then cultured at 2 days before the assessment of cell growth.

Migration assays

Cells (5×10^4) were seeded and cultured in the upper surface of Transwell 12-well plates (BD Bioscience) for 6 hours, as described previously (10). Specific inhibition was done with 1 μmol/L SU11274 or 200 nmol/L PHA665752. Cells treated with DMSO (1:1,000 dilution) were used as controls.

Phospho-RTK array analysis

Cells were cultured for 48 hours in six-well plates in serum-free DMEM or in DMEM containing 10% FBS. Cells were then lysed in NP40 lysis buffer [1% NP40, 20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail tablet (Roche)]. The human Phospho-RTK array kit (R&D Systems) was used according to the manufacturer's protocol.

Immunoblotting

Cell pellets were treated with lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail tablet]. The supernatants were assessed for protein concentration using the Bio-Rad DC protein assay kit II.

Downloaded from mcr.aacrjournals.org on October 21, 2011
Copyright © 2010 American Association for Cancer Research

Published OnlineFirst October 1, 2010; DOI:10.1158/1541-7786.MCR-10-0302

For the studies of the MEK/ERK and PI3K/Akt pathways, 20 μg of proteins from cell lysate were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were saturated in TBS–0.05% Tween 20 containing either 5% (w/v) nonfat dry milk or 5% bovine serum albumin (Sigma-Aldrich). Membranes were then incubated overnight at 4°C with the primary antibody, and then incubated at room temperature for 1 hour with a horseradish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibody. Analysis was done by chemiluminescence using the ECL-Plus Western blotting detection reagent (GE Healthcare) with Kodak film.

For the phospho-Met and Met detection, 100 μg of total proteins were subjected to SDS-PAGE. The membranes were then incubated with blocking buffer [0.2% (v/v) casein, 0.1% (v/v) Tween 20 dissolved in PBS] for 1 hour and probed for 1 hour at room temperature with appropriate antibodies diluted in blocking buffer according to the manufacturer's recommendations. After washing in PBS–Tween 0.2%, immune complexes were

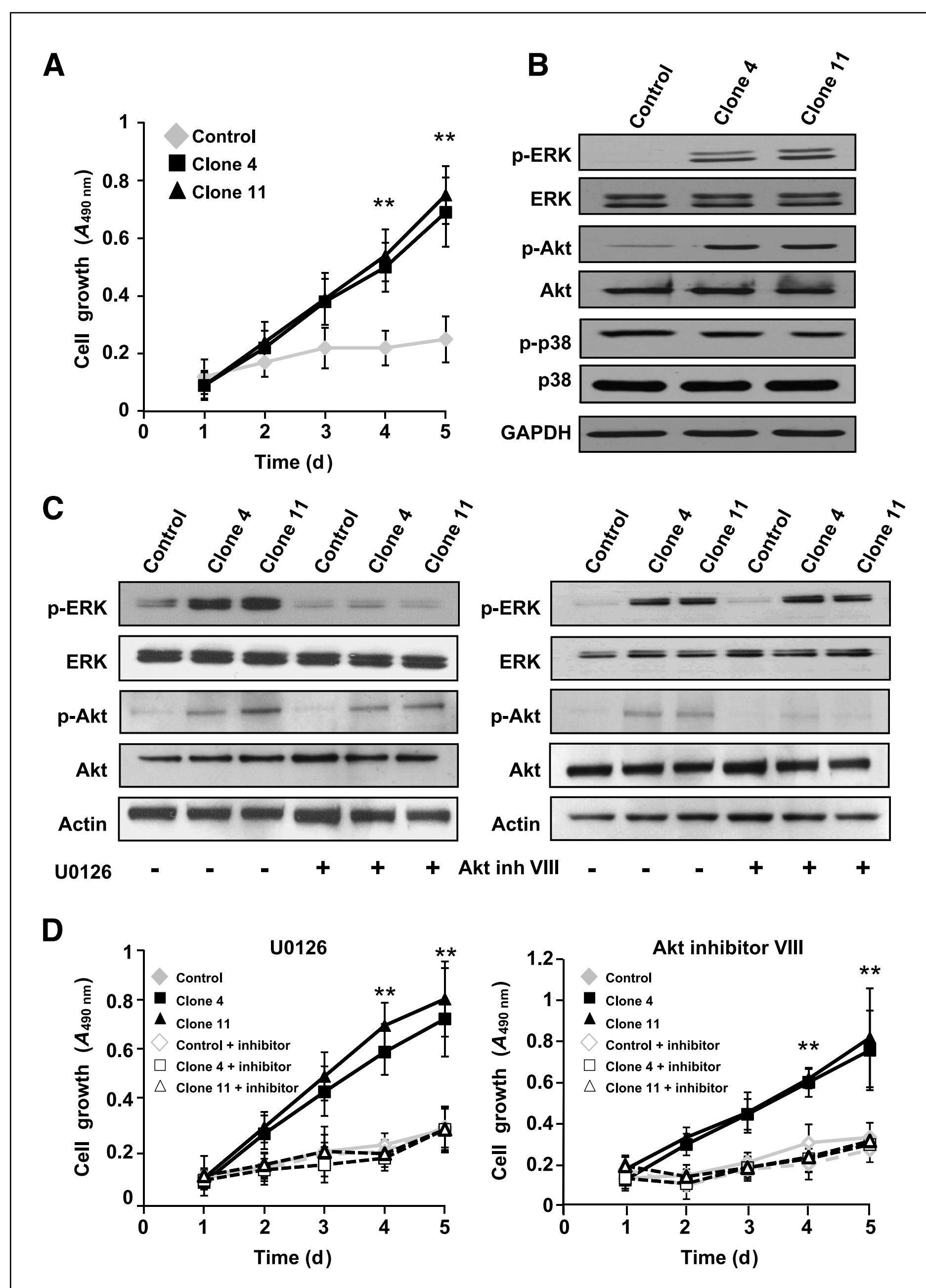


FIGURE 1. Involvement of MEK/ERK and PI3K/Akt pathways in the proliferation of GD3S$^+$ clones. A, cell proliferation assay on standard monolayer plates. GD3S$^+$ clones 4 and 11 and control cells were cultured in serum-free conditions for 5 d. Cell growth was determined by the MTS assay. Each measurement was done in 16 wells, and data are the means of four independent manipulations. **, $P < 0.01$, GD3S$^+$ cells versus control cells. B, Western blot analysis of ERK, Akt, and p38, and their phosphorylated forms in control and GD3S$^+$ MDA-MB-231 clones after 48 h of culture in serum-free conditions. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control for protein loading. C, cells were treated in serum-free medium for 48 h with 1 μmol/L U0126 or 1 μmol/L Akt inhibitor VIII. Lysates were subjected to immunoblotting to determine the decrease in pERK and pAkt proteins. D. Proliferation assay using MTS. GD3S$^+$clones 4 (■) and 11 (▲) and control cells (♦) were cultured in serum-free medium with the indicated concentration of DMSO as control. In parallel, clones 4 (□) and 11 (△) and control cells (◇) were cultured in the presence of U0126 or Akt inhibitor VIII for 5 d. **, $P < 0.01$, inhibitor-treated versus untreated GD3S$^+$ cells.

Downloaded from mcr.aacrjournals.org on October 21, 2011
Copyright © 2010 American Association for Cancer Research

Published OnlineFirst October 1, 2010; DOI:10.1158/1541-7786.MCR-10-0302

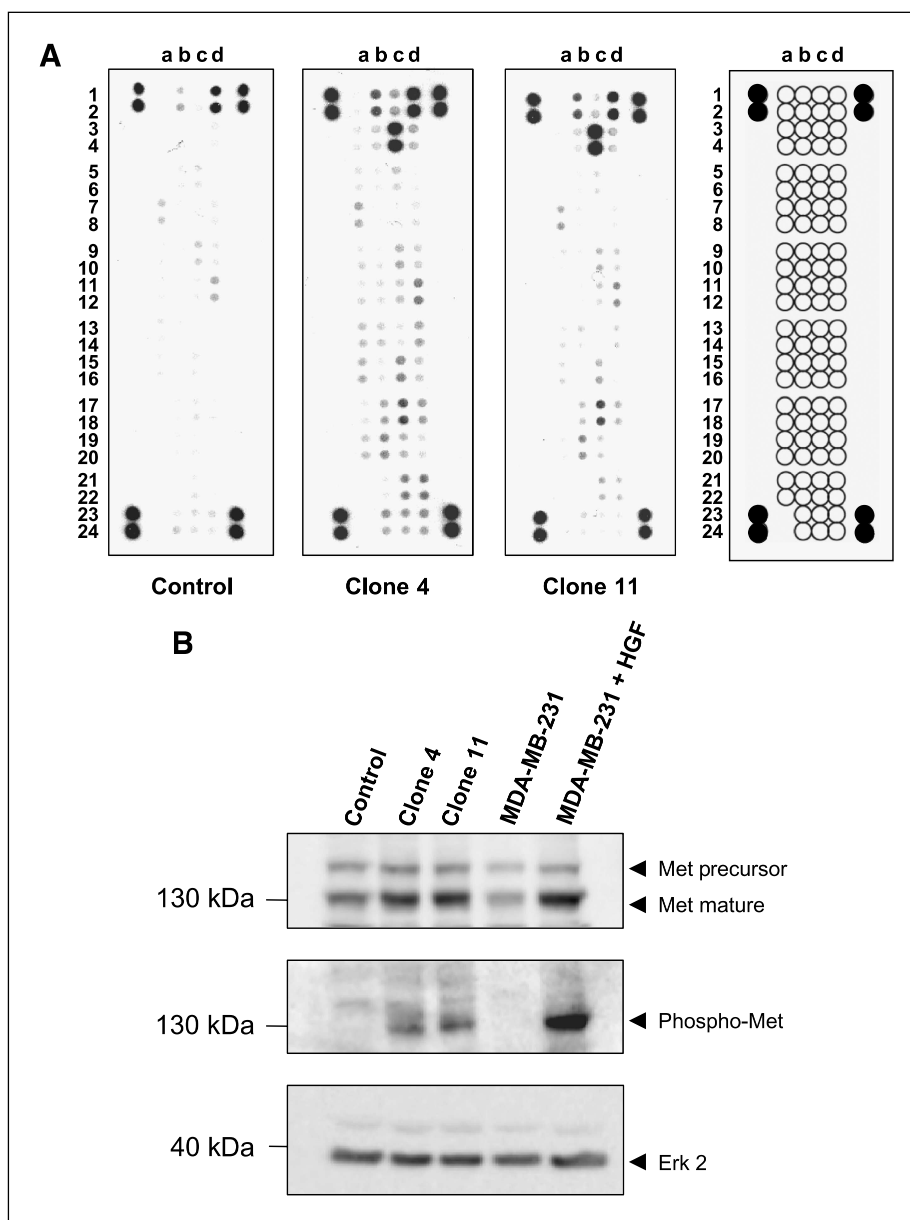


FIGURE 2. Activation of c-Met in GD3S⁺ clones in serum-free conditions. A, total cell lysates from control, clone 4, and clone 11, cultured for 48 h under serum-free conditions, were subjected to phospho-RTK array. The phospho-RTK array coordinates are given on the right side of the figure. Black dots represent phospho-tyrosine positive controls: a1, a2: EphA6; a3, a4: EphA7; a5, a6: EphB1; a7, a8: EphB2; a9, a10: EphB4; a11, a12: EphB6; a13, a14: mouse IgG1 negative control; a15, a16: mouse IgG2A negative control; a17, a18: mouse IgG2B negative control; a19, a20: goat IgG negative control; a21, a22: PBS negative control; b1, b2: Tie-2; b3, b4: TrkA; b5, b6: TrkB; b7, b8: TrkC; b9, b10: VEGFR1; b11, b12: VEGFR2; b13, b14: VEGFR3; b15, b16: MuSK; b17, b18: EphA1; b19, b20: EphA2; b21, b22: EphA3; b23, b24: EphA4; c1, c2: Mer; c3, c4: c-Met; c5, c6: MSPR; c7, c8: PDGFRα; c9, c10: PDGFRβ; c11, c12: SCFR; c13, c14: Flt-3; c15, c16: M-CSFR; c17, c18: c-Ret; c19, c20: ROR1; c21, c22: ROR2; c23, c24: Tie-1; d1, d2: EGFR; d3, d4: ErbB2; d5, d6: ErbB3; d7, d8: ErbB4; d9, d10: FGFR1; d11, d12: FGFR2α; d13, d14: FGFR3; d15, d16: FGFR4; d17, d18: insulin R; d19, d20: IGF-IR; d21, d22: Axl; d23, d24: Dtk. B, Western blot analysis using specific c-Met and phospho-c-Met antibodies. Control, GD3S⁺ MDA-MB-231, and wild-type MDA-MB-231 cultured in serum-free conditions were treated or not for 15 min with 30 ng/mL HGF/SF. Cell lysates were analyzed by Western blotting with antibody directed against human Met and the phosphorylated tyrosine residues of the kinase domain. Reprobing with anti-ERK2 antibody was done to assess loading.

detected with specific secondary antiserum conjugated with alkaline phosphatase followed by an enhanced chemiluminescence detection system (Amersham ECL Western Blotting Detection Reagents).

Tumor growth in SCID mice

Female SCID mice were purchased from the Pasteur Institute and acclimatized for at least 2 weeks. Mice were maintained under a 12 hours light/dark cycle at

Downloaded from mcr.aacrjournals.org on October 21, 2011
Copyright © 2010 American Association for Cancer Research

Published OnlineFirst October 1, 2010; DOI:10.1158/1541-7786.MCR-10-0302

a temperature of 20°C to 22°C. Food and water were available *ad libitum*. Mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. The local ethics committee approved the protocol used. MDA-MB-231 control and GD3S+ cells were harvested and resuspended in phosphate-buffered solution before s.c. injection into the flanks of mice (10 mice per group) as previously described (11).

Statistical analysis

Microarray data from two datasets previously published by van de Vijver et al. (ref. 12; Agilent platform) and Chin et al. (ref. 13; Affymetrix platform) were analyzed using Prism 5 (GraphPad Software). The probes considered for the analysis were UGID Hs.408614 (Agilent) and A.210073_at (Affymetrix) for *ST8SIA1* and A.203510_at (Affymetrix) for *MET*. The levels of expression of these genes were compared from one group to another by using the one-way ANOVA test. Correlation of gene expression was tested using Pearson's test. Statistical analysis of other results was done using Student's t test. $P < 0.05$ was considered statistically significant.

