Signal rewiring in Met-dependent cancers

by

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ABSTRACT

The Met receptor tyrosine kinase (RTK) plays critical roles in mammalian development and physiology. Met has been shown to activate diverse intracellular signaling pathways that modulate an invasive growth program upon stimulation with its ligand, hepatocyte growth factor (HGF). The Met RTK was initially discovered as a component of an oncogenically transforming fusion protein, TPR-Met, and elevated expression of Met has been associated with advanced and aggressive breast, lung, esophagogastric, colorectal and renal cancers. Attempts to improve patient outcomes in the clinic by targeting Met activity have not, however, improved patient outcomes in the majority of cases. Retrospective analyses have identified genetic aberrations that lead to Met constitutive activation, including *MET* gene amplification, as predictive of patients who are most likely to respond to Met-directed therapy in a clinical setting. Most epithelial and solid cancer derived cell lines undergo cell scattering or migration and invasion upon stimulation with HGF, but constitutive Met activity in *MET*-amplified cell lines has been shown to drive cell proliferation. Cancer-derived cell lines carrying *MET* genomic amplification have thus been established as a model system for studying oncogenic signaling in Met-dependent cancers.

Heterotypic activation of other receptor tyrosine kinases has been observed in *MET*-amplified cells, particularly those of the epidermal growth factor receptor (EGFR) family. Combinatorial targeting of EGFR and Met has been proposed as a mechanism of potentiating the effects of inhibitors against either receptor on its own, and while this so far is poorly successful in the clinic, preclinical models support that crosstalk between Met and EGFR-family RTKs contributes to

cancer-associated signaling downstream of Met. The manner in which heterotypic signaling between Met and EGFR family RTKs contributes to cancer cell fitness remains unclear.

We hypothesized that elevated Met kinase activity in *MET*-amplified cells leads to signal rewiring to promote a proliferative outcome over cell migration and invasion. This was examined using a panel of *MET*-amplified cancer cell lines to show that signaling between Met and EGFR family RTKs converges on HER3, a member of the EGFR family with impaired catalytic function that is thought to act as a pseudokinase. Depletion of HER3 by RNA interference impairs proliferation and early tumour outgrowth in *MET*-amplified cells, supporting a central role for Met-HER3 signaling in *MET*-amplified and Met-dependent cancers. While canonical Met-dependent intracellular signaling pathways are not impaired by loss of HER3, we identify a novel HER3-interacting protein, MPZL3, whose expression is HER3-dependent in *MET*-amplified cancer cells. Overexpression of MPZL3 partially rescues the loss of proliferation upon HER3 depletion, and loss of MPZL3 phenocopies loss of HER3. We conclude that a contribution of HER3 to proliferation in *MET*-amplified cells proceeds through a novel MPZL3-dependent signaling mechanism.

To identify candidate Met-dependent phosphosites that could additionally contribute to proliferative signaling we used mass spectrometry-based phosphoproteomics. We have observed the Met-dependent phosphorylation of several RTK-interacting proteins not previously associated with Met signaling as well as the phosphorylation of adhesion and junctional proteins, including the MPZL3 homologue MPZL1. In this thesis we also report the development of substrate-labeling mutants of the Met receptor as a tool to discover new substrates for the Met RTK. These results support a model in which the induction of noncanonical Met-dependent signaling is critical in promoting Met-dependent oncogenesis.

RÉSUMÉ

Le récepteur tyrosine kinase Met joue un rôle fondamental dans le développement et l'homéostasie tissulaires. L'activation par son ligand, l'HGF (*hepatocyte growth factor*), induit une variété de réponses biologiques telles que la prolifération et la migration cellulaire. L'implication de Met dans la tumorigenèse a été suggérée dès sa découverte en tant que protéine chimérique issue de la fusion avec le gène *Tpr*. De plus, la surexpression de Met est observée dans les cancers du sein, du poumon, colorectal, gastrique et rénal. Diverses altérations génétiques du gène *MET* peuvent induire une telle surexpression dont l'amplification du gène lui-même, favorisant ainsi une activité kinase constitutive et indépendante de sa stimulation par son ligand. Plusieurs stratégies d'inhibition de Met ont été développées par les laboratoires pharmaceutiques, considérant la surexpression de Met comme facteur prédictif de la réponse aux traitements. Toutefois, leur efficacité à améliorer la survie globale des patients reste à démontrer. Étant donné que l'activité constitutive de Met est fortement associée à la prolifération des cellules cancéreuses, des modèles cellulaires dérivés de cancers où le gène *MET* est amplifié ont été développés afin de caractériser la signalisation cellulaire oncogénique régulée par Met.

Une observation de ces modèles est l'influence du dialogue entre Met et les récepteurs tyrosine kinase comme l'EGFR (*epidermal growth factor receptor*), dont les réseaux de signalisation sont très similaires. Il a été montré que l'activation de Met permet de compenser l'inhibition de l'EGFR. Par conséquent, plusieurs essais cliniques visent à évaluer la combinaison d'inhibiteurs de ces deux récepteurs. Cependant, le dialogue complexe et dynamique entre Met et les autres récepteurs tyrosine kinase est loin d'être bien compris.

Nous proposons que l'activité constitutive de Met dans les cancers arborant une amplification du gène *MET* favorise les voies de signalisation cellulaire impliquées dans la prolifération plutôt

que dans la migration. Nous avons démontré que le dialogue entre Met et l'EGFR converge vers HER3, un récepteur tyrosine kinase dépourvu d'activité kinase, dans une variété de lignées cellulaires cancéreuses. En effet, l'inhibition de l'expression de HER3 par interférence à l'ARN altère la prolifération et diminue la croissance de xénogreffes de cellules cancéreuses arborant une amplification de *MET*, identifiant l'importance du dialogue Met-HER3 dans ce contexte. Bien que les voies de signalisation classiques de Met ne soient pas altérées par l'inhibition de HER3, nous avons identifié une nouvelle protéine associée à HER3, appelée MPZL3, dont l'expression est régulée par HER3 dans les cellules cancéreuses ayant une amplification du gène *MET*. De plus, nous avons démontré que la surexpression de MPZL3 permet de rétablir partiellement la prolifération cellulaire lorsque HER3 est inhibé, et que la diminution de l'expression de MPZL3 phénocopie l'inhibition de HER3. Ces résultats suggèrent que la régulation de la prolifération des cellules cancéreuses avec amplification de *MET* requiert la contribution MPZL3, un nouvel acteur dans le dialogue Met-HER3.

Afin de mieux caractériser les mécanismes impliqués dans la régulation de la prolifération par Met, nous avons réalisé une analyse du phosphoprotéome par spectrométrie de masse. Nous avons identifié la phosphorylation de plusieurs protéines associées aux récepteurs tyrosine kinase mais dont la relation avec Met n'a jamais été rapportée, incluant la protéine MPZL1 homologue de MPZL3. Nous avons développé une nouvelle approche moléculaire pour l'identification de substrats de la kinase Met basé sur le marquage par thiophosphate. Ces résultats mettent en lumière un réseau de signalisation cellulaire distinct dans un contexte d'amplification du gène *MET*, qui implique un dialogue entre Met, HER3 et MPZL3 et joue un rôle dans la prolifération des cellules cancéreuses.

ACKNOWLEDGEMENTS

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I deeply appreciate the friendship and collegial support I have received from my colleagues in the Park laboratory over the years. I have been privileged to work with fantastic scientists throughout my time as a student and have gotten to experience a diverse range of approaches to science through the changing environment of the laboratory as new people joined. I would particularly like to thank Monica Naujokas, Nancy Lateurrer, Camby Chhor and Valentina Ramos for all of your assistance as lab managers over the years—if I have helped anyone as much as each of you has helped me, I have done something right. I would like to thank Dr. Veena Sangwan for always showing an interest in my science and my thinking, and for all the mentorship and support she has offered over the years. I would like to thank Drs. Serhiy Havrylov, Pascal Peschard, Mat Gigoux, Tina Gruosso, Elena Kuzmin, Genevieve Deblois, Hellen Kuasne, and Anne-Marie Fortier for endlessly fascinating discussion, scientific expertise, friendship, encouragement and camaraderie. You have all been role models for me in many ways as a scientist and a professional, and your advice has proved invaluable in helping me navigate the world of biomedical research. Et merci beaucoup à Anne-Marie pour la traduction de mon résumé !

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PREFACE

This thesis is written in the traditional format. It is divided into five chapters as follows:

Chapter 1: Literature review

Chapter 2: Results

Chapter 3: Discussion

Chapter 4: Materials and methods

Chapter 5: References

Contribution to original knowledge

The work in this thesis describes signaling output of the Met RTK in the gene-amplified setting. We show that crosstalk between Met and the EGFR family of RTKs is a convergent feature of *MET*-amplified cells, and demonstrate that this primarily involves a Met-HER3 signaling axis. We further identify novel HER3-dependent transcripts in the *MET*-amplified setting, notably including the gene MPZL3, which is required for proliferation downstream of HER3 in *MET*-amplified cells. We further identify a novel protein-protein interaction between HER3 and MPZL3, as well as a large number of novel candidate Met-dependent phosphosites in *MET*-amplified cells. We show that HGF stimulation of *MET*-amplified cells elicits accelerated tumour growth and actin cytoskeletal remodeling in a manner that is not dependent on increasing Met-dependent phosphorylation, but may instead impact receptor trafficking. Finally, we generated and characterized novel Met mutants capable of utilizing modified ATPγS analogues for substrate-labeling assays.

Publications arising from this thesis

Chapter 2 contains material presented in the following articles:

Stern YE, Duhamel S, Al-Ghabkari A, Monast A, Fiset B, Walsh LA, Park M. Met-HER3 crosstalk supports proliferation via MPZL3 in *MET*-amplified cancer cells. *Manuscript in preparation.*

Stern YE, Duhamel S, Al-Ghabkari A, Park M. Novel substrates of the Met receptor via oncogenic gene amplification. *Manuscript in preparation*.

Contribution of authors

A. Monast performed the injections, measurments and necropsies for the *in vivo* experiments described in Figures 2.7 and 2.20. The patient-derived xenograft (PDX) GCRC1994 described in Figure 2.8 was collected by J. Spicer of the McGill University Health Centre and banked by M. Souleimanova and H. Zhao. PDX GCRC1994 tumours and tumour-derived cell line were generated by M. Dankner and P. Siegel. Initial characterization of Met protein levels and phosphorylation in PDX tumour lysates was performed by T. Golenar. Library preparation and RNA sequencing described in Figure 2.10 was performed by the Centre d'Expertise et de Services at Génome Québec, and sequencing data was aligned by B. Fiset and L. Walsh. Protein extraction and trypsinization, phosphotyrosine or titanium dioxide (Ti_2O) enrichment, mass spectrometry, and peptide mapping described in Figure 2.16 were performed by E. Kanshin and P. Thibault of the Université de Montréal. Haemotoxylin and eosin staining described in Figure 2.20 was performed by the Histology Innovation Platform of the Goodman Cancer Research Centre at McGill University. Immunofluorescence and cell-surface biotinylation described in figure 3.2 was performed by E. Bell. All other experiments described in this thesis were performed by Y. Stern.

Additional publications:

Sung VYC, Duhamel S, Knight JF, Johnson RM, **Stern YE**, Saleh SM, Savage P, Monast A, Zuo D, Park M. Co-dependency for MET and FGFR1 in mesenchymal basal triple negative breast cancers. *Manuscript under review*.

Kuasne H, Fortier AM, Pisano P, Zhao H, **Stern YE**, Zuo D, Souleimanova M, Pilon V, Monast A, Ramírez CM, Issac M, Bertos N, Bailey S, Cools-Lartigue J, Bass A, Ferri L, Sangwan V, Park M. 3D organoids drug screening for the identification of combined therapeutic strategies in esophagogastric adenocarcinoma. *Manuscript in preparation*.

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LIST OF ABBREVIATIONS AND NOMENCLATURE

Gene names are italicized while protein names are not, unless otherwise specified (in Table 1.1). Human gene names are capitalized, and are used unless a specific viral, murine or avian gene is referred to.

A	alanine
Ala	alanine
ALK	anaplastic lymphoma kinase
ANOVA	analysis of variance
ARF6	ADP ribosylation factor 6
Arg	arginine
Asn	asparagine
Asp	aspartate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATPγS	adenosine-5'-O-(3-thiotriphosphate)
BCR	breakpoint cluster region
BRAF	v-Raf murine sarcoma viral oncogene homolog B, gene encoding the B-Raf kinase
BRC	Bioresource Research Center
BSA	bovine serum albumin
C	cysteine
C-	carboxy (terminal)
Cbl	casitas B-lineage lymphoma

CCLE	Cancer Cell Line Encyclopedia
cDNA	complementary DNA
CEP7	centromere of chromosome 7
CML	chronic myelogenous leukemia
COSMIC	Catalogue of Somatic Mutations in Cancer
Crk	C10 regulator of kinase
CrkL	C10 regulator of kinase-like
CTK	cytoplasmic tyrosine kinase
Cys	cysteine
D	aspartate
Δ	delta (lost)
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribosenucleic acid
E3	enzyme 3 (of the ubiquitin ligase system)
ECL	electrochemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	egtazic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERRB2	Erb-B2 receptor tyrosine kinase, the gene encoding HER2
ERBB3	Erb-B3 receptor tyrosine kinase, the gene encoding HER3
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FLT3	fms-like tyrosine kinase 3
FLT4	fms-like tyrosine kinase 4, the gene encoding VEGFR3
FRS2	fibroblast growth factor receptor substrate 2
G	glycine
Gab1	growth factor receptor binding protein-2 associated binder 1
GDP	guanosine diphosphate
GFP	green fluorescent protein
GGA3	Golgi-associated, gamma adaptin ear containing, ARF-binding protein 3
Gln	glutamine
Gly	glycine
Grb2	growth factor receptor binding protein-2
GTP	guanosine triphosphate
H	histidine
H&E	hematoxylin and eosin
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	human epidermal growth factor receptor 2
HER3	human epidermal growth factor receptor 3
His	histidine

HGF	Hepatocyte Growth Factor
HGNC	HUGO gene nomenclature committee
HGS	the gene encoding hepatocyte growth factor receptor substrate
HRS	hepatocyte growth factor receptor substrate
HRP	horseradish peroxidase
I	isoleucine
Ig	immunoglobulin
IGF1	insulin-like growth factor 1
IL	interleukin
Ile	isoleucine
IRS2	insulin receptor substrate 2
JNK	Jun N-terminal kinase
K	lysine
kDa	kilodalton
KDR	kinase insert domain receptor, gene encoding VEGFR2
KIT	proto-oncogene c-KIT, the gene encoding the c-Kit/stem cell factor RTK
KRAS	Kirsten rat sarcoma gene, encoding the K-Ras protein
L	leucine
LC-MS/MS	liquid chromatography with tandem mass spectrometry
Leu	leucine
Lys	lysine
M	molar
MEM	modified Eagle medium
mg	milligram
μg	microgram
mM	millimolar
μM	micromolar
MMTV	murine mammary tumour virus
ml	milliliter
μl	microliter
MET	Met proto-oncogene, the gene encoding Met receptor tyrosine kinase
Met	Met receptor tyrosine kinase (or hepatocyte growth factor receptor)
MNNG	methyl-*N'*-nitro-*N*-nitroso-guanidine
MPZL1	myelin protein zero-like 1
MPZL3	myelin protein zero-like 3
MSP	macrophage-stimulating protein
MST1R	macrophage-stimulating protein 1 receptor
N	asparagine
N-	amino (terminal)
N-WASP	neural Wiskott-Aldrich syndrome protein
N6	adenine nitrogen 6
N6-Et-ATPγS	N6-phenethyl-adenosine-5'-O-(3-thiotriphosphate)
N6-Fu-ATPγS	N6-furfuryl-adenosine-5'-O-(3-thiotriphosphate)

N6-Ph-ATPγS	N6-phenethyl-adenosine-5'-O-(3-thiotriphosphate)
NCBI	National Center for Biotechnology Information
Nck	non-catalytic region of tyrosine kinase adaptor protein
NGF	nerve growth factor
nM	nanomolar
NSCLC	non-small-cell lung cancer
P	proline
PAX3	paired box gene 3
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PDK1	PIP_3-dependent kinase 1
PDX	patient-derived xenograft
PHA	PHA-665752
PI3K	phosphoinositide 3-kinase
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3 kinase catalytic subunit alpha
PIP_3	phosphatidylinositol-3,4,5-triphosphate
PKA	protein kinase A
PNBM	*p*-nitrobenzyl mesylate
Pro	proline
pS	phosphoserine
pSer	phosphoserine
PSI	plexin, semaphorin and integrin
pT	phosphothreonine
PTB	phosphotyrosine-binding
PTEN	phosphatase and tensin homolog
pThr	phosphothreonine
PTP1B	protein tyrosine phosphatase 1B
PTPRZ1	receptor-type tyrosine-protein phosphatase zeta
pTyr	phosphotyrosine
PVDF	polyvinylidene fluoride
pY	phosphotyrosine
PyMT	Py papovavirus middle T protein
Q	glutamine
qRT-PCR	real-time quantitative reverse-transcription polymerase chain reaction
R	arginine
RET	ret proto-oncogene
RPMI	Rosa Parks Memorial Institute 1640 medium
RNA	ribosenucleic acid
RON	Recepteur d'Origine Nantais
ROS1	proto-oncogene tyrosine-protein kinase ROS
RTK	receptor tyrosine kinase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shc	Src homology-2 domain-containing

shRNA	short hairpin RNA
SILAC	stable isotope labeling by amino acids in cell culture
SIMPL	split intein-mediated protein ligation
siRNA	short interfering RNA
SH2	Src-homology 2
SH3	Src-homology 3
SHP2	Src-homology 2 domain-containing phosphatase 2
SOS1	son of sevenless 1
STAT3	signal transducer and activator of transcription 3
TBST	Tris-buffered saline with Tween-20
TGH	Triton-glycerol-HEPES lysis buffer
TiO_2	titanium dioxide
TKI	tyrosine kinase inhibitor
TPR	translocated promoter region
TrkA	tropomyosin receptor kinase A
Tyr	tyrosine
V	valine
Val	valine
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
Y	tyrosine

CHAPTER 1: LITERATURE REVIEW

1.1 The discovery of receptor tyrosine kinases and their roles in mammalian biology

Receptor tyrosine kinases (RTKs) are a well-studied type of cell receptor with prolific and pleiotropic roles in metazoan biology. RTKs share structural homology and exhibit conserved mechanisms of action, and their aberrant activation has frequently been implicated in human disease pathology. The Met RTK, discovered as a component of a transforming fusion oncoprotein, is implicated in aggressive and advanced human cancers and has been an active target for drug development for over twenty years. This is supported by a large body of research demonstrating how RTKs in general, and Met in particular, operate in mammalian development, physiology, and disease.

1.2 RTKs comprise a conserved gene family with functional homology

RTKs comprise a major family of genes involved in signal transduction in the metazoan lineage. Humans and other mammals have 58 RTKs grouped in 20 families by both structural and genomic similarity, most of which have 1-4 paralogous members due to multiple rounds of genome duplication in the vertebrate lineage (Brunet et al., 2016; Holland and Ocampo Daza, 2018; Lemmon and Schlessinger, 2010). Related cytoplasmic tyrosine kinases (CTKs) comprise an additional 32 genes, bringing the total human tyrosine kinome to 90 genes (Manning, 2002). RTKs are characterized by the presence of an extracellular ligand-binding domain, a single-pass transmembrane helix, and a highly conserved cytoplasmic tyrosine protein kinase domain flanked by less-ordered juxtamembrane and C-tail regions (Lemmon and Schlessinger, 2010). RTKs are characteristically activated by the binding of soluble ligands to the extracellular ligand-binding domains, inducing receptor clustering at the membrane to promote kinase domain activation

(Schlessinger, 2014). This causes the *trans*-activation of the dimerized kinase domains, which has been shown to involve multiple mechanisms, including *trans*-phosphorylation of tyrosine residues in the kinase activation loop as well as the stabilization of the active kinase conformation through allosteric interactions involving the kinase and juxtamembrane domains (Hubbard et al., 1994; Jura et al., 2009; Zhang et al., 2006). RTK kinase activity promotes the further phosphorylation of tyrosine residues in the C-tail and juxtamembrane regions of the RTK. These serve as recruitment sites for proteins containing SH2-domains or other phosphotyrosine-binding motifs, which themselves frequently

Figure 1.1: Ligand-induced receptor clustering activates RTK signaling

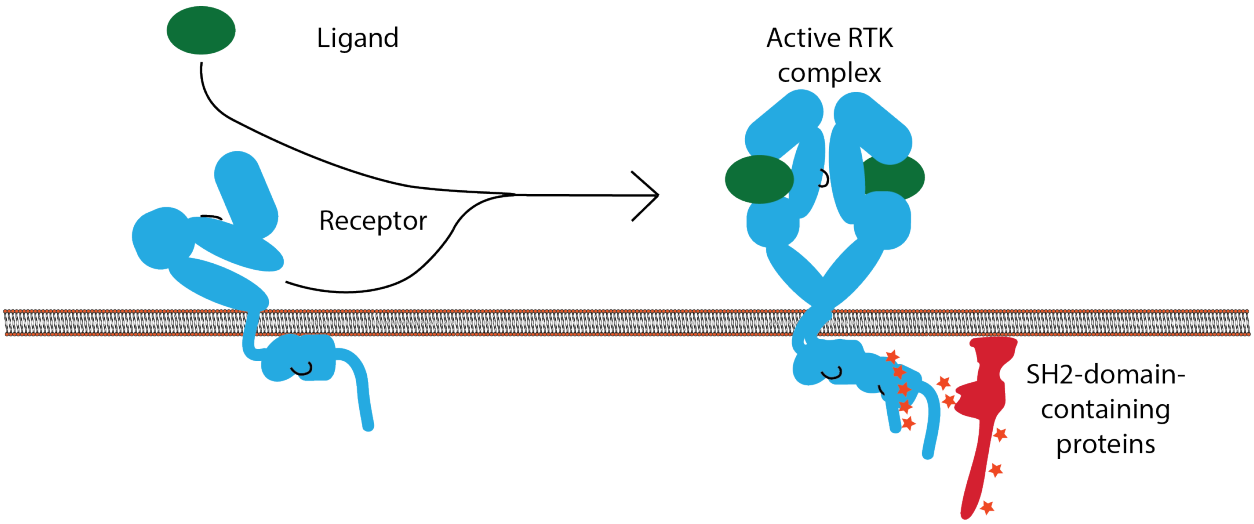


Figure 1.1: Receptor tyrosine kinase activation is characteristically driven by ligand-induced receptor clustering. *The extracellular domains of the receptor bind exogenous protein ligands such as growth factors to stabilize dimerized or oligomerized conformations of the cognate RTK. This induces the juxtaposition of the kinase domains in a conformation allosterically promoting kinase activation through interactions between the kinase and juxtamembrane domains of the receptors. The active kinase domains phosphorylate tyrosine residues in the cytoplasmic domains of the RTK, further stabilizing the activated conformation of the kinase and serving as recruitment sites for proteins containing SH2 and other phosphotyrosine-binding proteins. These can in turn be tyrosine phosphorylated, either modulating the activity of recruited substrates or serving as further binding sites to cluster and activate intracellular signal transduction proteins.*

contain tyrosines capable of being phosphorylated by the receptor (Lemmon and Schlessinger, 2010). The consequence of this series of actions is the recruitment of protein complexes to the

plasma membrane upon RTK activation, and the subsequent activation of recruited intracellular signal transduction proteins through a variety of mechanisms (Figure 1.1).

1.3 CTKs interact with RTKs and other extracellular cues

In contrast to RTKs, CTKs lack transmembrane domains and do not directly transduce extracellular cues. CTKs do not comprise a monophyletic class of tyrosine kinases themselves, and instead are broadly grouped into two sister clades, each of which also contains an RTK (Brunet et al., 2016; Manning, 2002). CTKs are generally characterized by the presence of SH2 and SH3 domains, facilitating their respective recruitment to phosphorylated tyrosine residues or proline-rich motifs in other cytoplasmic proteins. As these motifs are frequently found in RTKs or proteins recruited to the activated RTK, CTKs can themselves be recruited to activated RTK complexes to regulate their own tyrosine kinase activity. This can serve to enhance RTK-dependent tyrosine phosphorylation and downstream biological output, as observed in the recruitment of Src to the epidermal growth factor receptor (EGFR) (Maa et al., 1995; Tice et al., 1999). An additional role for CTKs downstream of RTKs may be to phosphorylate sequences inaccessible to the RTK itself. This may be due to the ability of CTKs to translocate to the nucleus or other cellular compartments more easily than RTKs (reviewed in Bagnato et al., 2020). In addition to this, differential substrate specificity for the CTKs ZAP-70 and Lck is critical to the sequential phosphorylation of substrates in T-cell receptor signalling, raising the possibility that CTKs expand the repertoire of tyrosine phosphorylation downstream of RTKs by activity towards weak substrates of the RTK itself (Shah et al., 2016). Thus, CTKs and RTKs are collectively responsible for catalyzing protein tyrosine phosphorylation in the eukaryotic cell and cooperate to achieve this at the molecular level.