Results

G_{D3} synthase expression promotes MDA-MB-231 proliferation through the activation of MEK/ERK and PI3K/Akt pathways

To determine the functional importance of the GD3S expression in breast cancer development and aggressiveness, we previously established a cellular model derived from MDA-MB-231 breast cancer cells expressing GD3S. The selected clones (named clones 4 and 11)

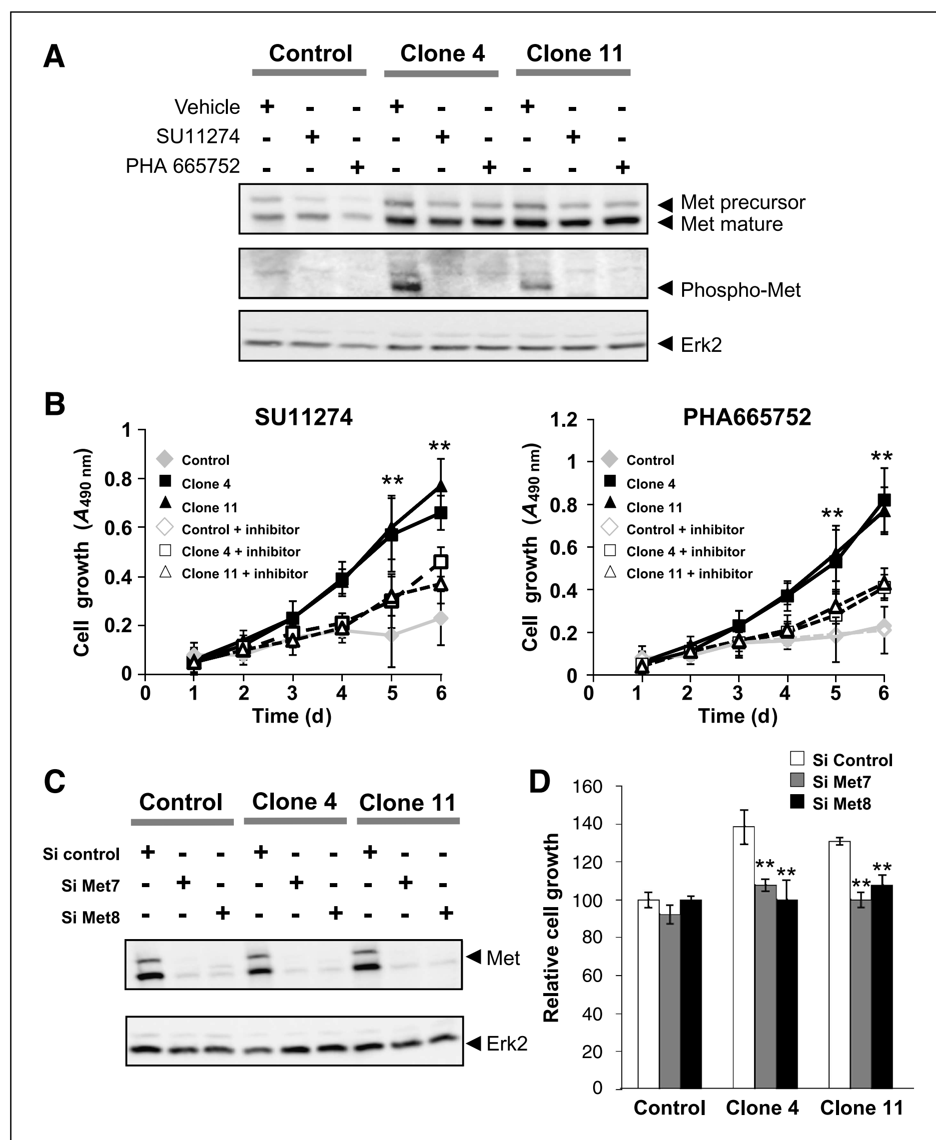


FIGURE 3. Reduction of the proliferation of the GD3S+ clones on c-Met inhibition. A, inhibition of c-Met phosphorylation by pharmacologic inhibitors of c-Met. Control cells and clones 4 and 11 were cultured in the presence of 500 nmol/L SU11274 or 100 nmol/L of PHA 665752 for 48 h before lysis and Western blot analysis. Expression of Erk2 was used as control for protein loading. B, cell proliferation assays. Clones 4 (■) and 11 (▲) and control cells (♦) were cultured for 5 d in serum-free medium containing 500 nmol/L SU11274, 100 nmol/L PHA 665752, or the indicated dilution of DMSO as the negative control (□, clone 4; Δ, clone 11; ◊, MDA-MB-231 control). **, $P < 0.01$, inhibitor-treated versus untreated GD3S+ cells. C, immunoblotting analysis of c-Met after siRNA transfection. Total cell lysates were extracted after one round of transfection with siRNA control or with siRNA targeting c-Met (named *Met7* and *Met8*). D, proliferation assay after c-Met siRNA transfection. One day after the transfection, cells were seeded in 96-well plates and cell growth was then analyzed after 48 h as described in Materials and Methods. Absorbance values were normalized to the control cells transfected with a scramble sequence, which was arbitrarily set as 100%. **, $P < 0.01$, GD3S+ cells versus control cells.

Downloaded from mcr.aacrjournals.org on October 21, 2011
Copyright © 2010 American Association for Cancer Research

Published OnlineFirst October 1, 2010; DOI:10.1158/1541-7786.MCR-10-0302

express b- and c- series gangliosides such as G_{D3}, G_{D2}, and G_{T3} at the cell surface (10). As shown in Fig. 1A, the modification of the ganglioside pattern of MDA-MB-231 cells was correlated with an increase of cell proliferation capacity in serum-free conditions. After 5 days of culture, the relative number of GD3S$^+$ cells is ~4-fold of control cells. The activation of the main signaling pathways involved in MDA-MB-231 proliferation (i.e., MEK/ERK, PI3K/Akt, and p38/mitogen-activated protein kinase pathways; ref. 14) was then determined by Western blot analysis. As shown in Fig. 1B, after 48 hours of culture in the absence of serum, higher levels of phospho-Akt (pAkt) and phospho-ERK (pERK) were observed in GD3S$^+$ cells compared with control.

In contrast, no difference in phospho-p38 was observed between clones and control cells. The involvement of MEK/ERK and PI3K/Akt pathways in cell proliferation was then determined using selective inhibitors against MEK (1 μmol/L U0126) and Akt (1 μmol/L Akt inhibitor VIII). The concentration used was based on the absence of toxicity in MDA-MB-231 cells, as determined by cell proliferation assay in serum-free medium for 48 hours (data not shown). Efficient inhibition of MEK or Akt (Fig. 1C) did not modify proliferation of control cells but strongly reduced that of GD3S$^+$ cells, with the relative number of GD3S$^+$ cells being similar to that of control ones (Fig. 1D). Taken together, these results indicate an essential function of MEK/ERK and PI3K/Akt pathways in the proliferative phenotype of GD3S$^+$ MDA-MB-231 cells.

c-Met receptor is constitutively activated in G_{D3} synthase–expressing cells

Several studies have shown that cellular ganglioside composition can modulate the activity of growth factor receptors, and consequently induce the activation of intracellular molecular pathways (15-17). In this context, the phosphorylation status of 42 RTKs was simultaneously examined using a phospho-RTK array in MDA-MB-231 cells. As shown in Fig. 2A, GD3S$^+$ MDA-MB-231 cells displayed a strong phosphorylation of c-Met receptor compared with control cells cultured in serum-free or complete medium (Supplementary Fig. S1). A weak increase of epidermal growth factor receptor (EGFR), Tie-2, and Ret phosphorylation was also observed. To confirm c-Met activation, we performed Western blot analysis using anti–phospho-c-Met antibodies directed against phosphorylated tyrosine residues 1234 and 1235 of c-Met. As shown in Fig. 2B, phospho-c-Met was detected only in GD3S$^+$ cells but not in control cells. As expected, HGF/SF stimulation induced efficient phosphorylation of c-Met in control MDA-MB-231 cells. In addition, confocal microscopy analysis of c-Met showed no significant difference in c-Met distribution between control and MDA-MB-231 GD3S$^+$ cells (Supplementary Fig. S2). Treatment of GD3S$^+$ MDA-MB-231 cells with 5D5 Fab, which inhibits ligand-dependent activation of c-Met through inhibition of HGF/SF-Met association (refs. 18, 19; Supplementary Fig. S3A), does not affect cell proliferation, excluding autocrine activation of c-Met by HGF/SF (Supplementary Fig. S3B). A slight increase of c-Met expression was also observed in both clones, in comparison with control cells. However, quantitative PCR analysis did not reveal modification of *MET* transcriptional expression in GD3S$^+$ MDA-MB-231 clones (Supplementary Fig. S4).

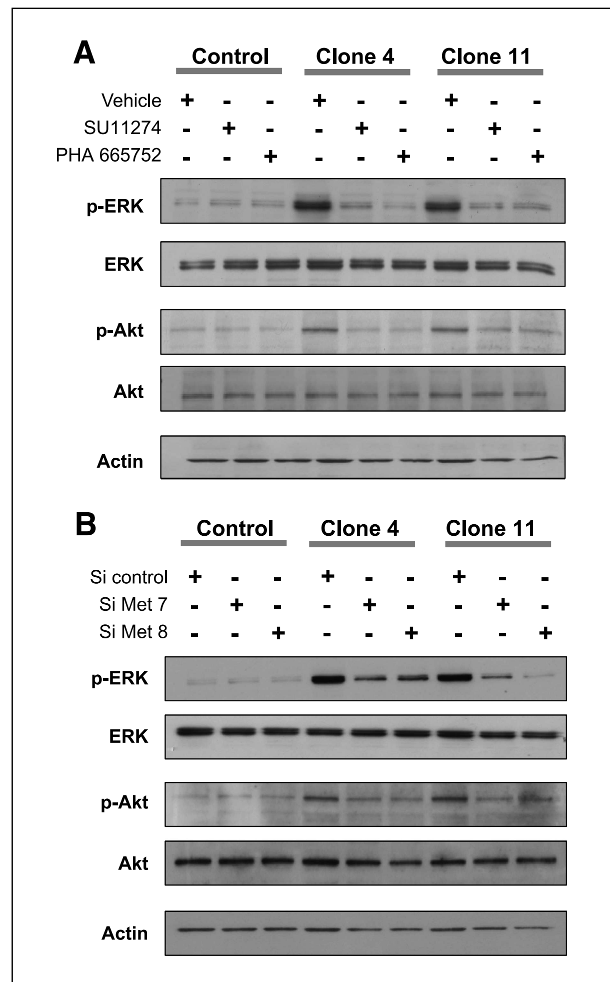


FIGURE 4. Western blot analysis of p-ERK and p-Akt on c-Met inhibition. A, Western blot analysis of pERK and pAkt in control cells and GD3S$^+$ clones after treatment with c-Met inhibitors: 500 nmol/L SU11274 or 100 nmol/L PHA 665752. B, Western blot analysis of MEK/ERK and PI3K/Akt pathways in control cells and GD3S$^+$ clones after treatment with 20 pmol of specific siRNA.

c-Met activation is involved in the increased proliferation of GD3S$^+$ MDA-MB-231 cells

To determine the potential involvement of c-Met in the increased proliferation of GD3S$^+$ MDA-MB-231 cells, we first inhibited c-Met activation using two selective ATP-competitive inhibitors of the c-Met kinase, SU11274 and PHA665752 (20, 21). Both inhibitors abrogated constitutive c-Met phosphorylation (Fig. 3A) and significantly

Downloaded from mcr.aacrjournals.org on October 21, 2011
Copyright © 2010 American Association for Cancer Research

Published OnlineFirst October 1, 2010; DOI:10.1158/1541-7786.MCR-10-0302

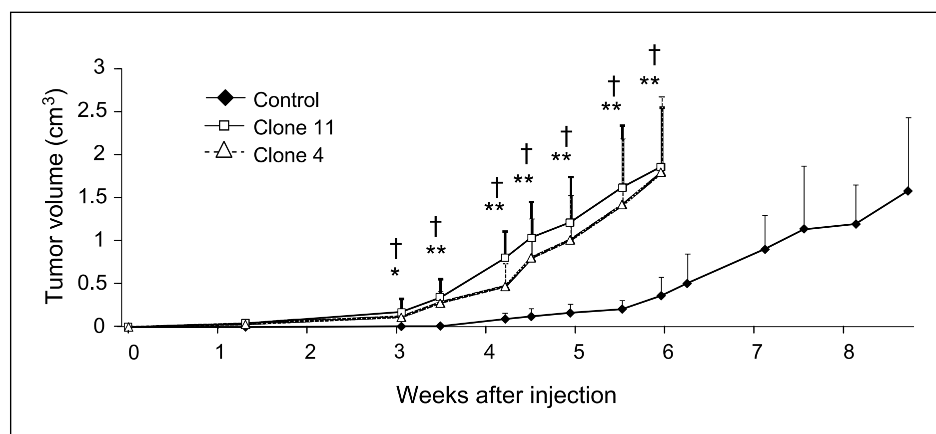


FIGURE 5. Xenograft tumor growth in SCID mice. Growth curve of tumors. Control and $GD3S^+$ MDA-MB-231 cells were s.c. injected into SCID mice. Ten animals were used in each group. Tumor volume was monitored every 2 or 3 d until tumor volume approached 2 cm^3. Student's *t* test was done between control and clone 11 (*, $P < 0.05$; **, $P < 0.01$), and between control and clone 4 (†, $P < 0.05$).

decreased the proliferation of both $GD3S^+$ clones (Fig. 3B). In addition, both inhibitors of the c-Met kinase activity reduced the migration capacity of both clones to the level of control cells (Supplementary Fig. S5). We then targeted c-Met with specific siRNA, which efficiently silenced c-Met expression in control and $GD3S^+$ MDA-MB-231 cells (Fig. 3C). Inhibition of c-Met expression prevented increased proliferation of clones 4 and 11, thus demonstrating the involvement of c-Met receptor in the high proliferative property of $GD3S^+$ cells in deprivation conditions. Moreover, both pharmacologic agents and c-Met siRNA also strongly decreased the phosphorylation of ERK and Akt in $GD3S^+$ clones (Fig. 4A and B). Altogether, these results show that the enhanced proliferation of $GD3S^+$ MDA-MB-231 cells is essentially due to c-Met activation, which in turn activates the downstream MEK/ERK and PI3K/Akt signaling pathways.

G_{D3} synthase overexpression increases tumor growth in SCID mice

MDA-MB-231 cells were s.c. injected into SCID mice. All animals formed a tumor at the injection site. However, tumor growth of $GD3S^+$ cells were significantly increased because tumors were palpable 1 week after injection and attained a size of 1.75 cm^3 in ~6 weeks. In contrast, empty vector–transfected cells formed palpable tumors with a latency of 3 weeks and formed tumors of similar size in ~8 weeks (Fig. 5).