1.4 The discovery of tyrosine kinases as transforming viral oncoproteins

Tyrosine kinases were initially discovered through the study of viral oncogenes, defined as genes capable of inducing cancer-like growth upon their expression in cells. Utilizing murine and avian viruses capable of inducing sarcomas in infected animals, biologists in the 1970s identified and characterized several genes capable of transforming cells upon viral transduction (Hunter, 2015). Among the genes initially identified by this method were the gene encoding the Py papovavirus middle T (PyMT) antigen and the gene encoding avian Rous sarcoma virus v-Src-transforming protein (v-Src) (Beemon and Hunter, 1977; Brugge and Erikson, 1977; Collett and Erikson, 1978; Hutchinson et al., 1978; Ito, 1979; Treisman et al., 1981; Turler, 1980). The phosphorylation of proteins to modulate their activity had been long established, but at the time this was thought to occur exclusively on serine and threonine residues. Tyrosine phosphorylation was first demonstrated in PyMT (Eckhart et al., 1979). The transforming activity of v-Src was linked to its enzymatic activity using proteins derived from viral subclones carrying temperature-sensitive mutations and transcribed *in vitro*, and this activity was shown to promote tyrosine phosphorylation of v-Src and its substrates, including PyMT (Erikson et al., 1980; Levinson et al., 1978). It was subsequently shown that the transforming capacity of PyMT was derived from the recruitment and activation of c-Src in transformed cells (Courtneidge, 1986; Courtneidge and Smith, 1983).

While the characterization of v-Src-dependent tyrosine phosphorylation and its transforming capacity progressed, the v-Src gene was discovered to have derived from an avian gene encoding a soluble protein tyrosine kinase with orthologues in the mammalian lineage (Stehelin et al., 1976). This cellular version of Src (*c-Src*) had been rendered constitutively activate upon its

inclusion in the viral genome. This was due to the loss of a tyrosine in the C-tail that inhibits the kinase upon its phosphorylation (Sefton and Hunter, 1986). In parallel to this work, a number of other murine and avian viral genomes were searched for transforming genes with tyrosine kinase activity (Hunter, 2015). One of these, the Abelson-murine leukemia virus, was also shown to encode a tyrosine kinase (Witte et al., 1980a; Witte et al., 1980b). This was similarly mapped to a gene found in humans, *c-Abl*, which was similarly shown to have lower kinase activity than the viral kinase (Witte et al., 1979) (Konopka et al., 1984). The convergent identification of tyrosine phosphorylation as a transforming event in these model systems led to an intensive effort to identify additional tyrosine kinases oncogenically transformed by viral capture and led to the identification of a number of potentially oncogenic tyrosine kinases.

The discovery that oncogenic murine viruses could transform cells using activated mutants of mammalian genes led to a search for additional mammalian proto-oncogenes that, upon activation, could transform cells. Of particular interest was the search for oncogenes in human cancers, which were known to be caused by genetic lesions within the human genome in the absence of viral transduction. The human orthologues of the cellular Ras proto-oncogenes were shown to transform human cells when overexpressed in 1982, confirming the discovery of the first human proto-oncogene (Chang et al., 1982). A second seminal discovery was that the Philadelphia chromosome, a translocation of chromosome 9q to chromosome 22 identified in patients with chronic myelogenous leukemia (CML), involved the human orthologue of the *c-Abl* kinase translocating to chromosome 22. This translocation drives oncogenic transformation through expression of the resulting BCR-Abl fusion protein (Klein et al., 1982; Nowell and

Hungerford, 1960; Rowley, 1973). This showed that in CML patients, the induction of gene fusions due to chromosomal translocation could produce transforming oncogenes.

1.5 Receptor tyrosine kinases encode proto-oncogenes with diverse roles in development

Many additional genes capable of transforming cells were identified via these methods from avian or rodent tumour viruses. Some of these genes encoded tyrosine kinase proteins like v-Src and v-Abl, while others encoded tyrosine-phosphorylated transforming proteins without intrinsic tyrosine kinase activity similar to the PyMT. Due to their ability to transform cells, these were considered to be oncogenes, and carried mutations or deletions enhancing their mitogenic capacity compared to their genomic homologues (Reddy et al., 1982; Scolnick and Parks, 1973; Shih and Weinberg, 1982; Weinberg, 1983). Unlike v-Src and v-Abl, several kinases identified in this manner in avian and murine oncogenic viruses, including the kinases v-fms, v-ros and v-Erb-B, were mapped to mammalian or avian genes containing transmembrane domains (Bishop, 1983). Ongoing experiments in the 1980s would identify additional mammalian RTKs in oncogenic viruses, such as v-Sea, derived from the avian *Sea* gene, known in mammals as *MST1R* (Smith et al., 1989). These discoveries provided an important biological context for understanding RTK function and would help show the potential for targeting RTK genes as oncogenic drivers in cancer.

Additional discoveries using viral oncogenes elucidated some of the mechanics of RTK signaling in cancer, and their relationship to mitogenic signaling through growth factors. The v-Erb-B kinase was shown to be a truncated form of the transmembrane receptor for the soluble protein epidermal growth factor (EGF) (Downward et al., 1984). EGF was known at the time as a potent

inducer of early mouse eyelid opening, linked to an ability to induce cell proliferation during development (Cohen, 2008). Excitingly, the proliferative activity of EGF had already been linked to its transforming potential, and it had similarly been shown that this proliferative capacity was dependent on tyrosine kinase activity (Cohen, 2008; Ushiro and Cohen, 1980). The identification of the human homologue of v-Erb-B as the gene encoding EGFR thus presaged the discovery of critical roles for EGFR signaling a variety of human cancers. Concurrently, the transforming viral oncoprotein v-Sis was shown to have homology to a mammalian gene encoding platelet-derived growth factor (Doolittle et al., 1983; Favera et al., 1981; Waterfield et al., 1983). This showed that human growth factors, already known to be important for their roles in development and tumourigenesis, could induce cell proliferation in both contexts through the activation of RTKs.

Another important early discovery in the field of RTK biology was that the receptor for insulin, a hormone critical to physiological metabolic regulation in mature mammals including humans, was also a receptor tyrosine kinase related to EGFR and to other proto-oncogenes discovered in tumour viruses such as v-Src and v-Ros (Ebina et al., 1985; Ullrich et al., 1985). Insulin had already been shown to promote tyrosine phosphorylation of its receptor, but stimulation of cells with insulin did not affect cell proliferation in a manner similar to EGF, and it remained unclear whether the kinase activity was present in the receptor itself (Avruch et al., 1982a; Avruch et al., 1982b; Petruzzelli et al., 1982). While the related mitogen insulin-like growth factor I (IGF1) was already known to bind to an RTK, the discovery that the non-mitogenic action of insulin also utilized an RTK for signal transduction showed that RTKs can perform diverse biological functions in addition to regulating cell proliferation, and that there are important physiological roles for RTKs in mature mammals including humans.

1.6 Oncogenic RTK activation induces cancer in mouse model systems

By the early 1990s, significant evidence had accumulated implicating RTKs in diverse developmental and physiological roles as hormone receptors. In addition to the role of insulin in metabolic regulation and of EGFR in developmental cell proliferation, other molecules such as nerve growth factor (NGF), a potent inducer of neuronal ganglion outgrowth in development, in fact mediated its mitogenic activity through TrkA, another RTK (Cordon-Cardo et al., 1991). Clearly, RTKs played important and specific roles in development, although they shared common mitogenic properties and enzymatic activity. It was further known through the association of RTK activity with viral or overexpression-mediated transforming capacity that the mitogenic action of RTKs was capable of inducing neoplasms, and RTKs as well as several of their ligands had been observed to be overexpressed in human cancers (Ullrich and Schlessinger, 1990). The specific role of each RTK in development, however, and the ways in which these developmental functions could be co-opted to drive neoplastic growth, were poorly understood on the basis of these experiments alone.

Key insights into both the developmental roles of RTKs in mammals as well as their capacity to induce spontaneous mammalian tumours were derived from experiments using genetically engineered mouse models. The expression and phosphorylation of *c-neu,* an RTK with homology to EGFR, was known to be associated with its oncogenic mutation in rat tumours (Bargmann et al., 1986; Padhy et al., 1982). A genetic cassette was designed to transgenically express oncogenic *neu* from the murine mammary tumour virus (MMTV) promoter in mice. Mice expressing *c-neu* developed polyclonal tumours involving the entire mammary epithelium with accelerated kinetics compared to tumours driven by other known oncogenes, including activated forms of

Ras. Surprisingly, expression of *c-neu* in other tissues such as the epididymis failed to induce fully malignant tumours, indicating that tissue-specific expression of oncogenic RTK mutants was required for their tumourigenicity (Muller et al., 1988). When wild-type alleles of *c-neu* were expressed in transgenic mice under the MMTV promoter, tumours formed focally with long latency compared to oncogenic *c-neu*, as seen with tumours formed by oncogenic Ras and c-Myc expression (Guy et al., 1992). Similarly, when activating mutations found in oncogenic *c-neu* were introduced into its human homologue, the human EGFR 2 (HER2), expression of this transgene in mice under the MMTV promoter caused early lethality due to multifocal preneoplastic lesions across a multitude of tissues, including epididymal, renal and lung tissue (Stocklin et al., 1993). HER2 had already been observed to be overexpressed in many human cancers and was considered to be a strong candidate human oncogene (Di Fiore et al., 1987; Slamon et al., 1987). Taken together, these results showed that mutationally activated forms of receptor tyrosine kinases could act as potent oncogenes when overexpressed within the genome of mammals.

1.7 Genetically engineered and spontaneous mutations in mice differentiate roles for RTKs in mammalian development and cancer

Major insights into the developmental roles of RTKs were driven by experiments in which individual RTKs were deleted from the mouse genome to generate knockout models. The deletion of *EGFR* from the mouse genome decreased embryonic viability and placental growth, impaired weight gain, and delayed eyelid closure and reopening in neonatal mice (Miettinen et al., 1995). The deletion of the homologue of *c-neu* gene in mice, *ERBB2,* caused embryonic lethality at day 11 due to a failure to form the trabecular myocardium in the developing heart, while also severely impairing development of the neural crest and reducing axonal projection

from neural crest-derived cells (Lee et al., 1995). By contrast, deletions of the insulin receptor resulted in normal uterine development, but neonatal mice were consistently born underweight, and died within days of birth due to diabetic ketoacidosis and a concomitant failure to gain post-natal weight at the rate of their control littermates. This was seen as confirmation that the insulin receptor primarily acts as a regulator of metabolic physiology, and plays less important roles during embryonic development (Accili et al., 1996; Joshi et al., 1996).

Additional mouse models continued to elaborate diverse roles for RTK signaling in animal development. The development of methods to generate viable mice lacking RTKs required for early stages of development provided critical insights into the pleiotropic roles of many RTK genes. The *KIT* gene, encoding the stem cell factor RTK (c-Kit), which was identified as the genomic complement of the viral oncogene v-Kit, was mapped to the *W* genomic locus, long-associated with early lethality in mice (Chabot et al., 1988; Zsebo et al., 1990). The lethality associated with this mutation could be rescued by the transplantation of fetal liver tissue from wild-type mice, rescuing the macrocytic anemia that was killing neonatal pups, but this method of studying adult phenotypes of *KIT*-deficient mice was not an experimentally robust system (Bernstein et al., 1990). The identification of a naturally-occurring strain of mutants whose progeny survived to adulthood showed that *KIT* mutants also lacked B- and T-cells, showing that *KIT* played a major role in lymphoid precursor specification, and not just in expansion of the hematopoeitic lineage generally (Waskow et al., 2002). While the discovery of spontaneously occurring mutants enabled the elucidation of tissue-specific roles for signaling downstream of c-Kit, for most other RTKs tissue-specific physiological and developmental roles have been reported

using tissue-specific expression of gene-editing proteins (discussed below in the context of the Met RTK).