Statistical analyses of microarray data

ST8SIA1 expression in breast cancer primary tumors was investigated by exploring two microarray datasets previously published by van de Vijver et al. (12) and Chin et al. (13). The first dataset consisted of 295 stage I or II breast cancers (12), and the second consisted of 118 stage I-III (mostly I-II) tumors (13). As previously reported (9), we found a significant association of *ST8SIA1* mRNA expression with ER negativity in both datasets (data not shown). To refine this result, we used the established subtype classification based on the 534 genes signature defined by Sorlie et al. (22). For both datasets, each tumor has been categorized as one of the five defined subtypes: basal, erbB2, normal-like, luminal A, and luminal B. This classification has been shown to reflect the clinical outcome of the patients with both basal and erbB2 subtypes being associated with poorer prognosis (22). We have analyzed the level of expression of *ST8SIA1* in the different subtypes and found it significantly higher in the basal subtype than in any other group in both datasets (Fig. 6A and B). Interestingly, higher expression of *MET* was also observed in the basal subgroup (Fig. 6B). We also found that the expression levels of *ST8SIA1* and *MET* were significantly correlated ($P < 0.001$ Pearson's correlation test) in the Chin et al. dataset. Taken together, these results suggest that *ST8SIA1* is overexpressed in poor-prognosis breast cancers (basal type), possibly in conjunction with *MET*.

Discussion

In the present study, we showed that the expression of GD3S and complex gangliosides in MDA-MB-231 cells leads to a proliferative phenotype in the absence of serum or growth factors. This phenotype is induced by the constitutive activation of c-Met and subsequent signal transduction MEK/ERK and PI3K/Akt pathways. GD3S expression not only promotes cell growth *in vitro* but also stimulates primary tumor growth in SCID mice and is associated with *MET* gene expression in the basal subtype of human breast tumors.

We previously shown that GD3S expression renders MDA-MB-231 cells independent of serum (10). The ability to survive and proliferate under serum-free conditions is one of the well-known features of aggressive cancer cells *in vitro* that can be mediated by a constitutive phosphorylation of RTKs, the key regulators of critical cellular processes such as cell growth and survival. Dysregulated activation of RTKs is implicated in the genesis and progression of a variety of cancers, including breast cancer (23). By using phospho-RTK array, we analyzed the activation pattern of various RTKs in $GD3S^+$ clones. We observed a strong and specific constitutive activation of

Downloaded from mcr.aacrjournals.org on October 21, 2011
Copyright © 2010 American Association for Cancer Research

Published OnlineFirst October 1, 2010; DOI:10.1158/1541-7786.MCR-10-0302

the c-Met receptor despite the absence of ligand-dependent stimulation. Moreover, the downregulation of c-Met induced a significant reversion of the phenotype, demonstrating the crucial function of c-Met in the proliferative capacity of GD3S⁺ clones. It has been well shown that during oncogenesis, abnormal c-Met activation results in a complex program called "invasive growth," consisting of the activation of a series of cellular processes such as proliferation, scattering, invasion, and survival (24). Thus, c-Met activation through HGF/SF stimulation induced MDA-MB-231 growth, motility, and invasiveness (25). c-Met is also known to induce survival of bladder carcinoma cells submitted to serum starvation (26); however, to our knowledge, nothing is described concerning the implication of c-Met in breast cancer cell proliferation in serum-free conditions.

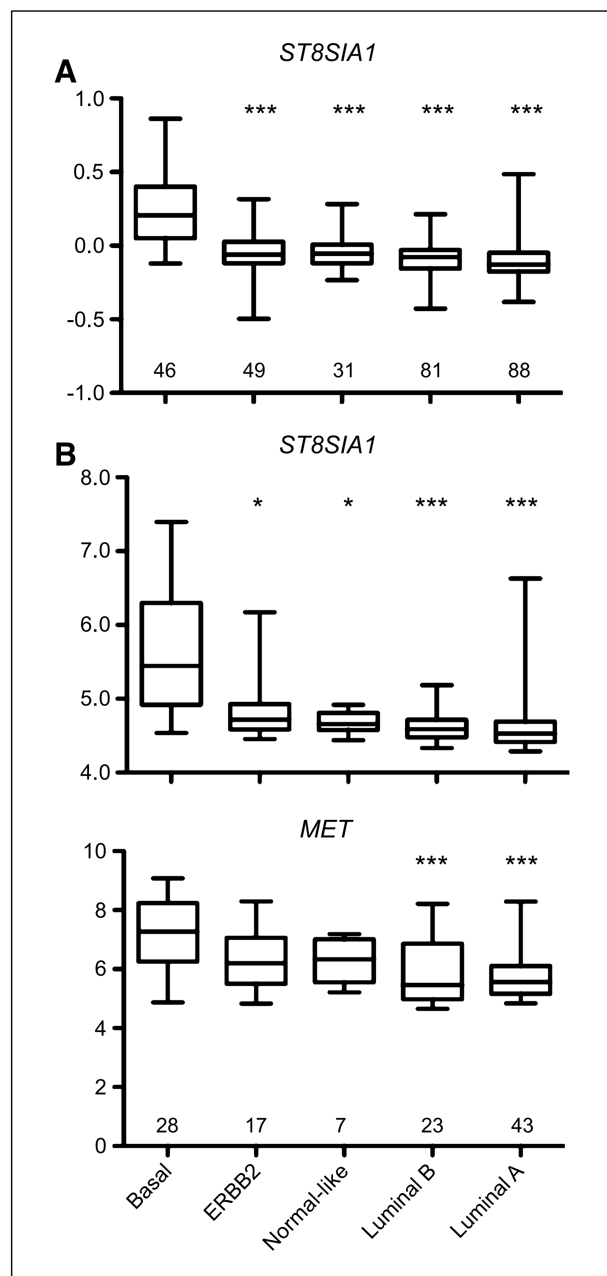


FIGURE 6. *ST8SIA1* and *MET* expression in breast cancer subtypes. A, *ST8SIA1* RNA expression is expressed as the difference to the median signal detected on Agilent microarray (12). B, *ST8SIA1* and *MET* expression is expressed as normalized signal detected on the Affymetrix microarray (13). For both dataset, the number of tumors in each subtype group is reported next to the *X* axis. Significant differences between basal and other subtypes are indicated: *, $P < 0.05$; ***, $P < 0.001$.

Perturbation of the HGF/Met signaling axis leads to enhanced signaling that occurs in a wide range of aggressive human cancers, correlated with poor prognosis. As previously described (27), the two intracellular pathways, MEK/ERK and PI3K/Akt, promoted by c-Met *trans*-phosphorylation serve as key downstream integrators of growth and survival signals in MDA-MB-231 cells (14). Signaling for mitogenesis and growth essentially occurs through the MEK/ERK pathway, whereas PI3K-Akt signaling has been shown to play a central role in the antiapoptotic responses induced by activated c-Met. Interestingly, in this context, we showed using specific pharmacologic inhibitors that the simultaneous activation of both pathways is necessary to maintain the proliferative phenotype of GD3S⁺ cells in serum-free conditions.

The GD3S expression leads to the accumulation of complex gangliosides from b- and c-series at the plasma membrane of MDA-MB-231 cells. It is commonly known that gangliosides are associated or complexed with signal transducers such as small G-proteins, Src family kinases, tetraspanins, and integrins (28). The modulation of ganglioside expression can therefore have deep effects on receptor-mediated signaling, notably RTKs, and regulate cell growth through inhibition/activation of signal transduction pathways (15-17). For example, tyrosine kinase activity of EGFR is inhibited by the monosialoganglioside G_{M3} through carbohydrate-carbohydrate interactions of G_{M3} with GlcNAc terminated *N*-glycan on EGFR, without interfering with EGF binding (29, 30). Moreover, G_{M3} has been described as an angiogenesis suppressor by regulating vascular endothelial growth factor receptor-2 phosphorylation (31, 32). Previously, it has been shown that a-series gangliosides act as negative regulators of c-Met. G_{D1a} inhibits HGF-induced motility and scattering of cancer cells through suppression of tyrosine phosphorylation of c-Met (33). G_{M3} and G_{M2} form heterodimers that specifically interact with tetraspanin (CD82) in glycosynapses, and such complexes inhibit c-Met activation and integrin cross talk (34, 35). However, to our knowledge, it is the first demonstration of ganglioside-induced c-Met constitutive activation in cancer cells.

Dysregulation of c-Met signaling through mutation, overexpression, or autocrine/paracrine loops has been well documented and is an important feature in various human cancers, especially in terms of metastasis development (36). To date, no mutation has been found either in human breast cancer biopsies or in breast cancer cell lines, including MDA-MB-231 (http://www.vai.org/met/). A slight

Downloaded from mcr.aacrjournals.org on October 21, 2011
Copyright © 2010 American Association for Cancer Research

Published OnlineFirst October 1, 2010; DOI:10.1158/1541-7786.MCR-10-0302

increase of c-Met expression was observed in both GD3S⁺ clones, but was not correlated to an increase of *MET* transcription, as revealed by quantitative PCR analysis. A majority of breast tumors aberrantly expresses HGF and its receptor (37, 38), which may activate c-Met in an autocrine manner. However, it has been shown that the autocrine activation of c-Met does not occur in MDA-MB-231 (39). Proliferation assay of GD3S⁺ clones with 5D5 Fab, which specifically blocks HGF binding and subsequent pathway activation, has confirmed ligand-independent activation of c-Met on GD3S expression. Thus, we described the original mechanism of c-Met activation independently of gene amplification or autocrine activation induced by complex gangliosides in MDA-MB-231 GD3S⁺ cells.

As shown in Fig. 3, downregulation of c-Met activation did not completely abolish the proliferative capacities of these cells after 5 days of culture in deprivation conditions. It is possible that other RTKs could contribute to the enhanced proliferation, such as EGFR, Ret, and Tie-2, which are also found to be slightly activated in GD3S⁺ cells. Consistent with this hypothesis, recent evidence shows that c-Met cross talk with other cell surface proteins, including RTKs, contributes to tumorigenesis (40, 41). For example, the synergistic action of c-Met and EGFR has been shown to enhance the malignant properties of cancer cells by promoting the resistance of tumors to therapy (42, 43).

In vivo experiments reveal that GD3S expression enhances the tumorigenicity of MDA-MB-231 cells in SCID mice. This suggests that modification of complex ganglioside patterns may provide some advantage for cancer cells to grow in the cellular environment of host tissues. Previous studies have shown that stable inhibition of G_{D3} and G_{D2} expression in human lung cancer cells by GD3S siRNA significantly suppressed cell growth in SCID mice (44). In parallel, *ST8SIA1* overexpression was associated with poor pathohistologic grading in ER-negative tumors and reduced overall survival of patients (8, 9). We report here a higher expression of *ST8SIA1* in the basal subtype of breast tumors compared with others. Interestingly, a similar pattern was found for *MET*, in accordance with previous reports (38, 45, 46). c-Met is also associated with poor clinical outcome and considered as a possible marker for earlier recurrence and shorter survival in breast cancer patients (47-49). Our results support the idea that the products of these two genes may cooperate within the same subtype of breast cancers: the basal type, which consists mainly of the triple-negative breast cancers (ER, progesterone receptor, and erbB2 negative). These cancers are of poor prognosis and cannot benefit from available targeted therapies (i.e., hormone therapy and Herceptin treatment; ref. 22).

It has been recently shown that the GD3S expression renders human hepatocarcinoma cells susceptible to hypoxia, which mediates ROS generation and subsequently cell death (50). Hepatocarcinoma GD3S⁺ cells display enhanced G_{D3} levels both at the cell surface and in internal organelles, most likely including mitochondria. This specific subcellular localization is consistent with the pro-apoptotic role of G_{D3} described in a variety of cell types. Therefore, the biological outcome of G_{D3} synthase expression in cancer cell biology also depends on the subsequent cellular localization and the relative amounts of complex gangliosides that localize to the mitochondria.

To conclude, our results show that GD3S and complex ganglioside expression may contribute to increase the malignant properties of breast cancer cells, by activating RTKs, especially c-Met. This mechanism underlies a new way for c-Met constitutive activation. Molecular targeted therapies have been developed for over a decade to specifically inhibit consistently activated oncogene products, including c-Met (25). However, their efficiency is limited to some patients because of the development of intrinsic or acquired resistance. The present results suggest that complex gangliosides are not only antigenic markers but could be also valid therapeutic targets in basal-type/triple-negative breast cancers. Although further studies are needed to precisely determine which type of gangliosides is responsible for the specific activation of c-Met, our results suggest that targeting both gangliosides and c-Met would lead to a more favorable clinical outcome than targeting RTKs alone.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the Microscopy-Imaging-Cytometry Facility of the Lille Pasteur Campus (MICPaL Facility) for access to instruments and technical advices.

Grant Support

Association pour la Recherche sur le Cancer grant nos. 7936 and 5023 (P. Delannoy), and 1137 (D. Tulasne), la Ligue régionale contre le Cancer (P. Delannoy and D. Tulasne), l'ANR-Young Investigator Program (D. Tulasne), and PAI Hubert Curien France-Korea STAR (P. Delannoy).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 07/09/2010; revised 09/09/2010; accepted 09/28/2010; published OnlineFirst 10/01/2010.

References

1. Hakomori SI. Inaugural article: the glycosynapse. Proc Natl Acad Sci U S A 2002;99:225–32.
2. Tettamanti G. Ganglioside/glycosphingolipid turnover: new concepts. Glycoconj J 2004;20:301–7.
3. Hakomori SI. Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv Exp Med Biol 2001;491:369–402.
4. Birklé S, Zeng G, Gao L, Yu RK, Aubry J. Role of tumor-associated gangliosides in cancer progression. Biochimie 2003;85:455–63.
5. Dyatlovitskaya EV, Kandyba AG. Role of biologically active sphingolipids in tumor growth. Biochemistry 2006;71:10–7.
6. Furukawa K, Hamamura K, Nakashima H, Furukawa K. Molecules in the signaling pathway activated by gangliosides can be targets of therapeutics for malignant melanomas. Proteomics 2008;8:3312–6.

Downloaded from mcr.aacrjournals.org on October 21, 2011
Copyright © 2010 American Association for Cancer Research

Published OnlineFirst October 1, 2010; DOI:10.1158/1541-7786.MCR-10-0302

7. Marquina G, Waki H, Fernandez LE, et al. Gangliosides expressed in human breast cancer. Cancer Res 1996;56:5165–71.
8. Ruckhäberle E, Karn T, Rody A, et al. Gene expression of ceramide kinase, galactosyl ceramide synthase and ganglioside G_{D3} synthase is associated with prognosis in breast cancer. J Cancer Res Clin Oncol 2009;135:1005–13.
9. Ruckhäberle E, Rody A, Engels K, et al. Microarray analysis of altered sphingolipid metabolism reveals prognostic significance of sphingosine kinase 1 in breast cancer. Breast Cancer Res Treat 2008;112:41–52.
10. Cazet A, Groux-Degroote S, Teylaert B, et al. GD3 synthase overexpression enhances proliferation and migration of MDA-MB-231 breast cancer cells. Biol Chem 2009;390:601–9.
11. Lagadec C, Meignan S, Adriaenssens E, et al. TrkA overexpression enhances growth and metastasis of breast cancer cells. Oncogene 2009;28:1960–70.
12. Van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002; 347:1999–2009.
13. Chin K, DeVries S, Fridlyand J, et al. Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. Cancer Cell 2006;10:529–41.
14. Ripple MO, Kalmadi S, Eastman A. Inhibition of either phosphatidylinositol 3-kinase/Akt or the mitogen/extracellular-regulated kinase, MEK/ERK, signaling pathways suppress growth of breast cancer cell lines, but MEK/ERK signaling is critical for cell survival. Breast Cancer Res Treat 2005;93:177–88.
15. Miljan EA, Bremer EG. Regulation of growth factor receptors by gangliosides. Sci STKE 2002;2002:re15.
16. Lopez PH, Schnaar RL. Gangliosides in cell recognition and membrane protein regulation. Curr Opin Struct Biol 2009;19:549–57.
17. Kaucic K, Liu Y, Ladisch S. Modulation of growth factor signaling by gangliosides: positive or negative? Methods Enzymol 2006;417: 168–85.
18. Martens T, Schmidt NO, Eckerich C, et al. A novel one-armed anti-c-Met antibody inhibits glioblastoma growth *in vivo*. Clin Cancer Res 2006;12:6144–52.
19. Jin H, Yang R, Zheng Z, et al. MetMAb, the one-armed 5D5 anti-c-Met antibody, inhibits orthotopic pancreatic tumor growth and improves survival. Cancer Res 2008;68:4360–8.
20. Berthou S, Aebersold DM, Schmidt LS, et al. The Met kinase inhibitor SU11274 exhibits a selective inhibition pattern toward different receptor mutated variants. Oncogene 2004;23:5387–93.
21. Christensen JG, Schreck R, Burrows J, et al. A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes *in vitro* and exhibits cytoreductive antitumor activity *in vivo*. Cancer Res 2003;63:7345–55.
22. Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 2003;100:8418–23.
23. Gschwind A, Fischer OM, Ullrich A. The discovery of receptor tyrosine kinases: targets for cancer therapy. Nat Rev Cancer 2004;4:361–70.
24. Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer 2002;2:289–300.
25. Beviglia L, Matsumoto K, Lin CS, Ziober BL, Kramer RH. Expression of the c-Met/HGF receptor in human breast carcinoma: correlation with tumor progression. Int J Cancer 1997;74:301–9.
26. Yamamoto N, Mammadova G, Song RX, Fukami Y, Sato K. Tyrosine phosphorylation of p145met mediated by EGFR and Src is required for serum-independent survival of human bladder carcinoma cells. J Cell Sci 2006;119:4623–33.
27. Eder JP, Vande Woude GF, Boerner SA, LoRusso PM. Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. Clin Cancer Res 2009;15:2207–14.
28. Kasahara K, Watanabe Y, Yamamoto T, Sanai Y. Association of Src family tyrosine kinase Lyn with ganglioside GD3 in rat brain. Possible regulation of Lyn by glycosphingolipid in caveolae-like domains. J Biol Chem 1997;272:29947–53.
29. Miljan EA, Meuillet EJ, Mania-Farnell B, et al. Interaction of the extracellular domain of the epidermal growth factor receptor with gangliosides. J Biol Chem 2002;277:10108–13.
30. Kawashima N, Yoon SJ, Itoh K, Nakayama K. Tyrosine kinase activity of epidermal growth factor receptor is regulated by GM3 binding through carbohydrate to carbohydrate interactions. J Biol Chem 2009;284:6147–55.
31. Mukherjee P, Faber AC, Shelton LM, Baek RC, Chiles TC, Seyfried TN. Ganglioside GM3 suppresses the pro-angiogenic effects of vascular endothelial growth factor and ganglioside GD1A. J Lipid Res 2008;49: 929–38.
32. Chung TW, Kim SJ, Choi HJ, et al. Ganglioside GM3 inhibits VEGF/VEGFR-2-mediated angiogenesis: direct interaction of GM3 with VEGFR-2. Glycobiology 2009;19:229–39.
33. Hyuga S, Kawasaki N, Hyuga M, et al. Ganglioside GD1a inhibits HGF-induced motility and scattering of cancer cells through suppression of tyrosine phosphorylation of c-Met. Int J Cancer 2001; 94:328–34.
34. Todeschini AR, Dos Santos JN, Handa K, Hakomori SI. Ganglioside GM2-tetraspanin CD82 complex inhibits met and its cross-talk with integrins, providing a basis for control of cell motility through glycosynapse. J Biol Chem 2007;282:8123–33.
35. Todeschini AR, Dos Santos JN, Handa K, Hakomori SI. Ganglioside GM2/GM3 complex affixed on silica nanospheres strongly inhibits cell motility through CD82/cMet-mediated pathway. Proc Natl Acad Sci U S A 2008;105:1925–30.
36. Corso S, Comoglio PM, Giordano S. Cancer therapy: can the challenge be MET? Trends Mol Med 2005;11:284–92.
37. Edakuni G, Sasatomi E, Satoh T, Tokunaga O, Miyazaki K. Expression of the hepatocyte growth factor/c-Met pathway is increased at the cancer front in breast carcinoma. Pathol Int 2001;51:172–8.
38. Kang JY, Dolled-Filhart M, Ocal IT, et al. Tissue microarray analysis of hepatocyte growth factor/Met pathway components reveals a role for Met, matriptase, and hepatocyte growth factor activator inhibitor 1 in the progression of node-negative breast cancer. Cancer Res 2003;63:1101–5.
39. Parr C, Jiang WG. Expression of hepatocyte growth factor/scatter factor, its activator, inhibitors and the c-Met receptor in human cancer cells. Int J Oncol 2001;19:857–63.
40. Lai AZ, Abella JV, Park M. Crosstalk in Met receptor oncogenesis. Trends Cell Biol 2009;19:542–51.
41. Xu AM, Huang PH. Receptor tyrosine kinase coactivation networks in cancer. Cancer Res 2010;70:3857–60.
42. Shattuck DL, Miller JK, Carraway KL III, Sweeney C. Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. Cancer Res 2008;68:1471–7.
43. Karamouzis MV, Argiris A. Update on the role of EGFR inhibitors in cancer therapeutics. Cancer Treat Res 2007;135:257–75.
44. Ko K, Furukawa K, Takahashi T, et al. Fundamental study of small interfering RNAs for ganglioside GD3 synthase gene as a therapeutic target of lung cancers. Oncogene 2006;25:6924–35.
45. Ponzo MG, Lesurf R, Petkiewicz S, et al. Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer. Proc Natl Acad Sci U S A 2009;106:12903–8.
46. Graveel CR, DeGroot JD, Su Y, et al. Met induces diverse mammary carcinomas in mice and is associated with human basal breast cancer. Proc Natl Acad Sci U S A 2009;106:12909–14.
47. Tolgay Ocal I, Dolled-Filhart M, D'Aquila TG, Camp RL, Rimm DL. Tissue microarray-based studies of patients with lymph node negative breast carcinoma show that met expression is associated with worse outcome but is not correlated with epidermal growth factor family receptors. Cancer 2003;97:1841–8.
48. Lengyel E, Prechtel D, Resau JH, et al. C-Met overexpression in node-positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu. Int J Cancer 2005;113:678–82.
49. Garcia S, Dalès JP, Charafe-Jauffret E, et al. Poor prognosis in breast carcinomas correlates with increased expression of targetable CD146 and c-Met and with proteomic basal-like phenotype. Hum Pathol 2007;38:830–41.
50. Lluis JM, Llacuna L, von Montfort C, et al. GD3 synthase overexpression sensitizes hepatocarcinoma cells to hypoxia and reduces tumor growth by suppressing the cSrc/NF-κB survival pathway. PLoS One 2009;4:e8059.

Downloaded from mcr.aacrjournals.org on October 21, 2011
Copyright © 2010 American Association for Cancer Research

C. R. Chimie xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Comptes Rendus Chimie

www.sciencedirect.com

Full paper/Mémoire

Expression of G_{D3} synthase modifies ganglioside profile and increases migration of MCF-7 breast cancer cells

Agata Steenackers [a,b,c,1], Aurélie Cazet [a,b,c,1], Marie Bobowski [a,b,c], Yoann Rombouts [a,b,c], Jonathan Lefebvre [a,d], Yann Guérardel [a,b,c], David Tulasne [a,d], Xuefen Le Bourhis [a,e], Philippe Delannoy [a,b,c,*]

[a] University Lille Nord de France, 59000 Lille, France
[b] USTL, UGSF, 59650 Villeneuve d'Ascq, France
[c] UMR CNRS 8576, Structural and Functional Glycobiology Unit, University of Sciences and Technologies of Lille, 59655 Villeneuve d'Ascq, France
[d] UMR 8161, CNRS, institut de biologie de Lille, institut Pasteur de Lille, 59021 Lille, France
[e] USTL, Inserm U908, 59650 Villeneuve d'Ascq, France

ARTICLE INFO

Article history:
Received 11 April 2011
Accepted after revision 19 May 2011

Keywords:
Gangliosides
G_{D3} synthase
Breast cancer
C-Met
MCF-7

ABSTRACT

B- and c-series of gangliosides are over-expressed in neuro-ectoderm-related cancers, including breast cancer. It has been shown that G_{D3} ganglioside is over-expressed in about 50% of invasive ductal breast carcinoma and the G_{D3} synthase (GD3S) gene displays higher expression among estrogen receptor (ER) negative breast tumors. We previously showed that GD3S expression in MDA-MB-231 breast cancer cells induces the expression of G_{D2} and increased cell proliferation and migration *via* a G_{D2}-dependent activation of c-Met receptor. Here, we show that in ER-positive MCF-7 breast cancer cells, GD3S expression resulted in an increase of G_{D1b}, which was associated with a decrease of G_{M1a} and G_{M2}. Meanwhile, GD3S expressing MCF-7 cells exhibited an increased migration without any modification of proliferation rate. Therefore, GD3S expression can result in different modifications of both ganglioside profiles and cell phenotypes depending on breast cell types.
© 2011 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

RÉSUMÉ

Les gangliosides des séries b- et c- sont surexprimés dans différents cancers d'origine neuro-ectodermique, dont le cancer du sein. Il a été montré que le ganglioside G_{D3} est surexprimé dans environ 50 % des carcinomes canalaires infiltrants et le gène de la G_{D3} synthétase (GD3S) est surexprimé dans les tumeurs estrogène-récepteur négatives. Nous avons précédemment démontré que l'expression de la GD3S dans les cellules cancéreuses mammaires MDA-MB-231 induisait l'expression du G_{D2} et augmentait la

Abbreviations: β3Gal T4, GM1a/GD1b synthase; β4GalNAc T1, GM2/GD2 synthase; BSA, Bovine Serum Albumin; Cer, ceramide; DMEM, Dulbecco's Modified Eagle's Medium; EDTA, Ethylene Diamine Tetra-acetic Acid; EGFR, Epithelial Growth Factor Receptor; ER, Estrogen Receptor; ERK, Extracellular Signal-Regulated Kinase; FBS, Fetal Bovine Serum; FITC, Fluorescein Isothiocyanate; GD3S, GD3 synthase; GSL, glycosphingolipid; HPRT, Hypoxanthine Phosphoribosyltransferase; LacCer, Lactosylceramide; mAb, monoclonal Antibody; MALDI-TOF, matrix assisted laser desorption-ionization time-of-flight; MEK, Mitogen-Activated Protein (MAP) Kinase/ERK kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, Phosphate Buffered Saline; PI3K, Phosphoinositide-3 Kinase; PR, Progesterone Receptor; QPCR, Quantitative real-time Polymerase Chain Reaction; RTK, Receptor Tyrosine-Kinase; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; ST3Gal V, GM3 synthase; ST8Sia I, GD3 synthase; ST8Sia V, GT3 synthase.

* Corresponding author.
E-mail address: philippe.delannoy@univ-lille1.fr (P. Delannoy).

[1] The two first authors have equally participated to this work.

1631-0748/$ – see front matter © 2011 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.crci.2011.05.004

Please cite this article in press as: Steenackers A, et al. Expression of G_{D3} synthase modifies ganglioside profile and increases migration of MCF-7 breast cancer cells. C. R. Chimie (2011), doi:10.1016/j.crci.2011.05.004

prolifération et la migration cellulaires par l'activation G_{D2}-dépendante du récepteur c-Met. Nous montrons ici que l'expression de la GD3S dans les cellules MCF-7 provoque une accumulation du G_{D1b} associée à une diminution du G_{M1a} et du G_{M2}. Parallèlement, les cellules MCF-7 GD3S positives montrent une augmentation de migration sans modification de la prolifération. Ce résultat montre que les modifications du profil gangliosidique et du phénotype induites par l'expression de la GD3S sont dépendantes du type cellulaire.

© 2011 Académie des sciences. Publié par Elsevier Masson SAS. Tous droits réservés.

1. Introduction

Gangliosides, the GSL carrying one or more sialic acid residues, are essentially located in the outer part of the plasma membrane in microdomains named ''glyco-synapses'', where they can interact with transmembrane receptors or signal transducers involved in cell proliferation and intracellular signaling [1,2].

GSL from ganglio-series are usually classified in four series according to the presence of 0 to 3 sialic acid residues linked to LacCer: β-Gal*p*-(1→4)-β-Glc*p*-Cer (LacCer, G_{A3}). The transfer of sialic acid residues is catalyzed in the Golgi apparatus by specific sialyltransferases (namely G_{M3} synthase, GD3S and G_{T3} synthase, respectively) that exhibit a high specificity toward their respective glycolipid substrates [3]. Thus, G_{A3}, G_{M3} (α-Neu*p*5Ac-(2→3)-β-Gal*p*-(1→4)-β-Glc*p*-Cer), G_{D3} (α-Neu-*p*5Ac-(2→8)-α-Neu*p*5Ac-(2→3)-β-Gal*p*-(1→4)-β-Glc*p*-Cer) and G_{T3} (α-Neu*p*5Ac-(2→8)-α-Neu*p*5Ac-(2→8)-α-Neu*p*5Ac-(2→3)-β-Gal*p*-(1→4)-β-Glc*p*-Cer) initiate 0-, a-, b- and c-series gangliosides and the biosynthesis of these compounds determines the relative proportion of gangliosides in each series (Fig. 1). The steady state level of membrane-associated gangliosides is therefore dependent on the activity of corresponding sialyltransferases, and the expression of b- and c- series gangliosides is strictly controlled by ST8Sia I, also named GD3S. Afterwards, further monosaccharides, including Gal*p*NAc, Gal*p* and Neu*p*5Ac, can be transferred in a stepwise manner by other glycosyltransferases [4].