1.8 Discovery of TPR-Met, an RTK-derived oncogene identified in human cells

The Met receptor tyrosine kinase was discovered by an experimental strategy designed to identify additional human oncogenes. Human osteogenic sarcoma (HOS) cells treated with *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine (MNNG) were presumed to harbour numerous cancer-associated oncogenic lesions including chromosomal translocations, and human DNA isolated from these cells was screened for transforming potential by focus formation in NIH-3T3 cells to isolate oncogenes (Cooper et al., 1984a). A gene isolated from MNNG-treated HOS cells that could reproducibly transform NIH-3T3 cells was shown to have kinase activity and arise from a translocation between chromosomes 1 and 7 (Cooper et al., 1984b; Park et al., 1986). The fusion product was named TPR-Met, after the translocated promoter region (TPR) from chromosome 1 found fused to the kinase mutagenized by MNNG (abbreviated as Met). TPR encodes a leucine zipper that dimerizes the fused Met kinase domain, constitutively activating the kinase (Rodrigues and Park, 1993). The proto-oncogene from which TPR-Met was derived was determined to encode a receptor tyrosine kinase by sequence similarity to other growth factor receptors identified at the time (Park et al., 1987). Subsequent experiments showed that the Met RTK was the receptor for hepatocyte growth factor (HGF) (Bottaro et al., 1991). HGF is an activator of cell proliferation in mature liver cells in cell culture and under wound-healing conditions (Nakamura et al., 1989; Nakamura et al., 1986). A separate line of research had led to the discovery of a fibroblast-derived secreted protein that induced epithelial cell scattering from colonies grown in tissue culture, with the concurrent downregulation of desmosomal junctions.

A majority of normal epithelial cell lines responded to stimulation with this "scatter factor," although tumour-derived cell lines largely did not exhibit this response (Stoker et al., 1987). It was discovered that both functions, the mitogenic capacity for which HGF was named and the scatter function assigned at the time to a "scatter factor," were activated by the same ligand binding to Met (Naldini et al., 1991). Met also showed high similarity to the avian viral oncogene *v-Sea*, derived from the avian gene *c-Sea*, which is similarly capable of transforming fibroblasts *in vitro* (Huff et al., 1993; Smith et al., 1989). The mammalian homologue of *c-Sea*, *MST1R*, encoding the RON RTK, regulate ciliary motility in response to their ligand macrophage stimulating protein, and together with Met constitutes one of the 20 RTK families conserved across vertebrates (Brunet et al., 2016; Lemmon and Schlessinger, 2010; Sakamoto et al., 1997). These results collectively confirmed that Met, similar to several other known RTKs, was a growth factor receptor capable of inducing cell proliferation through its tyrosine kinase activity upon activation by its ligand, HGF.

1.9 The Met and RON RTKs are structurally homologous and comprise an RTK family

The Met RTK, like all other members of the RTK family, is a single-pass transmembrane protein with an extracellular ligand-binding domain at the N-terminal portion of the mature protein, and a cytoplasmic region including a highly conserved tyrosine kinase domain (Figure 1.2). Met and RON share homology in their extracellular ligand-binding Sema domains and in their ligands, distinguishing them from other RTKs and characterizing the receptors as a family (Gaudino et al., 1994; Huff et al., 1993). The Sema domain contains a furin protease cleavage site, which is processed in the Golgi to generate a disulfide-linked heterodimer from the pro-Met peptide. The N-terminal alpha chain, consisting of part of the Sema domain, is required for the proper folding

of the receptor (Komada et al., 1993). The Met Sema domain interacts with the C-terminal serine protease homology domain of HGF at low affinity, while a high-affinity binding site for N-terminal and Kringle domains of HGF has been mapped to the 3rd and 4th Ig-like domains (Basilico et al., 2008; Stamos et al., 2004). In addition to this, Met and RON share an uncharacteristically long juxtamembrane including a tyrosine required for receptor ubiquitination, and two tyrosines in the short C-tail region that are required for downstream signaling (Fixman et al., 1995; Iwama et al., 1996; Peschard et al., 2004; Zhu et al., 1994). While the full-length Met protein has not been characterized using biophysical approaches, the domain architecture of the protein has been well-established using sequence analysis and structure-function approaches, and the structure of most of the extracellular domain has been established using a combination of X-ray crystallography, nuclear magnetic resonance and homology-based modeling (Gherardi et al., 2003; Kozlov et al., 2004; Park et al., 1987). Met and RON comprise a conserved family within RTKs, and are known as class VI RTKs in the 20-class subdivision of RTK genes (Robinson et al., 2000).

Early experiments showed that the full-length Met RTK constituted two disulfide-linked polypeptide chains arising from a single precursor through furin-mediated proteolysis(Giordano et al., 1989; Komada et al., 1993). This cleavage occurs in the 500 amino-acid Sema domain, which requires both chains to fold properly into a beta-propeller structure (Gherardi et al., 2003; Stamos et al., 2004).

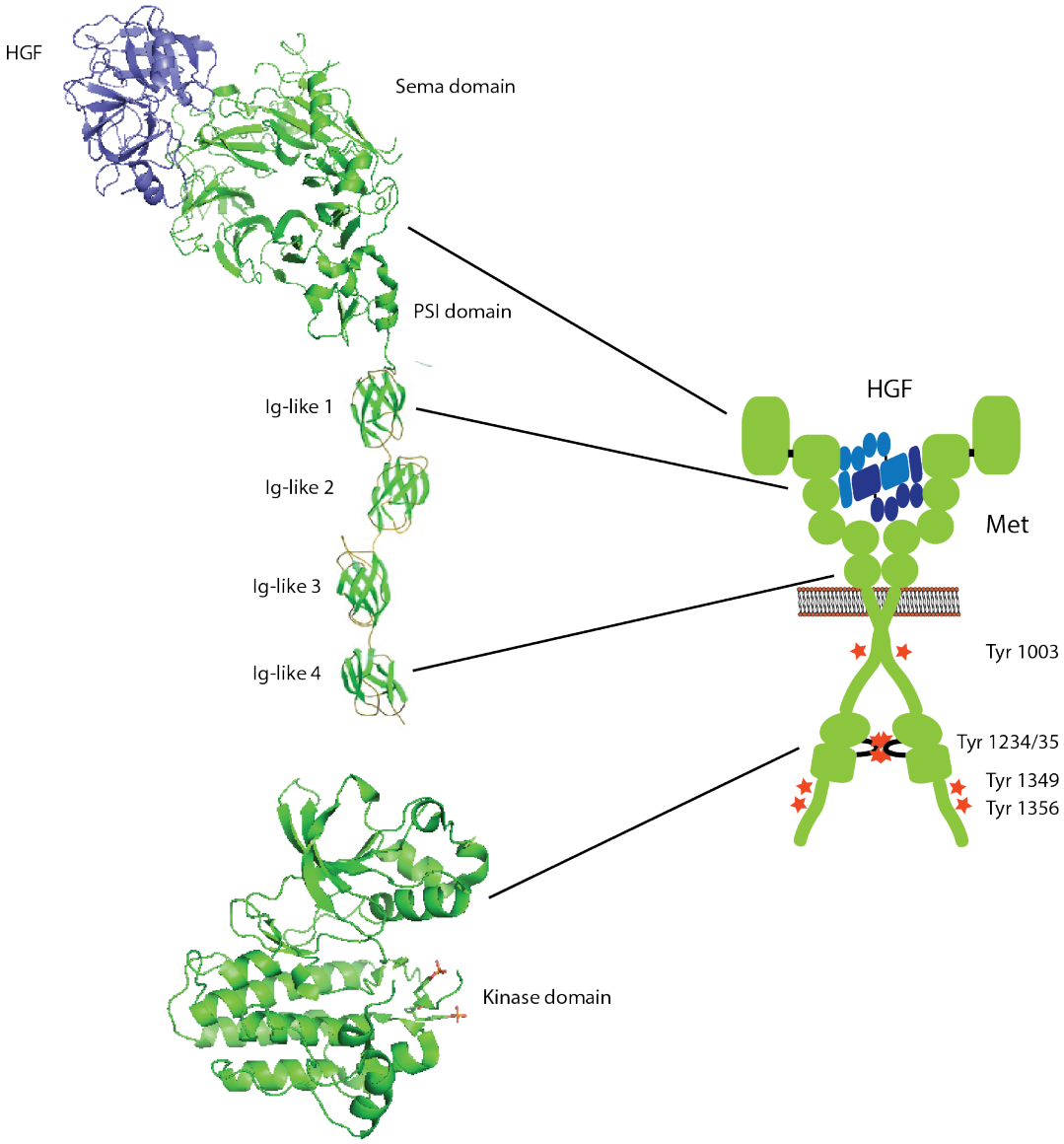


Figure 1.2: Met domain structure. *The Sema domain mediates contacts with the serine protease-like domain of the Met ligand HGF (blue), while the N-terminal and first Kringle domains of HGF bind to the 3rd and 4th Ig-like repeats in Met (Basilico et al., 2008; Stamos et al., 2004). A conserved PSI domain connects the Sema domain to the 4 Ig-like repeats. A transmembrane alpha helix connects the highly structured extracellular portion of the Met RTK to the largely soluble juxtamembrane cytoplasmic domain, which contains the conserved Cbl binding site (Tyr 1003). The kinase domain of Met is highly conserved and extensively characterized, containing two tyrosines phosphorylated in the fully activated state shown in this figure (PDB ID: 4IWD). The short C-tail region contains two tyrosines (Tyr 1349 and 1356) that mediate the recruitment of SH2-domain-containing adaptors, scaffolds and signaling effector proteins.*

Sema domains are primarily found in semaphorin secreted and transmembrane proteins involved in axon guidance and other cellular motility and adhesion processes. Sema domains are also found in transmembrane plexin molecules, which act as receptors for semaphorins, in addition

to Met and RON (reviewed in Alto and Terman, 2017). In the semaphorin-plexin signaling system, Sema domains of compatible proteins bind to each other to mediate heterophilic interactions. In the case of Met and RON, the Sema domain constitutes the primary binding site for their respective ligands, HGF and macrophage-stimulating protein (MSP) (Gherardi et al., 2004; Stamos et al., 2004). Met has also been shown to interact directly with plexin B1, leading to suppression of Met phosphorylation. The association between Met and plexin B1 may proceed through protein-protein contacts mediated by the Sema domains of both receptors, further establishing a critical role for this domain in regulating Met activity (Giordano et al., 2002; Lai et al., 2009; Soong and Scott, 2013).

1.10 The Met RTK initiates an invasive program during animal development

The activation of Met signaling by its ligand, HGF, also known as scatter factor, was initially studied *in vitro* using primary and immortalized cell lines, and primarily induced HGF-dependent cell dispersal in most epithelial cell lines (Stoker et al., 1987). Early experiments linked HGF-dependent epithelial cell scattering to cell invasiveness in cancer-derived cell lines, or to the formation of tubular structures in cells cultured in 3-dimensional collagen matrices with fibroblast-derived HGF (Montesano et al., 1991; Weidner et al., 1990). Further experiments showed that HGF could promote angiogenesis in endothelial cells, indicating a role for Met-dependent morphogenic signaling in homeostatic as well as developmental processes (Rosen and Goldberg, 1997). HGF-dependent angiogenesis was subsequently shown to synergize with signaling through vascular endothelial growth factor (VEGF) RTKs, and to play a role in tumour associated angiogenesis (Abounader et al., 2002; Xin et al., 2001). Conversely, VEGF-dependent activation of VEGFR2 suppresses Met phosphorylation and HGF-dependent tumour cell invasion

in glioblastoma, showing that HGF and VEGF can drive antagonistic biological outputs in some contexts (Lu et al., 2012). These experiments supported a role for dynamic HGF-Met signaling in the invasive biology of cancer cells associated with metastasis under pathological conditions, and with a likely role in tissue morphogenesis either during development or in adult homeostasis.

Genetically engineered knockout mice were used to confirm several roles for the Met RTK in mammalian development. Whole-body genetic deletion of *HGF* or *MET* results in early embryonic lethality with reduced proliferation and enhanced apoptosis in the liver, a failure of myogenic precursor cells to migrate from somites to the developing limb buds, and a failure of the trophoblast cells to invade the maternal decidua (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). Experiments using explant tissues from *HGF*-deficient mice further demonstrated that HGF acts as a chemoattractant for limb bud innervating axons, demonstrating a role for HGF-Met signaling in axon guidance as well (Ebens et al., 1996). Analysis of the expression of *HGF* and *MET* in the developing limb bud showed that *MET*-expressing epithelial somite cells were juxtaposed to *HGF*-expressing mesodermal tissue throughout their migration to the limb bud, and that deficient myogenesis in *PAX3*-deficient mice arises due to a lack of downstream *MET* expression (Yang et al., 1996). These collectively showed important roles for HGF-mediated Met signaling in promoting developmental invasion and chemotactic migration.

1.11 HGF-Met signaling regulates wound healing in adult homeostasis

The development of tissue-specific expression cassettes for the *Cre* recombinase system of gene editing permitted the study tissue-specific roles of essential genes in both development and adult homeostasis. This was particularly useful in elaborating the diverse developmental roles played

by essential RTK genes such as *EGFR* and *KIT*, and in identifying important roles for HGF-Met signaling in wound healing processes and immune function. Conditional expression of the *Cre* cassette in liver tissue was used to delete *MET* in adult mice, and wound-induced liver regeneration was impaired in *MET* knockout mice in comparison to controls, with a failure of liver cells to undergo proliferation (Borowiak et al., 2004).

In addition to its function in liver regeneration, HGF-dependent activation of Met has been shown to mediate the recruitment of neutrophils to murine tumours in mice with depletion of *MET* in the hematopoietic lineage. This effect has been shown to specifically occur in anti-tumour neutrophils, while inflammatory pro-tumour neutrophils are recruited by orthogonal signaling pathways (Finisguerra et al., 2015). Conditional deletion of *MET* in epithelial thymic cells has also been reported to impair the development of regulatory T cells (Su et al., 2018). The role of Met signaling in liver regeneration and in broader wound-healing processes has been extensively studied with the goal of therapeutic application. By contrast, the biology of Met signaling in the immune system is less fully understood and remains a priority for the field of cancer biology, given the increasing relevance of immunomodulatory agents in cancer therapy.

1.12 Mechanisms of RTK-dependent signaling

Coordinated action in biology requires the regulation of one domain of biological state by the conditions of a separate one; thus, mechanisms of signal transduction involve the capacity to sense biological changes in one dimension and relay this information to an orthogonal one. Kinases catalyze the phosphorylation of their various substrates, so they are capable of transmitting signals regulating their own activity into the induction of phosphorylation. Thus,

the variable mechanisms governing kinase activation determine what upstream information the kinase in question transduces, and the degree of phosphorylation, the identity of the substrates of the kinase, and the consequences of their phosphorylation collectively determine the signaling output of the kinase. RTKs operate by the transduction of extracellular signals, primarily via the binding of soluble protein ligands, to the cytosolic and nuclear domains of the cells expressing the receptor in question via the induction of tyrosine phosphorylation.

1.13 RTK activation is regulated by extracellular signals through ligand binding

RTK activation is characterized by the clustering of receptors at the plasma membrane in a manner promoting kinase domain *trans*-activation (Ullrich and Schlessinger, 1990). This was initially shown for EGFR using purified EGF and radiolabeled receptors *in vitro* (Yarden and Schlessinger, 1987). Early experiments showed that several modes of ligand binding existed among different RTK families, and that dimerization could be initiated either by ligand- or receptor-mediated interactions, but that receptor oligomerization was common to all RTKs. It was also established that this allowed for receptors to undergo heterodimerization with compatible RTKs, and hypothesized that such heterotypic RTK activation may alter signal output (reviewed in Ullrich and Schlessinger, 1990).

RTKs related to the insulin receptor are constitutively dimerized through disulfide bonds between receptor monomers formed in the secretory pathway, but insulin stimulation promotes receptor dimerization to activate the kinase domain in experimentally-dissociated insulin receptors (Sweet et al., 1987). Furthermore, chimeric receptors in which the insulin and EGF RTK ligand-binding and cytoplasmic domains were switched showed that both ligand-

binding modes could promote activation of the other cytoplasmic domain, confirming that the receptors share common mechanisms of activation (Ballotti et al., 1989; Riedel et al., 1989). This showed that RTKs require intermolecular association for kinase activation and indicated that even in the case of constitutively dimerized receptors, ligand binding could induce kinase activation through allosteric mechanisms. Recent advances in biophysical techniques have shown that binding of insulin or IGF1 to disulfide-linked receptors promotes a rearrangement of the kinase domains to promote their *trans*-activation through allosteric contacts (Cabail et al., 2015; Gutmann et al., 2018; Li et al., 2019). Ephrin receptors have similarly been shown to form oligomeric complexes of inactive receptors, but biophysical evidence again suggests that ligand binding is capable of rearranging kinase domain dimers within the complex to drive activation (reviewed in Taylor et al., 2017). In other cases, such as for the EGFR family, the existence of preformed inactive dimers has been reported, but these ligand-free dimers engage in a manner that inhibits kinase activity (Zanetti-Domingues et al., 2018). An elegant combination of cytometric, biophysical and molecular dynamics-based approaches were used by the Kuriyan and Shaw groups to show that EGF binding disrupts these autoinhibitory associations and promotes the formation of asymmetric, activating kinase dimers (Arkhipov et al., 2013; Nicholas et al., 2013). Thus, while RTKs can form dimeric or oligomeric complexes in the absence of ligand binding, ligand-driven dimerization remains the central mechanism of RTK activation.

As described above (Figure 1.1), the activation of RTKs by their cognate ligands induces a conformational change in the receptor, leading to activation of the cytosolic tyrosine kinase domain. Kinase domains are characterized by globular N- and C-lobes separated by a flexible peptide linker of variable length. The substrates of the phosphotransfer reaction catalyzed by

the kinase bind in the cleft between the N- and C-lobes. This was first demonstrated by solving the structure for the kinase domain of protein kinase A (PKA) using X-ray crystallography, and was later shown to operate by the same principle in tyrosine kinases with the crystallization of the kinase domain of the insulin RTK (Hubbard et al., 1994; Knighton et al., 1991a; Knighton et al., 1991b). Within the kinase domain, the catalytically active state is determined by the positioning of key residues to bind the beta and gamma phosphate groups of ATP near the catalytic His-Arg-Asp motif in the C-lobe, and the accessibility of both ATP and the substrate to the catalytic cleft between the lobes. Positioning of the ATP-binding residues is regulated by the movement of an alpha helix (alpha-C) in the N-lobe, while the positioning of the catalytic loop and access to the substrate binding site is regulated by a flexible loop without secondary structure called the activation loop. In many kinases, the activation loop contains serine, threonine or tyrosine residues that can be phosphorylated to maintain the "open" conformation of the loop, and the activity of the kinase (recently reviewed in Kim et al., 2017).

1.14 Kinase domain activity is tightly regulated by intra- and intermolecular constraints

Kinase domain activation can be regulated by a diverse array of intra- and intermolecular interactions. Within the kinase domain, rotation of the N-lobe toward the C-lobe, retraction of the activation loop, and extrusion of the alpha-C helix prevent the binding of substrates and fresh ATP, inactivating the kinase. Kinases can adopt a variety of "closed" conformations, as the tight evolutionary constraints required for catalytic activity are relaxed, with many stable inactive states for the kinase (Jura et al., 2011; Modi and Dunbrack, 2019). This has been exploited extensively in the development of selective ATP-competitive inhibitors, which can lock the kinase in a relatively unique inactive conformation instead of the common active, open state. Additional

strategies for selectively directing small molecules to specific kinase domains involve targeting unique pockets in the substrate-binding clefts of certain kinases, or the utilizing the side chains of specific amino acids in less-conserved positions in the catalytic pocket of the kinase (Noble et al., 2004). In CTKs, intramolecular associations between the phosphotyrosine-binding SH2 domain and phosphorylated tyrosines in the C-terminal region were identified early on as a major mechanism of kinase inactivation (Sefton and Hunter, 1986; reviewed in Shalloway and Taylor, 1997; Sicheri et al., 1997; Xu et al., 1997). In contrast, autoinhibitory association with the juxtamembrane domain of the RTKs c-Kit, FLT3 and several other RTKs are relieved by the phosphorylation of tyrosine residues within that region, and the mutation of these residues has been shown to activate c-Kit and FLT3 in cancer (reviewed in Hubbard, 2004). Thus, intramolecular associations can stabilize inactive conformations of kinases in general, including RTKs, and the autophosphorylation of residues involved in these interactions can promote either kinase activation or inactivation.

Diverse intermolecular interactions have also been shown to play a major role in regulating kinase activity. The activity of many cytoplasmic protein kinases is regulated allosterically by protein ligands, most notably in the formation of the PKA and cyclin-dependent kinase holoenzymes (reviewed in Jura et al., 2011). Seminal experiments using the EGFR kinase domain showed that in the case of RTKs, the C-lobe of one kinase domain (the “activator” kinase) can act as the activating protein ligand for the second kinase (the “receiver” kinase) in a similar manner (Zhang et al., 2006). Thus, the ligand-induced dimerization of RTKs directly leads to the association of the cytoplasmic kinase domains in an activator-receiver dimer, illustrating a molecular mechanism whereby dimerization activates the kinase domain. Further associations of

residues within the juxtamembrane and kinase domains with the plasma membrane regulate the activity of EGFR family kinases, and similar models of kinase-membrane interactions regulating kinase activity have been proposed for the entire family of RTKs (Abd Halim et al., 2015; Arkhipov et al., 2013; Hedger et al., 2015; Michailidis et al., 2011).

1.15 Heterotypic mechanisms of RTK activation

In addition to their canonical mechanisms of activation by ligand binding and subsequent homotypic clustering and kinase activation, many RTKs can be activated in a heterotypic manner, with their activity dependent on another RTK in addition to or instead of their own ligand (reviewed in Paul and Hristova, 2019). In the simplest example of this, the activation of a single RTK may induce the transcription of ligands for other RTKs. Thus, activation of the one RTK can indirectly induce ligand-mediated activation of a second RTK (Figure 1.3A). This mechanism of RTK crosstalk is observed extensively in development and homeostasis. Alternatively, the requirement for a ligand for the second RTK may be circumvented through the activation of a CTK, or alternatively through the inhibition of tyrosine phosphatase activity restricting the activation of the second RTK (Figure 1.3B). In this example, a cytoplasmic shuttle mechanism can again indirectly induce the phosphorylation of a second RTK, either activating its kinase domain or using it as a scaffold for CTK-catalyzed phosphorylation of downstream substrates. While this mechanism of RTK transactivation has been less frequently demonstrated than the ligand-dependent, indirect mechanism described above, many examples of CTK-mediated phosphorylation of RTKs or their major scaffolding proteins have been reported, particularly in cancer cell lines. Examples include Src-mediated phosphorylation of EGFR, which potentiates EGFR-dependent signaling (Maa et al., 1995; Tice et al., 1999). Recruitment of Src to the HER2-

HER3 RTK heterodimer has also been shown to potentiate downstream signaling, although the mechanism whereby this occurs has yet to be determined (Ishizawar et al., 2007). Src also mediates phosphorylation of the Met RTK downstream of EGFR in the absence of the Met ligand HGF (Dulak et al., 2011). Src has also been shown to phosphorylate Gab1, a large multisite docking protein downstream of both Met and EGFR, and to potentiate RTK-dependent signaling through Gab1 as a scaffold for the recruitment of additional phosphotyrosine-binding proteins (Chan et al., 2003; Furcht et al., 2015). Thus, phosphorylation of RTKs or their substrates by Src-family CTKs is an established mechanism of canonical and heterotypic RTK signaling.

Additionally, RTKs have been shown to form heterodimers capable of transactivating both receptors (Figure 1.3C). Closely related RTKs, in particular homologous receptors, frequently have been reported to form functional heterodimers. An example of this can be seen in the VEGF RTK family, where VEGFR2/VEGFR3 heterodimer formation increases angiogenic sprouting and elaboration (Tammela et al., 2008). Met and Ron have been shown to form productive heterodimers capable of efficient *trans*-activation, presumably with both kinase domains contributing to RTK phosphorylation (Follenzi et al., 2000). Additionally, the association of Tie1 and Tie2 receptors is thought to promote Tie1 transactivation, and is required for vascular remodeling later in the angiogenic process (reviewed in Jeltsch et al., 2013). Extensive receptor heterodimerization within RTK families has also been reported for the Eph family of RTKs, and is a hallmark of the EGFR family of receptors in particular (discussed below) (reviewed in Taylor et al., 2017; Yarden and Sliwkowski, 2001).