Normal human tissues usually express a-series gangliosides, mainly G_{M1a}, whereas complex gangliosides from b- and c-series are essentially found during embryogenesis, in developing tissues and are mostly restricted to the nervous system in adult [5]. Complex gangliosides expression also increases in different pathological conditions including cancers, such as melanoma [6], neuroblastoma [7], or small cell lung carcinoma [8]. In particular, disialogangliosides (G_{D3} and G_{D2}) play a key role in proliferation and migration of neuro-ectoderm-derived tumor cells [9] and are used as targets for development of anti-cancer vaccines [10]. In breast cancer, G_{D3} and 9-O-acetyl-G_{D3} are over-expressed in about 50% of invasive ductal carcinoma [11] and *ST8SIA1*, the gene that encodes GD3S, displays higher expression among ER negative breast cancer tumors [12,13]. We have previously shown that GD3S expression in the ER-negative MDA-MB-231 cells results in the accumulation of b- and c- series gangliosides [14] and mass spectrometry analysis of permethylated GSL has shown that G_{D2} is the main ganglioside expressed at the cell surface of MDA-MB-231 GD3S+ clones (unpublished data). The expression of G_{D2} is associated with an increased cell migration and with a proliferative phenotype of MDA-MB-231 GD3S+ clones in absence of serum or exogenous growth factors [14]. We have also shown that the proliferative capacities of MDA-MB-231 GD3S+ clones in serum-free conditions directly proceed from the specific and constitutive activation of c-Met receptor and the downstream MEK/ERK and PI3 K/Akt signaling pathways [15]. Moreover, GD3S expression also stimulates primary tumor growth in severe immunodeficient mice [15].Here, we describe the effect of GD3S expression in MCF-7 cells, a usual model of ER-positive breast cancer cells in culture. We show by mass spectrometry that the MCF-7 GD3S+ cells accumulate di- and trisialogangliosides, mainly G_{D1b}. Moreover, the MCF-7 GD3S+ clones showed an increased migration without any change in proliferation compared to control cells.

2. Material and methods

2.1. Antibodies and reagents

Anti-G_{M3} GMR6 (mouse IgM) and anti-G_{D2} (mouse IgG3) S220-51 mAbs were purchased from Seikagaku Corp. (Tokyo, Japan). Anti-G_{D3} R24 mAb was purchased from AbCam (Cambridge, UK). Anti-G_{D1b} GGR12 mAb (mouse IgG2b) [16] was kindly provided by Pr. Ronald L. Schnaar (Depart. of Pharmacology and Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, USA). Anti-G_{T3} A2B5 mAb [17] was kindly provided by Pr. Jacques Portoukalian (Depart. of Transplantation and Clinical Immunology, Claude Bernard University and Edouard Herriot Hospital, Lyon, France). FITC-conjugated cholera toxin B-subunit from *Vibrio cholerae* was from Sigma-Aldrich (Lyon, France) and FITC-conjugated sheep anti-mouse IgG was from GE Healthcare (Templemars, France). Alexa Fluor 488 donkey anti-mouse IgG (H + L), Alexa Fluor 488 donkey anti-mouse IgM and FITC-conjugated anti-mouse IgM were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Mouse mAb directed against the cytoplasmic region of human c-Met was purchased from Invitrogen, and rabbit polyclonal antibody against phosphorylated tyrosine 1234 and 1235 of the c-Met kinase domain was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase were purchased from GE Healthcare.

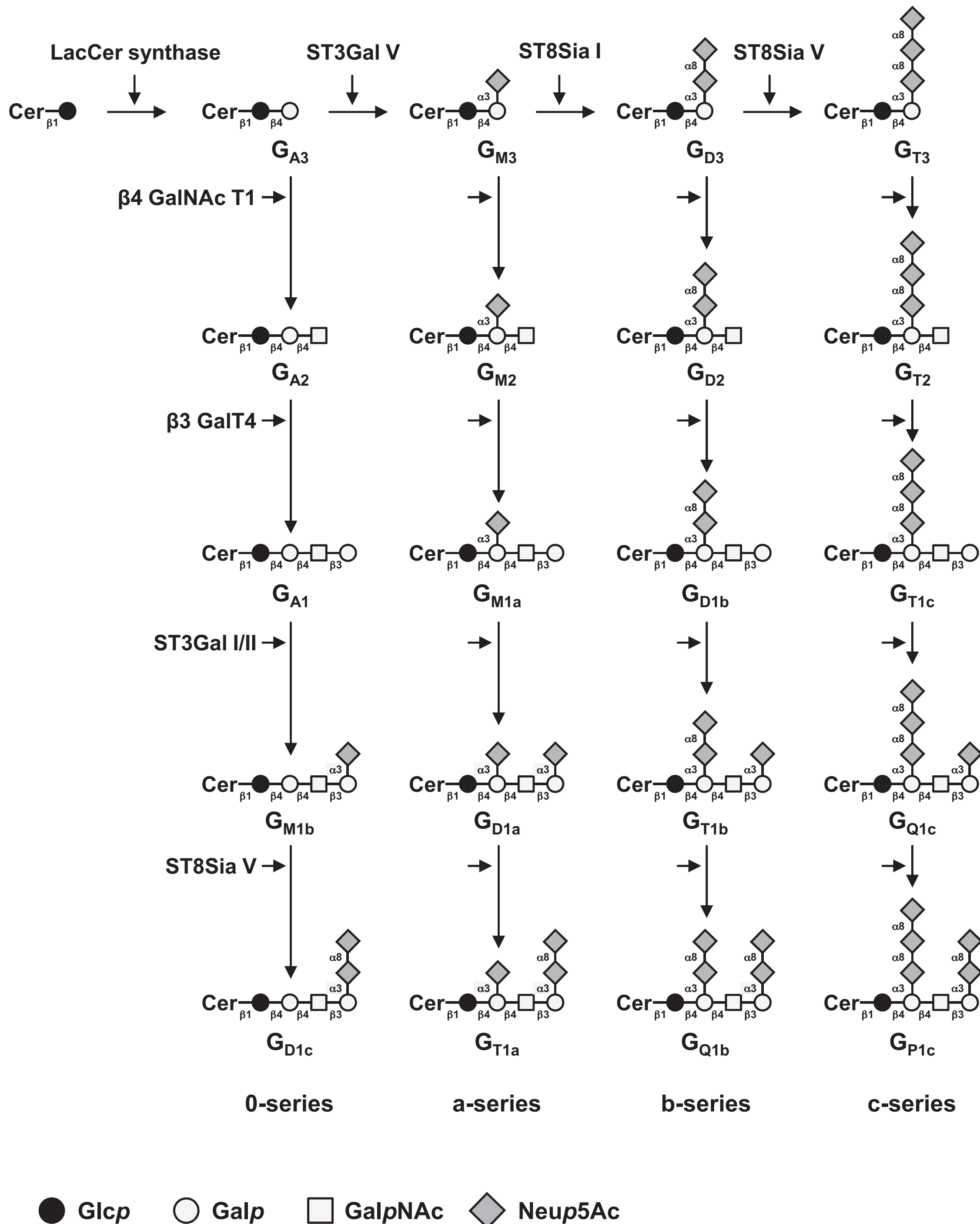


Fig. 1. Biosynthesis of gangliosides. The action of ST3 Gal V (G_{M3} synthase), ST8Sia I (G_{D3} synthase) and ST8Sia V (G_{T3} synthase) leads to the biosynthesis of the precursors of a-, b- and c-series gangliosides, respectively. The 0-series gangliosides are directly synthesized from lactosylceramide (G_{A3}). Elongation is performed by the sequential action of N-acetyl-galactosaminyltransferase (β4GalNAc T1), galactosyltransferase (β3 Gal T4) and sialyltransferases (ST3 Gal I, ST3 Gal II and ST8Sia V). Cer: ceramide; LacCer: lactosylceramide.

2.2. Cell culture

The breast cancer cell line MCF-7 and the melanoma cell line SK-Mel 28 were obtained from the American Type Cell Culture Collection. Cell culture reagents were purchased from Lonza (Levallois-Perret, France). Cells were routinely grown in monolayer and maintained at 37 °C in an atmosphere of 5% CO_2, in DMEM supplemented with

10% FBS, 2 mM L-glutamine, and 100 units/mL penicillin-streptomycin. MCF-7 control (empty vector transfected) and MCF-7 GD3S+ clones #1 and #4 were cultured in the presence of 1 mg/mL G418 (Invitrogen).

2.3. Stable transfection

The pcDNA3-GD3S expression vector encoding the full-length human G_{D3} synthase [18] was obtained from Pr. C.H. Kim (Molecular and Cellular Glycobiology Unit, Department of Biological Science, SungKyunKwan University, Suwon, Korea). Transfections were performed by electroporation using the Nucleofection technology according to Amaxa Biosystem protocol (Lonza). Briefly, 2×10^6 cells were resuspended in 100 μl of Cell Line Nucleofector™ Solution V and cell suspension was mixed with 2 μg of pcDNA3 or pcDNA3-GD3S vector. The sample was transferred into an electroporation cuvette and transfection was performed using the program E-014 according to manufacturer's instructions. After nucleofection, cells were transferred into prewarmed complete DMEM medium and maintained at 37 °C in an atmosphere of 5% CO_2. After 48 h, the transfected cells were cultured in the presence of 1 mg/mL G418 (Invitrogen). After 21 days of culture in the selective medium, individual G418-resistant colonies were isolated by limit dilution. Two positive clones (#1 and #4), expressing GD3S and complex gangliosides, as determined by real-time PCR and flow cytometry analysis, respectively, were used for further study.

2.4. Extraction and preparation of glycolipids

20 dishes (10 cm diameter) of cultured cells were washed twice with ice-cold PBS and cells were scraped and homogenized. Cells were suspended in 200 μL of water and sonicated on ice. The resulting material was dried under vacuum and sequentially extracted by $CHCl_3/CH_3OH$ (2:1, v/v), $CHCl_3/CH_3OH$ (1:1, v/v) and $CHCl_3/CH_3OH/H_2O$ (1:2:0.8, v/v/v). Supernatants were pooled, dried and subjected to a mild saponification in 0.1 M NaOH in $CHCl_3/CH_3OH$ (1:1) at 37 °C for 2 h and then evaporated to dryness [19]. Samples were reconstituted in CH_3OH/H_2O (1:1, v/v) and applied to a reverse phase C_{18} cartridge (Waters, Milford, MA) equilibrated in the same solvent. After washing with CH_3OH/H_2O (1:1, v/v), GSLs were eluted by CH_3OH, $CHCl_3/CH_3OH$ (1:1, v/v) and $CHCl_3/CH_3OH$ (2:1, v/v).

2.5. Mass spectrometry analysis of GSL

Prior to mass spectrometry analysis, GSL were permethylated according to Ciucanu and Kerek [20]. Briefly, compounds were incubated 2 h in a suspension of 200 mg/mL NaOH in dry DMSO (300 μL) and CH_3I (200 μL). The methylated derivatives were extracted in $CHCl_3$ and washed several times with water. The reagents were evaporated and the sample was dissolved in $CHCl_3$ in the appropriate dilution. MALDI-MS and MS/MS analyses of permethylated GSL were performed on 4800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) mass spectrometer, operated in the reflectron mode. For MS acquisition, 5 μL of diluted permethylated samples in $CHCl_3$ were mixed with 5 μL of 2,5-dihydroxybenzoic acid matrix solution (10 mg/mL dissolved in $CHCl_3/CH_3OH$ (1:1, v/v)). The mixtures (2 μL) were then spotted on the target plate and air-dried. MS survey data comprises a total of 50 subspectra of 1500 laser shots. Peaks observed in the MS spectra were selected for further MS/MS. CID MS/MS data comprises a total of 100 subspectra of 3000 laser shots. Two or more spectra can be combined postacquisition with mass tolerance set at 0.1 Da to improve S/N ratio. The potential difference between the source acceleration voltage and the collision cell was set to 1 kV and argon was used as collision gas.

2.6. Analysis of cell surface ganglioside by flow cytometry

Cells were washed in cold PBS and detached by EDTA 4 mM. Cells were incubated at 4 °C during 1 h with anti-ganglioside mAbs: anti-G_{M3} GMR6 (1:75), anti-G_{D3} R24 (1:100), anti-G_{D2} S220-51 (1:100), anti-G_{D1b} GGR12 (1:100) and anti-G_{T3} A2B5 (1:10), diluted in PBS containing 0.5% PBS-BSA (Sigma-Aldrich). After washing with PBS-BSA, cells were incubated on ice during 1 h with FITC-conjugated anti-mouse IgM or IgG. To analyze G_{M1a} expression, cells were incubated with a FITC-conjugated cholera toxin B-subunit (1:1000). Control experiments were performed using secondary antibody alone. Cells were analyzed by flow cytometry (FACScalibur, Becton Dickinson).

2.7. Analysis of ganglioside by confocal microscopy

Cells were cultured on glass coverslips in DMEM supplemented with 10% FBS. After 24 h, cells were washed twice in PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature. After washing with PBS, cells were blocked in PBS-BSA 0.2% for 30 min. Cells were incubated for 1 h at room temperature with anti-G_{D3} R24 (1:100), anti-G_{D2} S220-51 (1:100), anti-G_{D1b} GGR12 (1:100) and anti-G_{T3} A2B5 (1:10), diluted in PBS-BSA. Cells were then washed three times in PBS-BSA and incubated with Alexa Fluor® 488 anti-IgG or anti-IgM (1 h at room temperature; dilution 1:2000 in PBS-BSA). After three washes in PBS-BSA, PBS and deionised water, cells were mounted in Mowiol. Stained slides were examined under a Leica SP5 spectral microscope (IRI CNRS USR 3078, Villeneuve d'Ascq, France) with a 63X oil immersion lens at room temperature. Data were therefore collected using the LAS 6000 AF software.

2.8. Quantitative real-time-PCR (QPCR) analysis of G_{D3} synthase

Total RNA was extracted using the Nucleospin RNA II kit (Macherey Nagel, Hoerdt, France), quantified using a NanoDrop spectrophotometer (Thermo Scientifics, Wilmington, USA) and the purity of the preparation was checked by ratio of the absorbance at 260 and 280 nm. The cDNA was synthesized using 2 μg of RNA (GE Healthcare). PCR primers for GD3S and HPRT were previously described

Please cite this article in press as: Steenackers A, et al. Expression of G_{D3} synthase modifies ganglioside profile and increases migration of MCF-7 breast cancer cells. C. R. Chimie (2011), doi:10.1016/j.crci.2011.05.004

[14,21] and synthesized by Eurogentec (Seraing, Belgium). PCR reactions (25 μL) were performed using 2X SYBR® Green Universal QPCR Master Mix (Stratagene, Amsterdam, The Netherlands), with 2 μL of cDNA solution and 300 nM final concentration of each primer. PCR conditions were as follows: 95 °C for 30 s, 51 °C for 45 s, 72 °C for 30 s (40 cycles). Assays were performed in triplicate and GD3S transcript expression level was normalized to HPRT using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen [22]. Serial dilutions of the appropriate positive control cDNA sample were used to create standard curves for relative quantification and negative control reactions were performed by replacing cDNA templates by sterile water.

2.9. Proliferation assays

Control and clones cells (2×10^3) were seeded in 96-well plates (Thermo Fisher Scientific, Rockford, IL, USA) and grown in DMEM containing 5, 1, 0.5% FBS, or in FBS-free medium. Cell growth was analyzed at different times using the MTS reagent (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions.