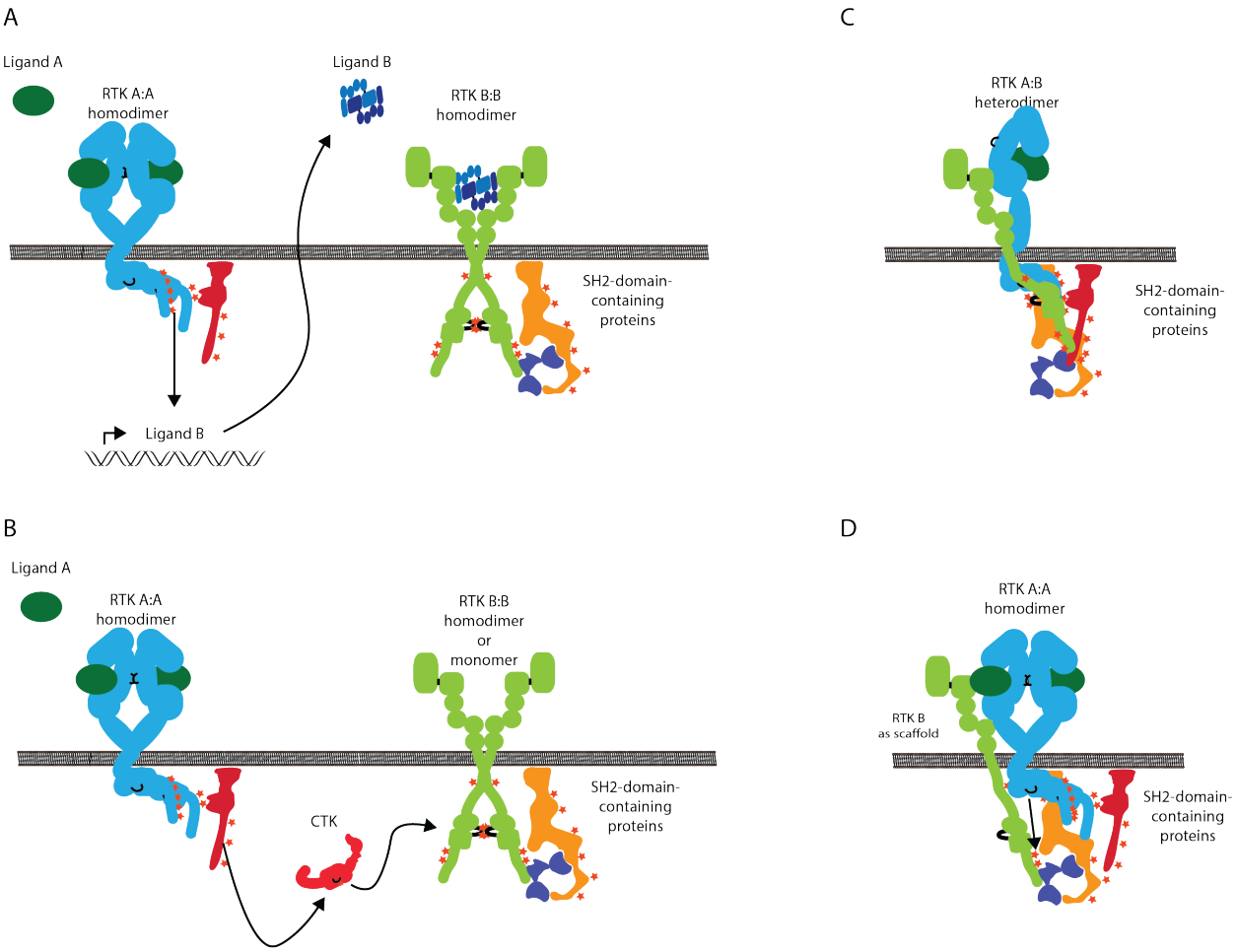


Figure 1.3: Heterotypic mechanisms of RTK activation. *(A) The activation of a second RTK (RTK B) by an upstream receptor (RTK A) can be accomplished by the induction of a ligand for RTK B upon RTK A activation, in a ligand-dependent, dissociated mechanism that depends on the kinase activity of RTK B. (B) RTK co-activation can also proceed through a cytoplasmic shuttle, in which a CTK can phosphorylate the intracellular domains of RTK B in a ligand-independent manner without interacting with RTK A. This can also be accomplished by the inhibition of cytoplasmic phosphatases. (C) The formation of RTK A:B heterodimers can lead to direct co-activation of both kinases, leading to crosstalk that is dependent on the interaction and kinase activity of RTK A and RTK B. (D) Alternatively, RTK A can recruit molecules of RTK B to the activated RTK A:A complex to act as a downstream substrate, in a mechanism that depends on RTK A:B complex formation but is independent of the kinase activity of RTK B.*

Finally, heterotypic activation of RTK signaling can involve a second receptor used as a scaffold for an associated RTK (Figure 1.3D). This mechanism of crosstalk does not require activation of the kinase domain in the second RTK, and functions similarly to the cytoplasmic shuttle mechanism using CTKs to phosphorylate the receptor and associated proteins. As few experiments directly distinguish between cooperative heterodimerization by heterotypic RTKs and the use of a second RTK as an inert scaffold, this may in fact be a major mechanism governing heterotypic RTK signaling.

1.16 The pseudokinase HER3 is phosphorylated in a heterotypic manner

A key example of the recruitment of a second RTK as a scaffold is the activation mechanism for the EGFR family member HER3, an RTK lacking intrinsic kinase activity. HER3 was identified early as a homologue of EGFR and HER2 whose kinase domain contains substitutions in key conserved residues, impairing its catalytic activity (Kraus et al., 1989; Plowman et al., 1990). Unlike the other two receptors, which primarily contain binding sites for the Grb2 adaptor protein and the SHC family of adaptor proteins (SHC1-4), the HER3 C-tail predominantly contains binding sites for the p85 regulatory subunit of the growth-factor stimulated phosphoinositide 3-kinase (PI3K) complex (Figure 1.4A) (Hellyer et al., 1998). Experiments in cells expressing the *neu* oncogene showed that cooperativity between HER2 and HER3 underlies the potent oncogenic potential of the *neu* gene (Alimandi et al., 1995; Pinkas-Kramarski et al., 1996). HER3 recruits EGFR or HER2 in response to binding its own ligand, neuregulin, and can also be recruited to EGFR homodimeric complexes in response to EGF stimulation, or to the constitutively-activated HER2 upon its genomic amplification and overexpression (Junttila et al., 2009; Littlefield et al., 2014; Sliwkowski et al., 1994; Soltoff et al., 1994; Van Lengerich et al., 2017).

This has the effect of modulating the signal transduction downstream of different dimers, with EGFR homodimers or EGFR/HER2 heterodimers strongly activating their own phosphorylation and downstream Ras-mitogen activated protein kinase (MAPK) signaling, while only transiently activating the PI3K-Akt pathway through HER3 as a scaffold. In contrast, neuregulin-induced EGFR/HER3 or HER2/HER3 heterodimers preferentially show HER3 phosphorylation and activation of the PI3K-Akt pathway (Figure 1.4B) (Van Lengerich et al., 2017). Some evidence exists for a catalytic role played by the HER3 kinase domain. HER3 has been shown to bind ATP

and catalyze phosphotransfer to dimerized RTKs *in vitro* (Shi et al., 2010; Steinkamp et al., 2014). Mice with a mutation in the ATP-binding pocket of HER3 preventing nucleotide binding are phenotypically normal, but show reduced outgrowth of neuregulin-dependent intestinal organoids, pointing to a role for HER3 catalytic activity in cancer-associated proliferation (Nguyen et al., 2020). In general, phosphorylation of tyrosine residues in HER3 requires association with EGFR or HER2 and their catalytic function, as the catalytically-impaired HER3 kinase domain is incapable of efficient tyrosine autophosphorylation or phosphorylation of exogenous substrates (Kovacs et al., 2015; Shi et al., 2010). Generally speaking, heterotypic activation of the EGFR family kinases is associated with diversifying and amplifying signal output, and can be considered as a model for obligate RTK crosstalk in physiology and disease (reviewed in Yarden and Sliwkowski, 2001).

1.17 Recruitment of signaling effector proteins to the activated RTK complex

RTKs mediate signal transmission to other molecules via tyrosine phosphorylation. All RTKs contain tyrosine residues in their own cytoplasmic regions that can be phosphorylated upon receptor activation (Lemmon and Schlessinger, 2010). Autophosphorylation of tyrosine residues in the activation loop of the kinase domain were identified early as major regulators of catalytic activity, and crystal structures of the insulin RTK showed that phosphorylation of these residues stabilized the open conformation of the receptor (Hubbard, 1997; Hubbard et al., 1994; White et al., 1988). In contrast to this mechanism, in which phosphorylation primarily induces conformational change through charge alterations, most signal transduction downstream of RTKs is mediated by the recruitment of intracellular signaling effector proteins upon RTK activation

(Kazlauskas and Cooper, 1989; Kazlauskas et al., 1990; Kypta et al., 1990; Margolis et al., 1989; Meisenhelder et al., 1989b).

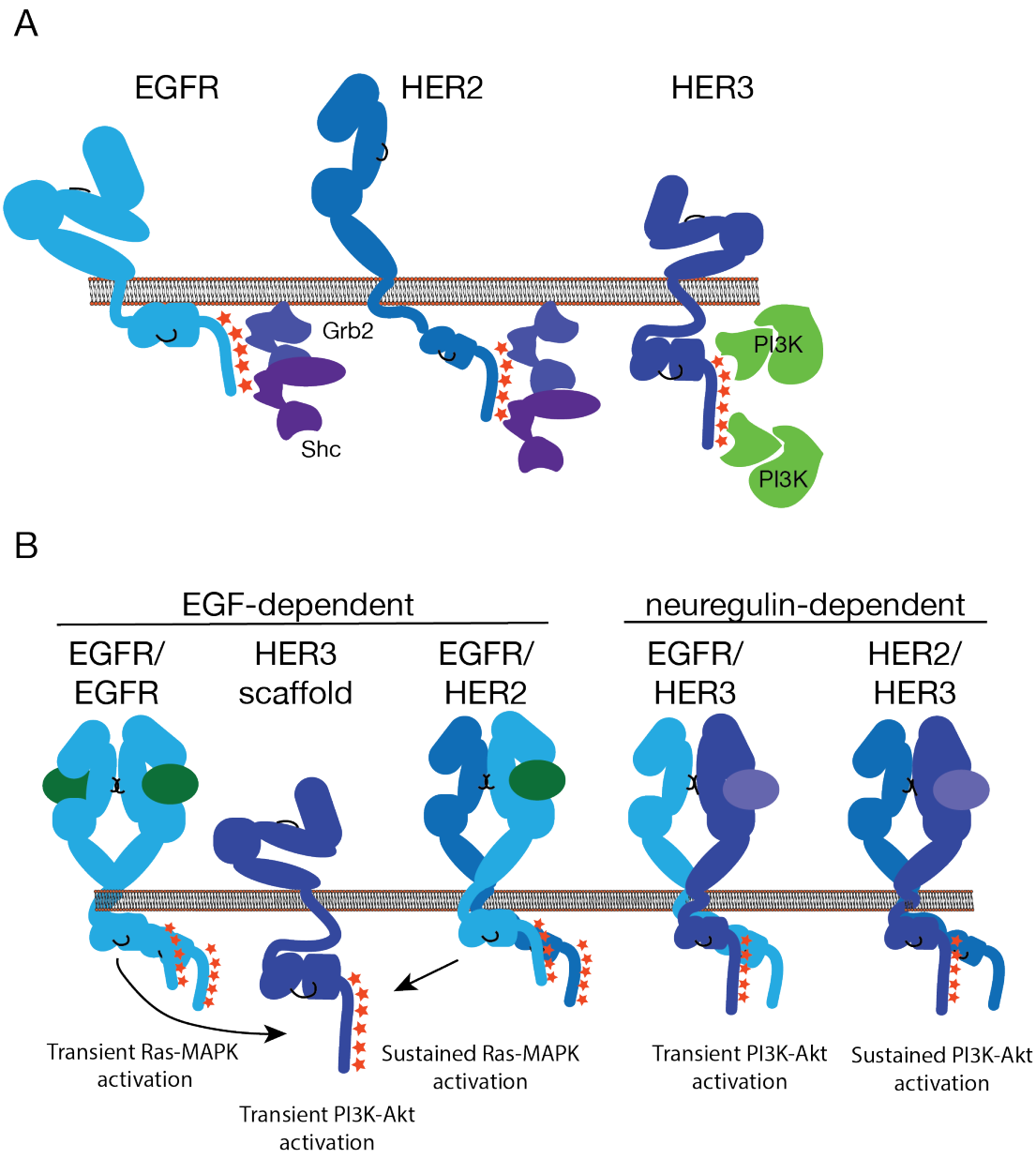


Figure 1.4: HER3 activation exemplifies heterotypic signaling within the EGFR family. *(A) EGFR and HER2 both efficiently recruit the Grb2 and Shc adaptor proteins to phosphorylated tyrosine residues in their C-tails, and primarily differ in that EGFR is ligand-activated, while HER2 is constitutively primed for dimerization and activation but has no known ligands. HER3, by contrast, preferentially recruits and activates the p85 activator of the PI3K complex. (B) Combinatorial signaling by different dimers within the EGFR family has differential signal output. The formation of particular dimers depends on RTK expression levels as well as the ligand inducing complex formation. HER3 can be recruited either as a scaffold to activated EGFR and HER2 or as an activator of the kinase domain of either RTK. EGF or other EGFR ligands are shown in green, the HER3 ligand neuregulin is shown in purple.*

The recruitment of these proteins is predominantly induced by phosphorylation of tyrosines inside binding motifs for SH2 domains, a specialized phosphotyrosine-binding domain responsible for protein-protein interactions (Koch et al., 1991; Sadowski et al., 1986). SH2 domains comprise two antiparallel beta sheets sandwiched between short alpha helices. Conserved residues within each SH2 domain interact with the phosphorylated tyrosine itself as well as the amino acid side chains immediately C-terminal to the phosphorylated tyrosine (Figure 1.4A) (Cantley and Songyang, 1994). In addition to this, the more flexible loops within the SH2 domain contribute to binding specificity (Kaneko et al., 2010). Thus, different SH2 domains can selectively recognize distinct tyrosine-phosphorylated sequences based on side chain chemistry, thereby mediating selective recruitment to specific tyrosine phosphorylated motifs.

RTK activation results in the tyrosine phosphorylation of residues in the usually disordered juxtamembrane and C-tail regions flanking the kinase domain. In addition, tyrosines within proteins that are recruited to the receptor can recruit additional SH2-domain-containing proteins upon their phosphorylation by the active RTK (Lemmon and Schlessinger, 2010). Several additional domains have been shown to bind phosphorylated tyrosines using distinct mechanisms from the SH2 domain, including phosphotyrosine-binding (PTB) domains and a specialized SH2 domain known as the tyrosine kinase binding (TKB) domain, found in the Cbl family of E3 ligases. These engage tyrosine-phosphorylated substrates with a specificity for an Asn-Pro-X-pTyr sequence, with selectivity determined by the identity of the amino acid preceding the phosphotyrosine (Zhou and Fesik, 1995). Additional mechanisms of binding to phosphotyrosine have been reported in several cases. A family of related large multisite docking

proteins with mostly disordered structures containing many tyrosines are frequently recruited to activated RTKs to act as scaffolds for further protein recruitment. Structured N-terminal pleckstrin homology, phox homology or phosphotyrosine binding domains mediate recruitment of these proteins to the activated RTK at the membrane, while a unique linear sequence within the scaffold Gab1 has been shown to bind selectively to the Met RTK C-tail (Lock et al., 2003; Weidner et al., 1996). Phosphorylation of tyrosines within exposed loops of the disordered regions of these proteins expands the repertoire and concentration of tyrosine-phosphorlyated motifs in the activated receptor complex (reviewed in Liu and Rohrschneider, 2002; Simister and Feller, 2012). Thus, the activation of a particular RTK results in the recruitment of SH2-domain-containing proteins specific to tyrosines within the receptor, as well as those recruited to phosphorylated tyrosines in other proteins recruited to the complex.

Additional mechanisms are involved in the recruitment of proteins to activated RTKs, and for the downstream activation of those proteins. While tyrosine phosphorylation can directly regulate the activity of a variety of kinases, it can also regulate the activity of RTK substrates by modulating SH2 domain interactions. Src-family CTKs are characterized by phosphotyrosine-dependent allosteric kinase inhibition, mediated by the binding of the CTK SH2 domain to a tyrosine in its C-tail (Figure 1.4B). Displacement of the CTK's SH2 domain by other phosphotyrosine motifs with better binding kinetics to release the inhibition of the kinase is known as a mechanism of Src-family CTK activation, and presumably occurs upon recruitment of these proteins to activated RTKs (Alexandropoulos and Baltimore, 1996; Filippakopoulos et al., 2009; Liu et al., 1993). The tyrosine phosphatase SHP2, recruited to many activated RTKs, can similarly be activated by the relief of allosteric inhibition upon engagement of their SH2 domains (Barford and Neel, 1998).

Thus, the recruitment of proteins via SH2 domains can directly facilitate their activation by an RTK.

1.18 Activation of downstream signaling effectors by RTKs

The clustering of SH2-domain-containing proteins can induce the association of activator and effector components of downstream signaling pathways. A key example of this is shown by the activation of MAPKs by the GTPase Ras. Ras is targeted to the plasma membrane by farnesylation and requires extrinsic activator proteins to exchange bound GDP for GTP, thereby activating the protein (Overbeck et al., 1995). Binding of the SH2-domain-containing protein Grb2 to an activated RTK induces recruitment of the Ras guanine exchange factor SOS1 to the RTK complex, through the constitutive association of SOS1 with Grb2 (Chardin et al., 1993; Egan et al., 1993; Gale et al., 1993). This is mediated by a Src-homology 3 (SH3) domain, also found in Src-family CTKs, that binds to exposed proline-rich motifs within proteins. In Src-family CTKs, this domain participates in the autoinhibition of the kinase with the SH2 domain (Figure 1.4B). SH3 domains mediate both constitutive interactions between proteins such as Grb2 and SOS1, or interactions that can be modulated by conformational change as seen in Src-family CTKs and some scaffold proteins (Simister and Feller, 2012).

Another example of this is observed in the activation of PI3K-dependent signalling downstream of RTKs. The PI3K enzyme activated by RTKs at the plasma membrane functions as a heteromeric complex composed of distinct catalytic and regulatory subunits. The recruitment the 85-kilodalton (kDa) regulatory subunit, p85, to phosphorylated tyrosine residues in the RTK complex occurs via its SH2 domain. This clusters the regulatory subunit at the membrane and allosterically

relieves inhibition of the associated 110-kDa catalytic subunit, thus inducing local accumulation of phosphatidyl-inositol-3,4,5 triphosphate (PIP_3) (Burke and Williams, 2013; McGlade et al., 1992).

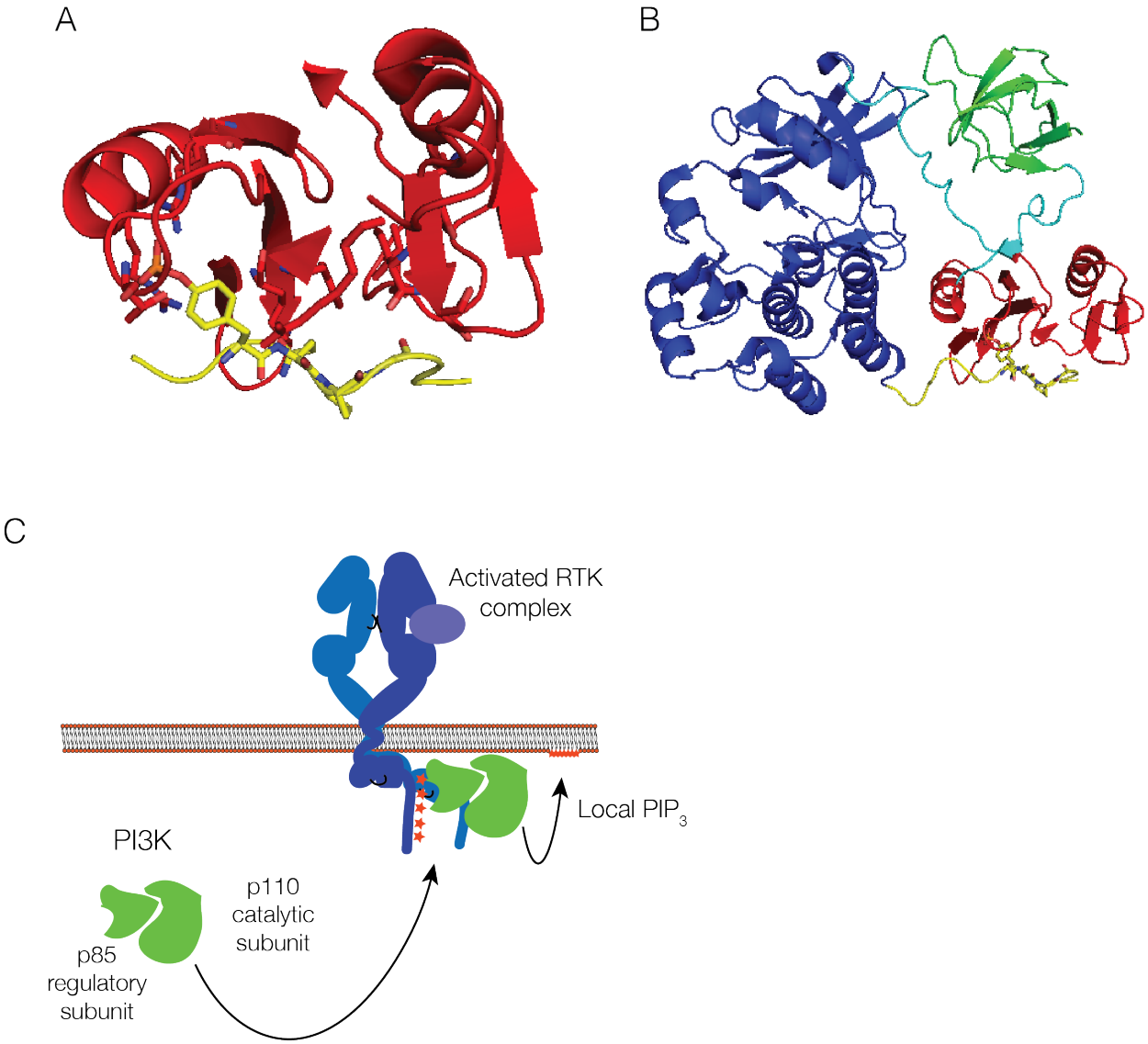


Figure 1.5: SH2 domains mediate the recruitment and activation of effectors downstream of RTKs. *(A) SH2 domains bind phosphotyrosine residues and the downstream flanking sequence. Shown is the Src SH2 domain (red) binding the autoinhibitory tyrosine (Tyr527) in the Src C-tail (yellow) (1KSW). Residues in the SH2 domain determining sequence specificity, and the residues in the C-tail they interact with, are shown. (B) The Src SH2 domain (red) cooperates with the SH3 domain (green) to allosterically inhibit the kinase domain (blue) when it is engaged to the C-tail (yellow). Engagement of the SH2 domain with competing pTyr residues displace the autoinhibitory interactions, activating the kinase. (C) PI3K activation downstream of RTKs is also dependent on engaging SH2 domains in the p85 regulatory subunit to relieve allosteric autoinhibition of the catalytic domain, and to localize catalytic activity to the plasma membrane. Pools of PIP_3 at the membrane then recruit and activate downstream effectors including PDK1 and Akt.*

As many proteins can be recruited to PIP_3-rich membrane regions through a variety of lipid-interacting domains, induction of PIP_3 at the membrane represents a further mechanism whereby RTKs can localize effectors of downstream signaling, such as the Akt serine-threonine kinase and its activating kinase, PDK1, to the membrane. RTKs also frequently induce the cleavage of membrane lipids into free inositol phosphate and diacylglycerol by the SH2-domain-dependent recruitment of phospholipase C gamma to the plasma membrane. This can in turn induce calcium-dependent signaling through the opening of gated channels in the plasma membrane or endoplasmic reticulum (Hajicek et al., 2019; Margolis et al., 1989; Meisenhelder et al., 1989a; Morrison et al., 1990; Sekiya et al., 2004).

RTK signaling is characterized by the combination of the above mechanisms to broadly activate downstream signaling proteins. As many RTKs share downstream effectors, the specificity of the signal derived from activation of a particular RTK likely depends on the particular combination of effectors recruited to the active complex (Tan and Kim, 1999; Yarden and Sliwkowski, 2001). Tyrosine phosphorylation driven by the RTK can directly or indirectly activate RTK substrates, and it can also feed forward into alternative signaling mechanisms including serine or threonine phosphorylation, lipid phosphorylation and cleavage and the release of calcium into the cytosol. Thus, tyrosine-dependent recruitment of proteins to the active RTK complex mediates the activation of downstream signaling by diverse mechanisms.