2.10. Cell migration analysis

Cells (5×10^4) were seeded in the upper surface of Transwell 12 plates (BD Biosciences, Le-Pont-de-Claix, France) and cultured for 24 h in 10% FBS-containing medium. After incubation, cells were fixed in 4% paraformaldehyde (20 min at room temperature) and stained with Hoescht 33528 (Sigma-Aldrich) during 20 min in the dark at room temperature. Then, the upper chamber of the well's porous membrane was removed by scraping with a cotton swab and washed several times with PBS. The membrane was mounted on slide with Glycergel mounting medium (Dako, Trappes, France). The migrating cells were counted on the overall membranes under × 200 magnification and the mean number of cells was evaluated in three independent experiments.

2.11. Immunodetection of c-Met and phospho-c-Met

Cell pellets were treated with lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 1 mM sodium orthovanadate, and protease inhibitor cocktail tablet). The supernatants were assessed for protein concentration using the Bio-Rad DC protein assay kit II. One hundred microgram of total proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were saturated in TBS containing 5% BSA and 0.05% Tween-20. Membranes were then incubated with blocking buffer (0.2% casein, 0.1% Tween-20 in PBS) for 1 h and probed for 1 h at room temperature with appropriate antibodies diluted in blocking buffer according to the manufacturer's recommendations. After washing in PBS-Tween 0.2%, immune complexes were detected with specific secondary antiserum conjugated with alkaline phosphatase followed by an enhanced chemiluminescence detection system (Amersham ECL Western Blotting Detection Reagents).

2.12. Statistical analyses

Student's *t*-test was used for statistical analysis. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Establishment of GD3S+ MCF-7 breast cancer cells

MCF-7 cells were transfected with the pcDNA3-GD3S expression vector containing the full-length cDNA of human GD3S or the empty pcDNA3 vector as control. Transfected cells were cultured 21 days in the presence of 1 mg/mL G418. As previously shown [14], QPCR analysis of GD3S expression (Fig. 2A) indicates that GD3S mRNA is expressed at a very low level in wild-type and control (empty vector transfected) MCF-7 cells compared to SK-Mel 28 melanoma cells used as positive control for GD3S expression [23]. Meanwhile, GD3S expression was significantly increased in pcDNA3-GD3S transfected MCF-7 cell population. Flow cytometry analysis of gangliosides in both control and pcDNA3-GD3S transfected cells shows that GD3S stable transfectants do not expressed G_{D3} or G_{D2} and only a low percentage of cells (about 15%) expresses G_{D1b} concomitantly with a decrease of G_{M1a} expression, a larger proportion of cells being recognized by the anti-G_{T3} A2B5 mAb (Fig. 2B).

Given the fact that the pcDNA3-GD3S transfected cell population was heterogenous, individual G418-resistant colonies were isolated by limiting dilution cloning. Forty-three different clones were obtained and analyzed for the expression of GD3S and gangliosides. Positive clones for G_{D1b} and G_{T3} expression were selected and two of them, clones #1 and #4, were used for this study. The pattern of gangliosides was monitored in the two clones by flow cytometry using different anti-ganglioside mAbs. As shown in Fig. 3A, the two selected clones expressed high levels of G_{D1b} and G_{T3}. In contrast, G_{M1a} expression level was reduced (Fig. 3A). G_{D1b} expression appeared to be slightly higher in clone #1 than in clone #4. Control cells showed no change in the ganglioside profile compared with wild-type MCF-7 (data not shown). Finally, immunofluorescence and confocal microscopy confirmed that G_{D1b} and G_{T3} were expressed at the cell surface of both clones (Fig. 3B).

3.2. Mass spectrometry analysis of GD3S+ MCF-7 cells GSL composition

In order to confirm the modifications in gangliosides composition observed by flow cytometry analysis in MCF-7 GD3S+ cells, we compared the GSLs profiles from control and GD3S+ MCF-7 cells by MALDI-TOF-MS and MALDI-TOF-MS/MS sequencing (Figs. 4 and 5). The total glycolipids were extracted and subjected to mild alkalinolysis. GSLs were further purified on reversed-phase C_{18} column and analyzed by mass spectrometry as permethylated derivatives. As shown in Fig. 4, the MALDI-MS analysis revealed that MCF-7 cells expressed a complex pattern of GSLs. Individual signals were identified by calculating

Please cite this article in press as: Steenackers A, et al. Expression of G_{D3} synthase modifies ganglioside profile and increases migration of MCF-7 breast cancer cells. C. R. Chimie (2011), doi:10.1016/j.crci.2011.05.004

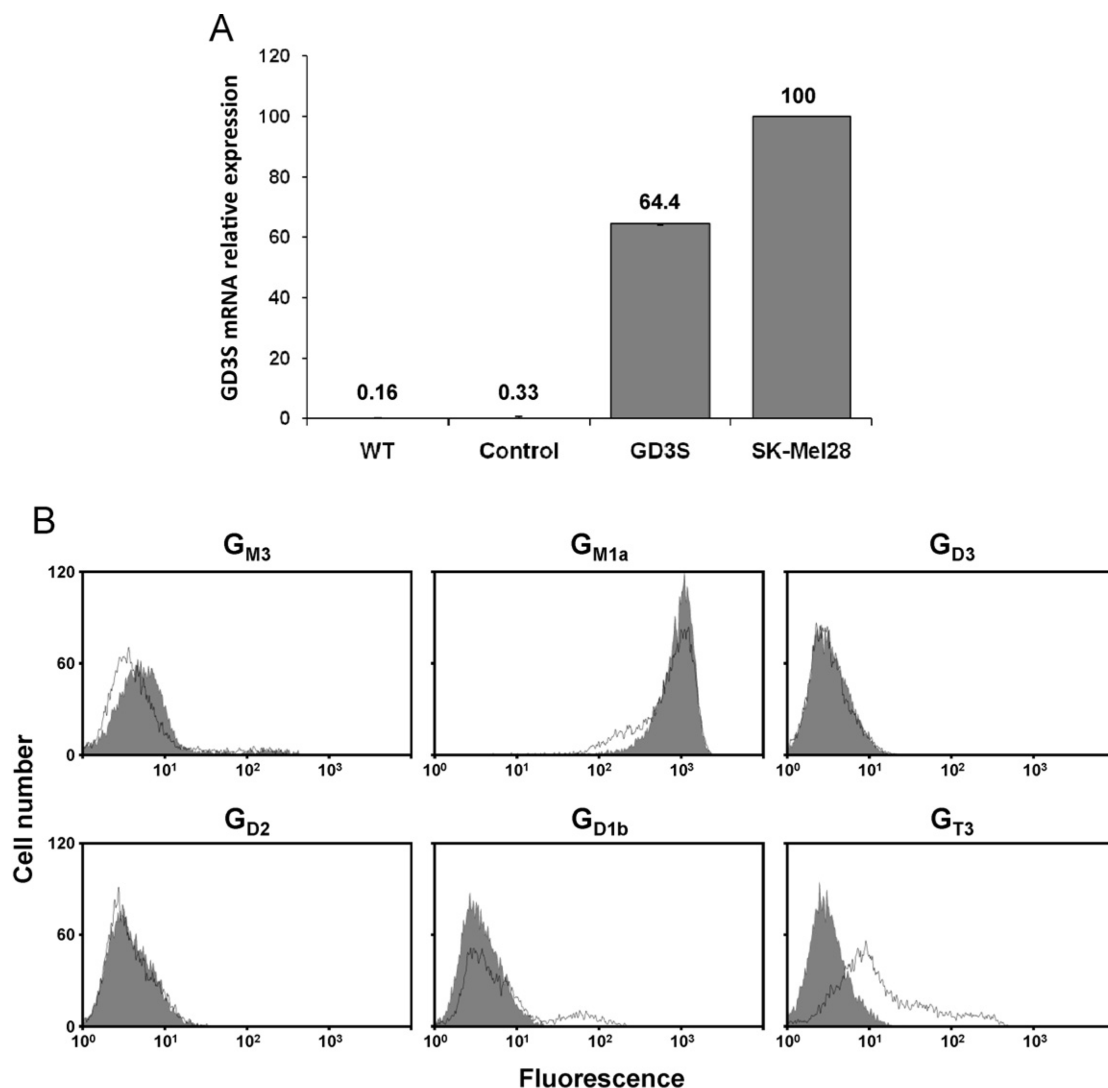


Fig. 2. A: QPCR analysis of GD3S mRNA in pcDNA3-GD3S transfected MCF-7 cell population. Results were normalized to the expression of HPRT mRNA and expressed relative to GDS3 mRNA in SK-Mel 28 melanoma cells, which was regarded as 100%. The quantification was performed by the method described by Livak and Schmittgen [22]. WT: MCF-7 wild-type; Control: empty vector transfected MCF-7; GD3S: pcDNA3-GD3S transfected MCF-7. B: Flow cytometry analysis of gangliosides expression in pcDNA3-GD3S transfected MCF-7 cell population. The immunodetection of G_{M3}, G_{D3}, G_{D2}, G_{D1b}, G_{T3} and G_{M1a} was performed using GMR6, R24, S220-51, GGR12, A2B5 mAbs and cholera toxin B-subunit from *Vibrio cholerae*, respectively. The grey peaks correspond to control MCF-7 cells (empty vector transfected) and black lines correspond to pcDNA3-GD3S transfected MCF-7 cells.

monosaccharide and Cer compositions and by establishing monosaccharide sequences by MALDI-TOF-MS/MS fragmentation. Then, the final attribution of individual GSLs to existing series was based on the known biosynthetic pathways of GSLs in human cells. Altogether, the data established that MCF-7 cells expressed GSLs from the globo- and the ganglio-series. Each GSL, irrespective of their series, were identified as two distinct signals exhibiting 112 mu differences, which established that they are substituted by two Cer moieties differing by eight $–CH_2–$ groups. Indeed, two Cer isoforms are usually expressed in human tissues due to the substitution of the sphingosine by palmitic acid C16:0 or lignoceric acid C24:0. Globo-series GSLs were characterized by the presence of simple G_{b3} and G_{b4} at *m/z* 1215/1327 and 1460/1572, respectively. Ganglio-series GSLs were characterized by the presence of a single neutral G_{A1} at *m/z* 1215/1327, monosialylated G_{M2} and G_{M1} at *m/z* 1616/1728 and 1821/1933, as well as disialylated at *m/z* 2182/2294. MS/MS fragmentation of individual signals allows one to differentiate isobaric molecules such as G_{b4}/G_{A1} and G_{M1a}/G_{M1b} (data not shown). It is noteworthy that the MS/MS sequencing established that the disialylated G_{D1} expressed in empty vector-transfected MCF-7 cells was exclusively constituted by the a-series ganglioside. Indeed, the intense B- and C-cleavage ions at *m/z* $[M+Na]^+$ 847 and 620 unambiguously established that the terminal Gal residue of the Hex-HexNAc motif was monosialylated (Fig. 5B). Accordingly to the attribution as G_{D1a}, Y-cleavage ion at *m/z* $[M+Na]^+$ 1469 and the Y2α/C5 double cleavage ion at *m/z*

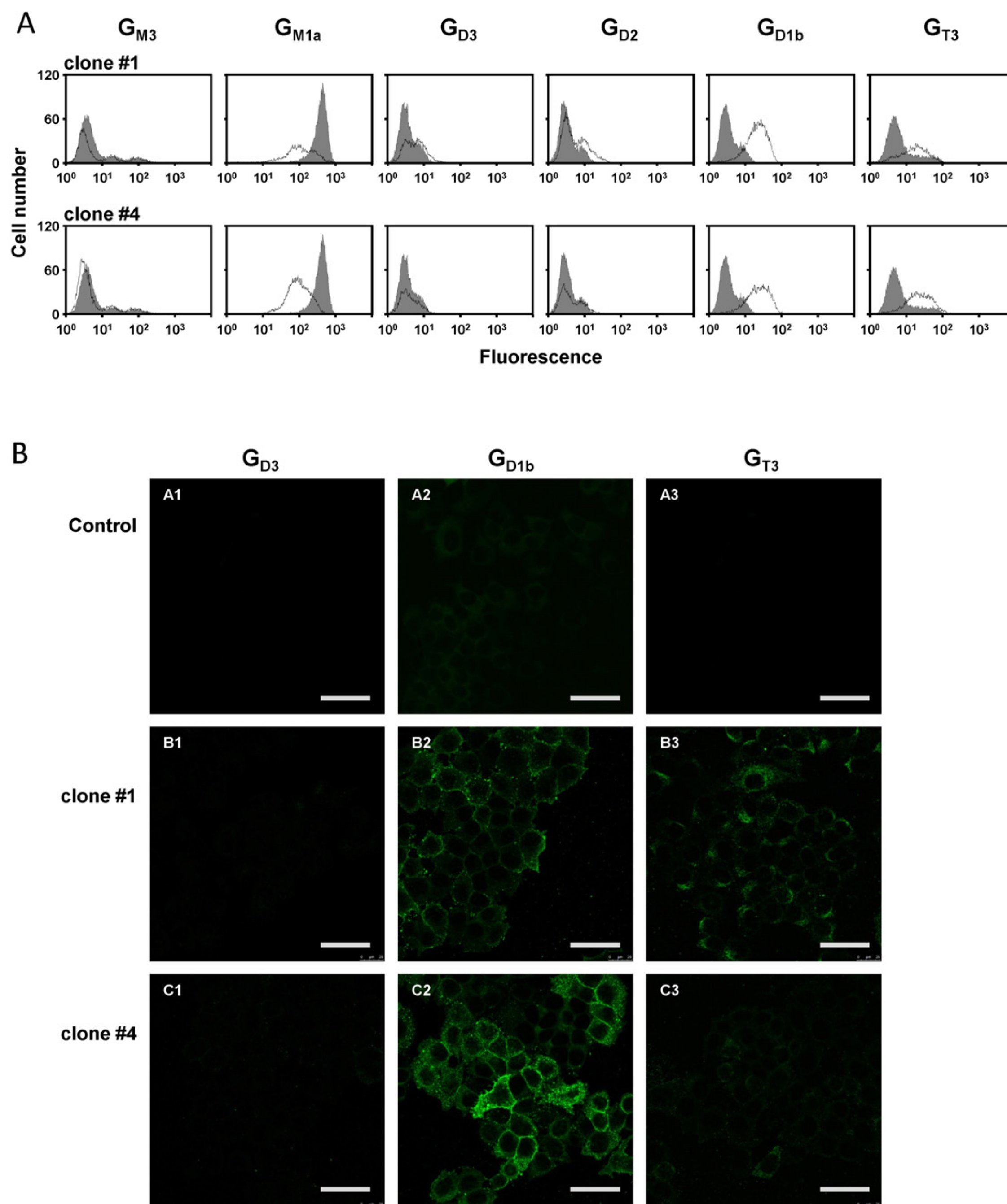


Fig. 3. Analysis of the expression of gangliosides in MCF-7 GD3S+ clones. A: flow cytometry analysis of ganglioside expression. Gangliosides G_{M3}, G_{D3}, G_{D2}, G_{D1b}, and G_{T3} were revealed with anti-G_{M3} GMR6, anti-G_{D3} R24, anti-G_{D2} S220-51, anti-G_{D1b} GGR12, and anti-G_{T3} A2B5 mAbs, respectively. G_{M1a} expression was analyzed with FITC-labeled cholera toxin B from *Vibrio cholerae*. The grey peaks correspond to control MCF-7 cells, whereas black lines correspond to MCF-7 GD3S+ cells. Results are representative of three independent experiments. B: Analysis of ganglioside expression at the membrane surface MCF-7 cells by immunocytochemistry and confocal microscopy. Ganglioside were revealed with anti-G_{D3} R24 (A1, B1, C1), anti-G_{D1b} GGR12 (A2, B2, C2) or anti-G_{T3} A2B5 (A3, B3, C3) mAbs and anti-mouse IgM or anti-mouse IgG labeled with Alexa 488. Bars: 50 μm.

810 $[M + Na]^+$ confirmed that Gal*p*-(1→4)-β-Glc*p* disaccharide was also mono-sialylated.

As expected, the gangliosides profiles of MCF-7 GD3S+ clones established by MALDI-MS significantly differed from those of the wild-type (data not shown) and empty vector-transfected MCF-7 (control) cells (Fig. 4). Indeed, whereas MCF-7 cells expressed simple a-series gangliosides including G_{M2}, G_{M1a} and G_{D1a}, GD3S+ clones accumulated di-, tri- and tetra-sialylated gangliosides of higher complexity including G_{D3}, G_{D2}, G_{D1}, G_{T2}, G_{T1} and G_{Q1}. In parallel, monosialylated gangliosides totally disappeared in MCF-7 GD3S+ cells compared to control cells.

Please cite this article in press as: Steenackers A, et al. Expression of G_{D3} synthase modifies ganglioside profile and increases migration of MCF-7 breast cancer cells. C. R. Chimie (2011), doi:10.1016/j.crci.2011.05.004

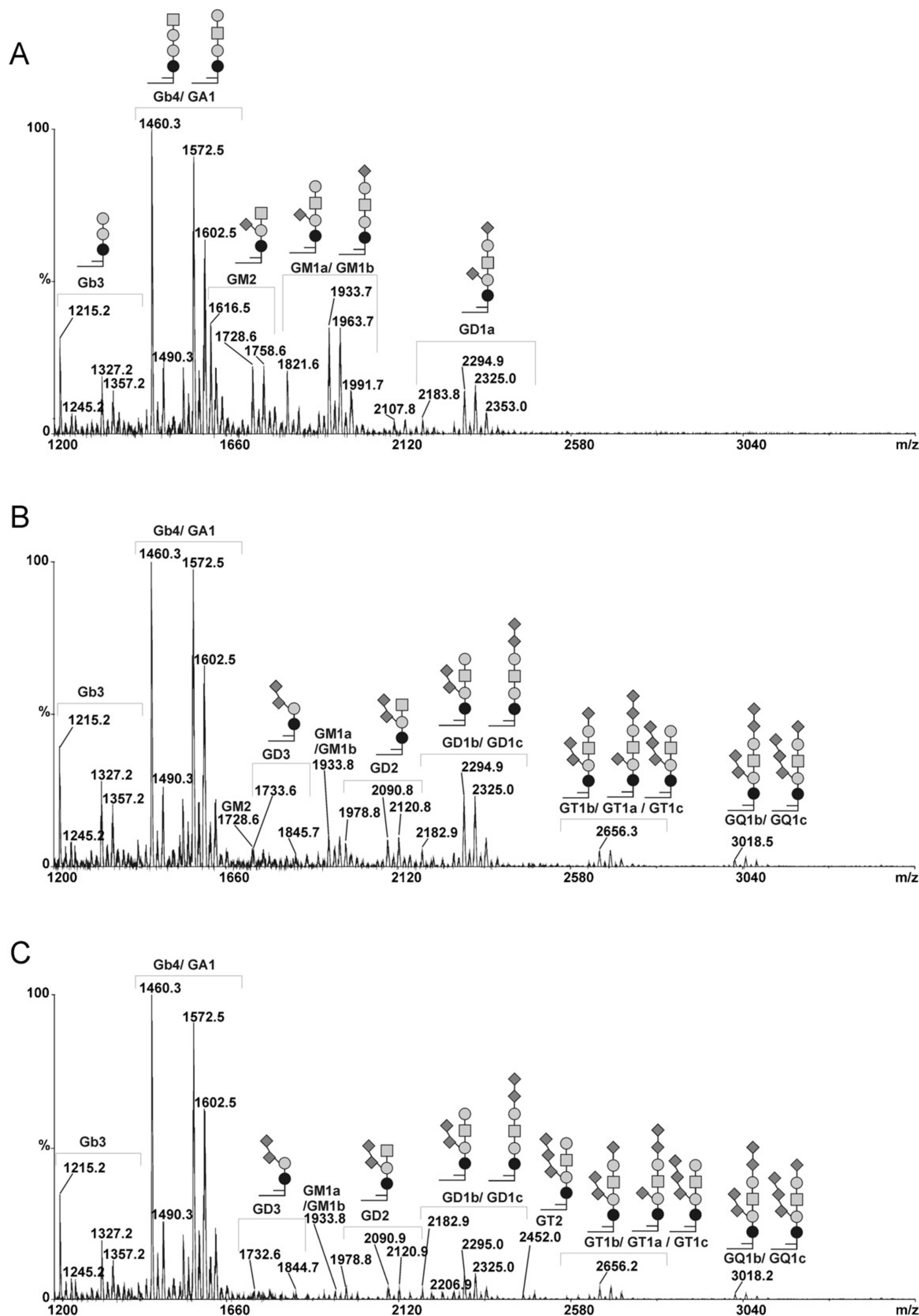


Fig. 4. MALDI-TOF-MS profiles of permethylated GSLs from control and GD3S+ MCF-7 cells. Mass Spectra of permethylated GSLs isolated from control MCF-7 transfected with empty vector (A), GD3S+ MCF-7 cells clone #1 (B) and clone #4 (C). Gangliosides from b- or c-series were not detected in control cells, whereas complex gangliosides containing 2 to 4 sialic acids residues (i.e. G_{D3}, G_{D2}, G_{D1b}, G_{T2}, G_{D3}, $G_{T1b/c}$, $G_{Q1b/c}$) were detected in GD3S+ MCF-7 cells. ● Glc*p*; ○ Gal*p*; □ Gal*p*NAc; ◇ Neu*p*5Ac; ⅂ Ceramide.

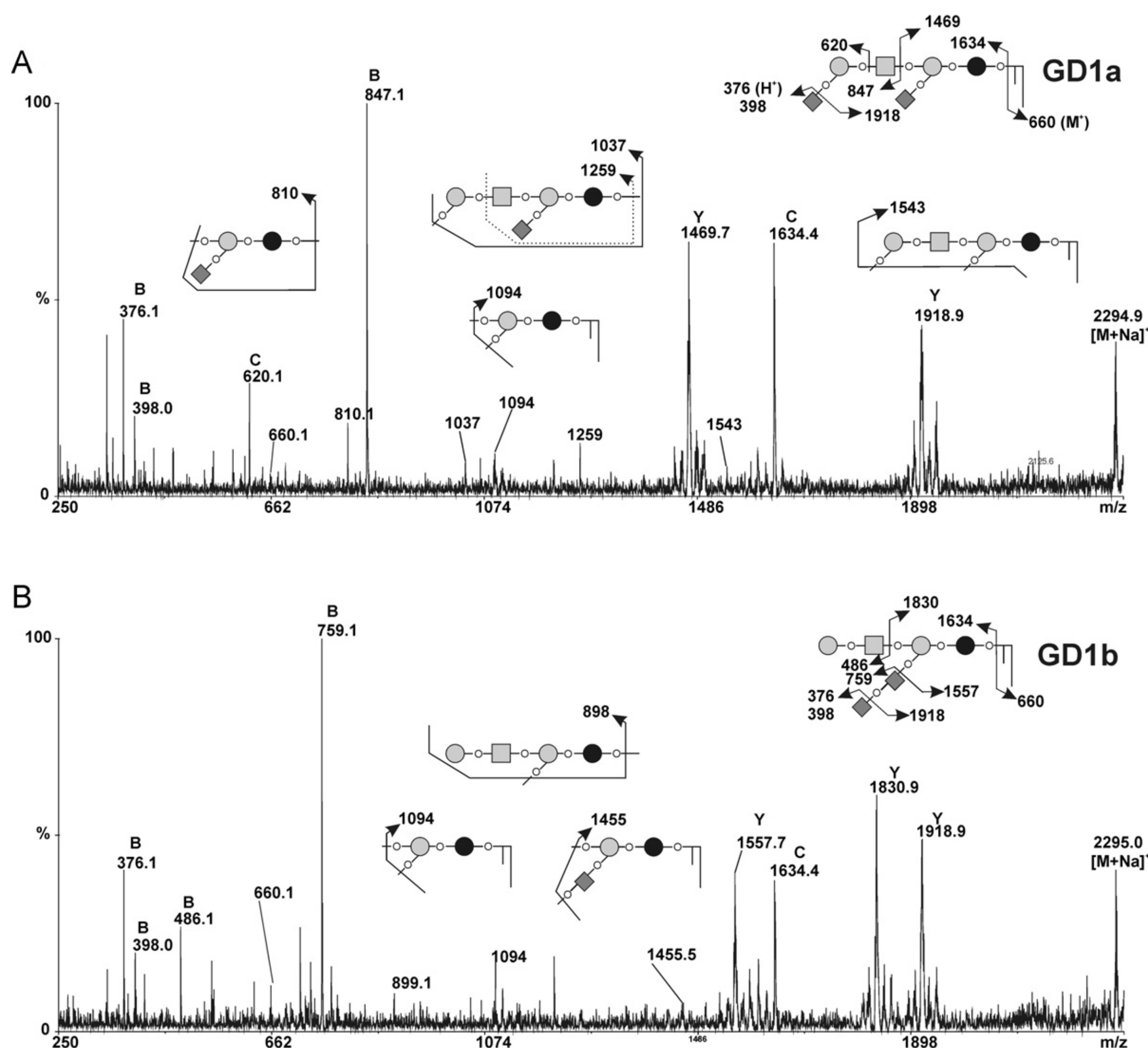


Fig. 5. MALDI-MS/MS sequencing of the permethylated gangliosides from control and GD3S+ MCF-7 cells. MALDI-MS/MS spectra of the $[M + Na]^+$ molecular ion *m/z* 2495 corresponding to permethylated G_{D1} gangliosides in MCF-7 transfected with empty vector (**A**) and GD3S+ MCF-7 clone #1 (**B**). Fragment ions were annotated according to nomenclature of Domon and Costello [31]. All molecules are detected as $[M + Na]^+$ ions with the exception of those into brackets {}. The fragmentation profiles of permethylated G_{D1} ganglioside typifies G_{D1a} in control MCF-7 cells and G_{D1b} in GD3S+ MCF-7 clones. The C/Z couple fragment ions at *m/z* 1634 $[M + Na]^+$ and 660 $[M + H]^+$ typify the ceramide moiety as sphingosine C18:1 substituted by lignoceric acid C24:0. ● Glc*p*; ○ Gal*p*; □ Gal*p*NAc; ◆ Neu*p*5Ac; ⅂ Ceramide.

Then, MS/MS fragmentation analysis of individual compounds in MCF-7 GD3S+ cells established that all GSLs are b- and c-series, which permitted to precisely identify G_{D1b}, $G_{T1b/c}$ and $G_{Q1b/c}$. In particular, comparison of the fragmentation patterns of G_{D1} signals from control MCF-7 cells and GD3S+ clones revealed that G_{D1} isolated from GD3S+ cells exclusively contained the b-series isomer whereas control MCF-7 cells only contained the a-series isomer (Fig. 5B). The presence of the di-sialylated motif on the lactose moiety typifying b-series was unambiguously established by the presence of Y/B couple cleavage ions at *m/z* 1830/486 and B ion at *m/z* 759.

3.3. Growth and migration of GD3S+ MCF-7 cells

Cell growth was determined by MTS assay in culture media containing decreasing concentrations of FBS. As shown in Fig. 6A, no difference in cell growth was observed between control and GD3S+ cells. Similar result was obtained with wild-type MCF-7 cells (data not shown). In all cases, cell proliferation decreased with serum concentration. Similar result was obtained by direct cell counting experiments (data not shown). Interestingly, cell migration assay using Transwell Boyden chambers showed that GD3S expression induced twice as much cell migration to the bottom chamber compared to control cells (Fig. 6B).

3.4. Immunodetection of c-Met and phospho-c-Met in MCF-7 GD3S+ clones

The expression and phosphorylation of c-Met were analyzed in control and MCF-7 GD3S+ clones by Western blotting and immunodetection using anti-c-Met and anti–phospho-c-Met antibodies directed against phosphorylat-

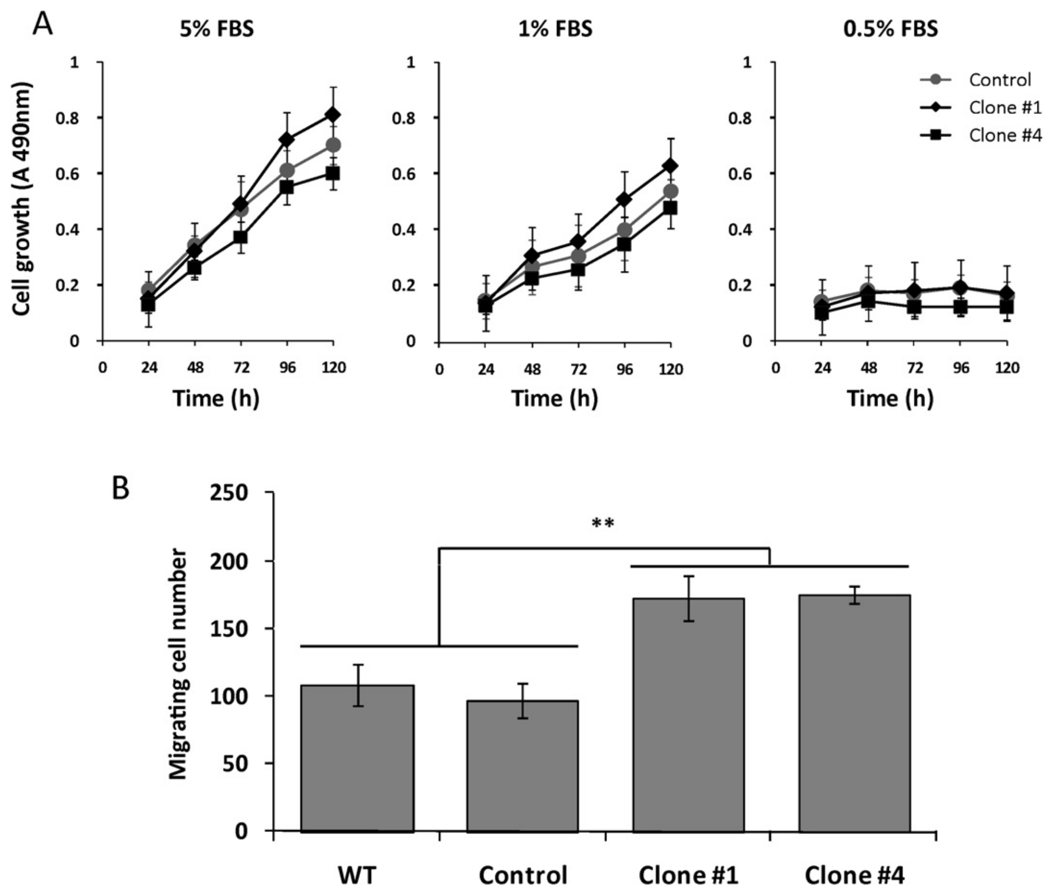


Fig. 6. Analysis of the effect of GD3S expression on MCF-7 cell proliferation and migration. A: Proliferation assays were performed by MTS in 96-wells plates and cells were grown in DMEM supplemented with 5, 1 or 0.5% FBS. Cells were counted after 24, 48, 72, 96 and 120 h. Each measure was performed in 16 wells and data are mean of four independent manipulations. ●: control cells; ◆: clone #1; ■: clone #4. B: Transwell cell migration analysis. Cells were counted after 24 h of culture in DMEM medium supplemented with 1% FBS. ** $p < 0.02$.

ed tyrosine residues 1234 and 1235 of the receptor. As shown in Fig. 7, c-Met was detected at the same expression level in both control and GD3S+ clones (Fig. 7). Phosphorylation of c-Met was revealed neither in control nor in untreated GD3S+ clones, while HGF/SF induced efficient Met phosphorylation in MCF7 cells (Fig. 7).

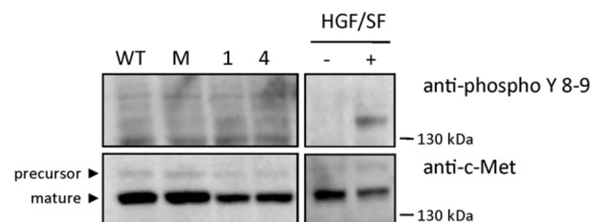


Fig. 7. Western blot analysis using specific c-Met and phospho-c-Met antibodies. Untransfected MCF-7 (WT), control MCF-7 transfected with empty vector, GD3S+ MCF-7 cells clone #1 and clone #4 were cultured in serum free condition. Untransfected MCF-7 (WT) were treated or not 15 min with 30 ng/ml HGF/SF. Cell lysates were analyzed by Western blotting with antibodies directed against human c-Met and the phosphorylated tyrosine residues of the c-Met kinase domain. The positions of molecular weight markers are indicated. Arrowheads indicate the positions of precursor and mature c-Met.

4. Discussion

Recent evidences have proposed that GD3S could be associated with breast cancer development and aggressiveness. Clinical studies using breast tumors microarray datasets have shown that the GD3S gene (*ST8SIA1*) displayed higher expression among ER negative tumors [12]. *ST8SIA1* expression is associated with poor histopathological grading and tumor size in ER negative tumors [12,13]. We have also recently showed that *ST8SIA1* expression is significantly higher in the basal-subtype [24] breast cancer tumors [15], indicating that GD3S could be over-expressed in aggressive breast cancer tumors. However, in comparison with tumors, breast cancer cells do not express complex gangliosides from b- and c-series in culture conditions [14]. To analyze the effect of GD3S expression in breast cancer, we have established and characterized cellular models deriving from two breast cancer cell lines: the triple negative (ER-, PR- and Her2-) basal-subtype MDA-MB-231 and the luminal-A subtype ER+ MCF-7 breast cancer cells, that both express GD3S. We have shown that MDA-MB-231 GD3S+ clones exhibit a proliferative phenotype in absence of serum or growth factors, and an increased tumor growth in severe

immunodeficiency mice. This phenotype results from the constitutive activation of c-Met receptor and subsequent activation of MEK/ERK and PI3 K/Akt pathways [15]. Here, we reported the phenotype of MCF-7 GD3S+ clones.

MALDI-TOF analysis has confirmed that wild-type MCF-7 and MDA-MB-231 have different GSL composition. GSL composition of MDA-MB-231 and MCF-7 cell lines was previously examined [25], showing that there is twice as much neutral GSL in MCF-7 as in MDA-MB-231 cells and that ganglioside content is four-fold higher in MDA-MB-231 cells compared to MCF-7 cells. Our data confirm that MCF-7 express large amount of globosides such as G_{b3} and G_{b4} and lower amount of gangliosides compared to MDA-MB-231 [14]. This difference in GSL composition may be explained by the lowest expression of the G_{M3} synthase (ST3 Gal V) in MCF-7, the enzyme that regulates the first step of gangliosides biosynthesis [14]. Similarly to MDA-MB-231 cells, wild-type MCF-7 cells only express a-series gangliosides, but mainly accumulate G_{M1a} instead of G_{M2} in MDA-MB-231, probably due to the 5-fold higher expression of the β3 Gal T4 in MCF-7 cells (data not shown). In MCF-7 clones, GD3S expression resulted in the conversion of a-series in b- and c-series gangliosides absent from control cells, G_{D1b} being the main compound instead of G_{D2} in MDA-MB-231 GD3S+ clones (unpublished data). These data are in good agreement with ganglioside profiles of wild-type MCF-7 and with the expression of related enzymes, G_{D2} being efficiently converted in G_{D1b} by the β3 Gal T4 in MCF-7 cells. It is noteworthy that GD3S+ clones also express c-series gangliosides whereas the G_{T3} synthase ST8Sia V is weakly expressed in both MDA-MB-231 and MCF-7 cells. This may be due to the G_{T3} synthase activity of GD3S that also uses G_{D3} as acceptor substrate to synthesize G_{T3} [26].

As previously shown, GD3S expression in MDA-MB-231 induces a proliferative phenotype in serum-free conditions, resulting from the constitutive activation of c-Met receptor and subsequent activation of MEK/ERK and PI3 K/Akt transduction pathways [15]. Moreover, the silencing of the G_{M2}/G_{D2} synthase (β4GalNAc T1) reverses the proliferative phenotype and c-Met phosphorylation, underlying the role of G_{D2} in c-Met activation. It is commonly known that ganglioside expression can have important effects on RTKs-mediated signaling and regulates cell growth through activation or inhibition of intracellular transduction pathways [27]. For example, EGFR tyrosine kinase activity is inhibited by G_{M3} through interactions of G_{M3} with terminal β-Glc*p*NAc of EGFR *N*-glycans [28]. However, no change in the proliferative capacity of GD3S+ MCF-7 clones was observed and no activation of c-Met was detected in these cells. The absence of phenotype of GD3S+ MCF-7 clones can be related to the expression of G_{D1b} instead of G_{D2}, and reinforces the role of G_{D2} in c-Met activation. In parallel, MCF-7 GD3S+ cells show an increased migration as observed for MDA-MB-231 clones. This seems to indicate that proliferation and migration are activated by different mechanisms in GD3S+ cells.

In conclusion, the present data together with our previous results show that the expression of GD3S in breast cancer cells results in different ganglioside profiles and phenotypes according to the cell type and the cell-dependent expression of other glycosyltransferases involved in ganglioside biosynthesis (i.e. ST3 Gal V, β4GalNAc T1, and β3 Gal T4). Recently, it has been shown that breast cancer cell gangliosides can serve as functional E-selectin ligands and be involved in cell adhesion to vascular endothelium, favoring metastasis [29]. Moreover, the expression of ST6GalNAc V, a sialyltransferase catalyzing α-ganglioside biosynthesis, has been associated to brain metastasis of breast cancer. Normally restricted to the brain, the expression of ST6GalNAc V in breast cancer cells enhances their adhesion to brain endothelial cells and their passage through the blood-brain barrier [30]. We have also previously shown that *ST8SIA1* expression is significantly higher in the basal-subtype breast cancer tumors and that G_{D2} expression induces a growth factor independent proliferative phenotype *via* the constitutive activation of c-Met [15]. Nevertheless, *ST8SIA1* alone is not sufficient to induce G_{D2} accumulation and c-Met activation, as low expression of β3 Gal T4 is also required. Altogether, these data indicate that the fine analysis of ganglioside glycosyltransferase genes expression should give new insights on proliferation capacity and tissue-specific metastasis targeting of basal-subtype breast cancer tumors.

Acknowledgments

We thank Pr. Jacques Portoukalian (Depart. of Transplantation and Clinical Immunology, Claude Bernard University and Edouard Herriot Hospital, Lyon, France) and Pr. Ronald L. Schnaar (Depart. of Pharmacology and Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, USA) for the kind gift of anti-G_{T3} and anti-G_{D1b} mAbs, respectively. We also thank Pr. C.H. Kim (Molecular and Cellular Glycobiology Unit, Depart. of Biological Science, Sung Kyun Kwan University, Suwon, Korea) for providing the pcDNA3-GD3S expression vector. This work was supported by the University of Sciences and Technologies of Lille, the Association pour la Recherche sur le Cancer (P. Delannoy grant no. 7936 and 5023, D. Tulasne grant no. 1137), La Ligue Régionale contre le Cancer and the ANR-Young Investigator Program (D. Tulasne).

References

[1] S.I. Hakomori, Proc. Natl. Acad. Sci. U S A 99 (2002) 225.
[2] A.R. Todeschini, S.I. Hakomori, Biochim. Biophys. Acta. 1780 (2008) 421.
[3] G. Zeng, R.K. Yu, Curr. Drug Targets. 9 (2008) 317.
[4] G. Tettamanti, Glycoconj. J. 20 (2004) 301.
[5] T. Yamashita, R. Wada, T. Sasaki, C. Deng, U. Bierfreund, K. Sandhoff, R.L. Proia, Proc. Natl. Acad. Sci. U S A 96 (1999) 9142.
[6] C.S. Pukel, K.O. Lloyd, L.R. Travassos, W.G. Dippold, H.F. Oettgen, L.J. Old, J. Exp. Med. 155 (1982) 1133.
[7] N. Baumann, M.L. Harpin, J.C. Turpin, Biomedicine 35 (1981) 215.
[8] D.A. Cheresh, J. Rosenberg, K. Mujoo, L. Hirschowitz, R.A. Reisfeld, Cancer Res. 46 (1986) 5112.
[9] K. Furukawa, K. Hamamura, W. Aixinjueluo, K. Furukawa, Ann. N Y Acad. Sci. 1086 (2006) 185.
[10] S.I. Hakomori, Adv. Exp. Med. Biol. 491 (2001) 369.
[11] G. Marquina, H. Waki, L.E. Fernandez, K. Kon, A. Carr, O. Valiente, R. Perez, S. Ando, Cancer Res. 56 (1996) 5165.
[12] E. Ruckhäberle, A. Rody, K. Engels, R. Gaetje, G. von Minckwitz, S. Schiffmann, S. Grösch, G. Geisslinger, U. Holtrich, T. Karn, M. Kaufmann, Breast Cancer Res. Treat. 112 (2008) 41.
[13] E. Ruckhäberle, T. Karn, A. Rody, L. Hanker, R. Gätje, D. Metzler, U. Holtrich, M. Kaufmann, J. Cancer Res. Clin. Oncol. 135 (2009) 1005.

[14] A. Cazet, S. Groux-Degroote, B. Teylaert, K.M. Kwon, S. Lehoux, C. Slomianny, C.H. Kim, X. Le Bourhis, P. Delannoy, Biol. Chem. 390 (2009) 601.
[15] A. Cazet, J. Lefebvre, E. Adriaenssens, S. Julien, M. Bobowski, A. Grigoriadis, A. Tutt, D. Tulasne, X. Le Bourhis, P. Delannoy, Mol. Cancer Res. 8 (2010) 1526.
[16] H. Ozawa, M. Kotani, I. Kawashima, T. Tai, Biochim. Biophys. Acta 1123 (1992) 184.
[17] C. Dubois, J.C. Manuguerra, B. Hauttecoeur, J. Maze, J. Biol. Chem. 265 (1990) 2797.
[18] S.K. Moon, H.M. Kim, Y.C. Lee, C.H. Kim, J. Biol. Chem. 279 (2004) 33063.
[19] R.L. Schnaar, Methods Enzymol. 230 (1994) 348.
[20] I. Ciucanu, F. Kerek, Carbohydr. Res. 131 (1984) 209.
[21] X. Zhang, L. Ding, A.J. Sandford, BMC Mol. Biol. 6 (2005) 4.
[22] K.J. Livak, T.D. Schmittgen, Methods 25 (2001) 402.
[23] S. Ruan, B.K. Raj, K.O. Lloyd, J. Neurochem. 72 (1999) 514.
[24] T. Sorlie, R. Tibshirani, J. Parker, T. Hastie, J.S. Marron, A. Nobel, S. Deng, H. Johnsen, R. Pesich, S. Geisler, J. Demeter, C.M. Perou, P.E. Lønning, P.O. Brown, A.L. Børresen-Dale, D. Botstein, Proc. Natl. Acad. Sci. U S A 100 (2003) 8418.
[25] K. Nohara, F. Wang, S. Spiegel, Breast Cancer Res. Treat. 48 (1998) 149.
[26] J. Nakayama, M.N. Fukuda, Y. Hirabayashi, A. Kanamori, K. Sasaki, T. Nishi, M. Fukuda, J. Biol. Chem. 271 (1996) 3684.
[27] P.H. Lopez, R.L. Schnaar, Curr. Opin. Struct. Biol. 19 (2009) 549.
[28] N. Kawashima, S.J. Yoon, K. Itoh, K. Nakayama, J. Biol. Chem. 284 (2009) 6147.
[29] V.S. Shirure, K.A. Henson, R.L. Schnaar, L. Nimrichter, M.M. Burdick, Biochem. Biophys. Res. Commun. 406 (2011) 423.
[30] P.D. Bos, X.H. Zhang, C. Nadal, W. Shu, R.R. Gomis, D.X. Nguyen, A.J. Minn, M.J. van de Vijver, W.L. Gerald, J.A. Foekens, J. Massagué, Nature 459 (2009) 1005.
[31] B. Domon, C.E. Costello, Glycoconj. J. 5 (1988) 397.

Please cite this article in press as: Steenackers A, et al. Expression of G_{D3} synthase modifies ganglioside profile and increases migration of MCF-7 breast cancer cells. C. R. Chimie (2011), doi:10.1016/j.crci.2011.05.004