1.19 Structure-function and genetic experiments delineate the Met-dependent signaling pathway

Signaling by the Met RTK depends on the recruitment of an array of downstream effectors through phosphotyrosine-dependent protein-protein interactions. The phosphorylation of two tyrosines in the activation loop of the kinase, Tyr1234 and Tyr1235, promotes a fully-active conformation of the kinase domain (Rodrigues and Park, 1994).

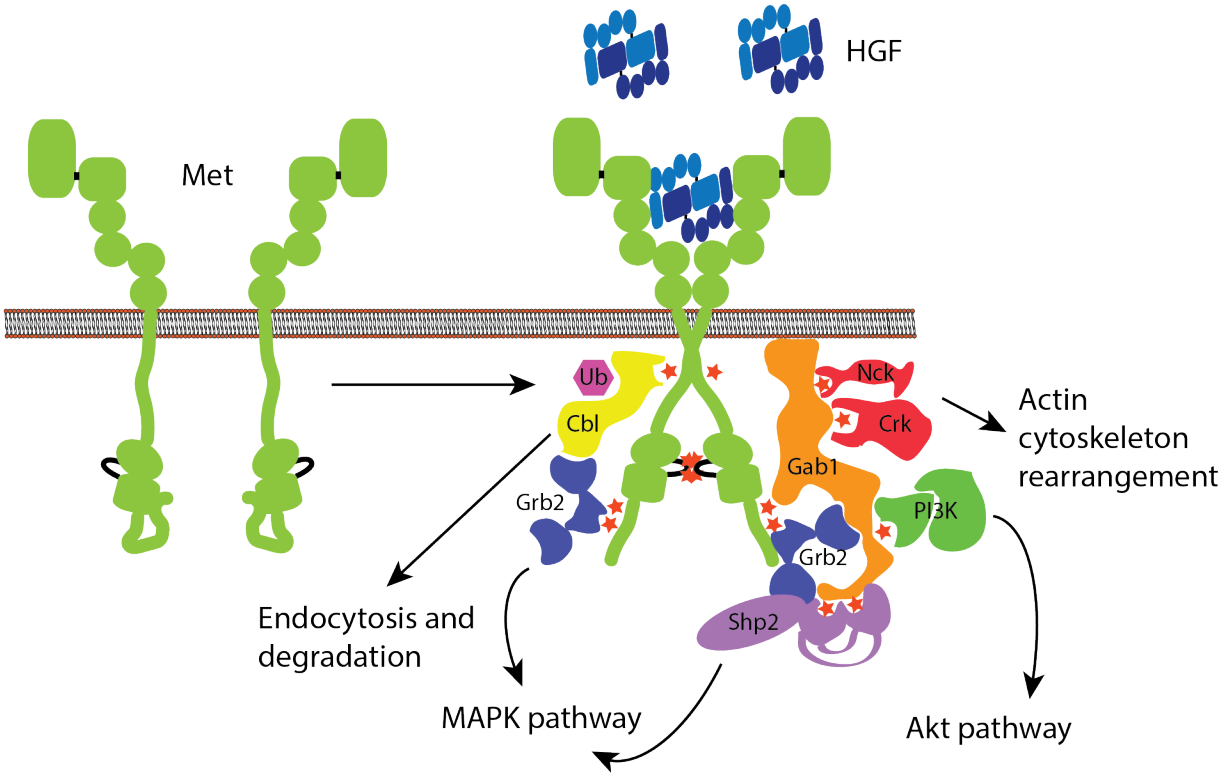


Figure 1.6: HGF-dependent signaling activated by the Met RTK*. Activation of the Met kinase domain is driven by the binding of its unique ligand, HGF, and the dimerization-induced* trans*-autophosphorylation of tyrosines within the activation loop, including the twin tyrosines Tyr 1234/35. Subsequent tyrosine phosphorylation of Tyr 1003 in the juxtamembrane region and Tyr 1349 and 1356 in the C-terminal tail mediate recruitment of the major adaptor and scaffolding proteins known to interact with Met. Recruitment of the E3 ligase Cbl induces Met ubiquitination and signal termination, while Met-dependent signal output is largely dependent on the recruitment of Grb2 to Tyr1356 in the C-tail, and the Grb2-dependent recruitment and phosphorylation of the large multisite docking protein Gab1 as a scaffold. Phosphorylated tyrosines in Gab1 then serve as recruitment sites for effectors such as p85, SHP2 and Src-family CTKs, while Grb2-dependent recruitment of SOS1 contributes to Ras-MAPK activation directly. Gab1 also recruits additional adaptor proteins including those of the Crk family and mediators of actin dynamics such as NCK1/2 to the activated complex to promote cytoskeletal rearrangement required for cell dispersal and invasion.*

Subsequent phosphorylation of Tyr1356 in the C-tail provides a consensus binding site for the recruitment of Grb2, the PTB-domain-containing SHC1 adaptor, and the majority of downstream effectors through these adaptors, including the E3 ubiquitin ligase Cbl and the scaffolding protein Gab1 (Fixman et al., 1996; Fixman et al., 1997; Ponzetto et al., 1994; Weidner et al., 1996). Mutants of TPR-Met in which the corresponding site was replaced with the non-phosphorylatable amino acid phenylalanine had no transforming capacity, while experiments with chimeric receptors showed that this site was required for cell invasion (Fixman et al., 1997; Fixman et al., 1995; Zhu et al., 1994). Grb2-dependent recruitment of Gab1 results its hyperphosphorylation and the recruitment of a large number of downstream effector proteins, including p85, the adaptor proteins Crk and CrkL, and the tyrosine phosphatase SHP2 (Lamorte et al., 2000; Maroun et al., 1999a; Maroun et al., 2000; Nguyen et al., 1997; Schaeper et al., 2000) (Figure 1.6).

Gab1 recruitment to Met also involves engagement of phosphorylated Tyr1349 in the C-tail of Met. A proline-rich linear motif referred to as the Met-binding site in Gab1 has been shown to associate uniquely with Met among a panel of RTKs by yeast-two-hybrid screen, and Gab1 is required for all known Met-dependent developmental signaling *in vivo* (Frigault et al., 2008; Lock et al., 2003; Lock et al., 2002; Lock et al., 2000; Sachs et al., 2000; Weidner et al., 1996). The Met-dependent phosphorylation of Gab1 is prolonged compared to the phosphorylation of Gab1 by other RTKs such as EGFR. This is associated with the efficient recruitment of Gab1 to PIP_3-rich regions at the plasma membrane upon Met activation, as ectopically targeting Gab1 to the membrane induces prolonged EGF-dependent Gab1 phosphorylation and the induction of Met-like cell invasion (Maroun et al., 1999a; Maroun et al., 1999b). Mass spectrometry identified distinct tyrosine phosphorylation patterns in Gab1 in response to EGF and HGF signaling, and

these correlated with activation of distinct downstream effectors (Johnson et al., 2013). Engagement of Tyr1349 with the Met binding motif in Gab1 may contribute to sustained Gab1 phosphorylation by stabilizing the open conformation of the Met C-tail and the Met-Gab1 interaction (Frigault et al., 2008; Lock et al., 2003; Lock et al., 2002).

A diverse array of proteins have been shown to be recruited to the activated Met RTK complex, primarily through Gab1-dependent mechanisms. Structure-function experiments demonstrated that Grb2- and SHC1-dependent activation of the Ras-MAPK pathway mediates cell transformation, and Gab1-dependent activation of the SHP2 tyrosine phosphatase by SH2 domain displacement is required for the sustained MAPK activation and efficient cell invasion (Fixman et al., 1996; Fournier et al., 1996; Maroun et al., 2000; Saucier et al., 2002). Gab1 contains three SH2-binding motifs corresponding to the p85 binding site, has been shown to contribute to PI3K -Akt activation upon EGF or NGF stimulation, and is required for PI3K-dependent functions downstream of Met (Fan et al., 2001; Holgado-Madruga et al., 1997).

Gab1 has also been shown to mediate interactions with a variety of proteins involved in the regulation of actin cytoskeletal dynamics. The regulatory proteins NCK1 and Pak4 and the actin nucleating factors cortactin and N-WASP are recruited to Gab1 in response to HGF and contribute to cell morphogenesis and invasion (Abella et al., 2010b; Paliouras et al., 2009; Rajadurai et al., 2012). Crk-family adaptor proteins are recruited to six sites in Gab1, and mediate activation of the JNK MAPK pathway to promote cell invasion and anchorage-independent growth through the regulation of adherens junctions and the actin cytoskeleton (Garcia-Guzman et al., 2000; Lamorte et al., 2000; Lamorte et al., 2002b). Crk expression is required for the sustained phosphorylation of Gab1 downstream of Met, and its overexpression promotes epithelial cell invasion in response

to EGF in a manner comparable to HGF (Lamorte et al., 2002a; Watanabe, 2006). Crk is required for invasion, migration and metastatic growth in breast cancer cell lines, and has been reported to play similar roles in pancreatic and bladder cancer (Fathers et al., 2012; Matsumoto et al., 2015; Uemura et al., 2020). Thus, in addition to activating the major pathways required for transformation downstream of Met, Gab1-dependent signaling contributes extensively to cytoskeletal remodeling and resultant invasion and metastasis.

In addition to these pathways, a number of other signaling effectors have been linked to Met-dependent signaling. Src-family CTKs have been shown to phosphorylate Gab1 downstream of Met and EGFR as well as other receptors, and Src may cooperate with Met to regulate Gab1 phosphorylation (Chan et al., 2003; Chan et al., 2010; Furcht et al., 2015). Signal transducer and activator of transcription 3 (STAT3), a signaling effector and transcription factor involved in immunomodulatory signaling, has been shown to bind Met and is required for Met-dependent proliferative signalling in cancer, although the precise mechanism of Met-dependent STAT3 phosphorylation in cancer is disputed and may depend on induction of autocrine or paracrine signaling through interleukin-6 (Boccaccio et al., 1998; Lai et al., 2014; Lee et al., 2009). Recent work by our group has shown that Met cooperates with the RTK FGFR1 to promote tumour initiating capacity in a murine model of Met-dependent breast cancer, and that this involves the phosphorylation of the Gab1-related large multisite docking protein FRS2 (Sung, 2019; Sung et al., In press). Thus, tyrosine phosphorylation of Met induces the activation of a diverse array of downstream signaling proteins that collectively mediate the biological response to Met activation, primarily through the recruitment and phosphorylation of the Grb2-Gab1 complex.

1.20 Met signaling dynamics and output are regulated by endocytosis and degradation

Like other RTKs, activation of Met induces negative feedback that is responsible for signal termination. The major mechanisms of signal termination impinging on Met involve trafficking of the receptor from the plasma membrane to intracellular compartments. Met activation promotes the recruitment and phosphorylation of Cbl and the subsequent phosphorylation of hepatocyte growth factor receptor substrate (HRS), a component of the ESCRT complex, which is responsible for the maturation of late endosomes to degradative multivesicular bodies (Abella et al., 2005; Fixman et al., 1997). Loss of the Cbl binding site in the juxtamembrane region flanking the kinase domain facilitates Met-dependent transformation, either through mutation of Tyr1003 to which Cbl is recruited or by exon skipping affecting the site (Peschard et al., 2001; Peschard et al., 2004; Peschard and Park, 2003). Indeed, in *MET*-amplified gastric cancer cells with no mutations uncoupling Cbl binding from Met, phosphorylation and degradation of Cbl is associated with Met-dependent transformation and renders the cells hypersensitive to EGF-dependent signaling as well (Lai et al., 2012). Tyrosine phosphorylation is directly antagonized by phosphatase activity and Met activation has been shown to induce the trafficking of the phosphatase PTP1B to the membrane to attenuate Met-dependent phosphorylation and promote Met trafficking to endosomal sorting machinery (Sangwan et al., 2011; Sangwan et al., 2008; Stuible et al., 2010).

Internalized Met is not always directed to be degraded. Sorting in early endosomal vesicles directs Met to be recycled to the plasma membrane via engagement with the adaptor protein GGA3, which is required for the sustained activation of Met-dependent Ras-MAPK signaling in HeLa cells (Parachoniak et al., 2011). This is dependent on the activation of the GTPase ARF6,

whose regulation of RTK trafficking has also been linked to cell junction stability and crosstalk with the Wnt-β-catenin signaling pathway (Pellon-Cardenas et al., 2013). Activation of ARF6 through the guanine exchange factor cytohesin-2 is required for HGF-dependent cell migration, linking the recycling-dependent prolonged activation of signaling to known Met-dependent biology (Ratcliffe et al., 2019). Thus, the dynamic regulation of Met trafficking and degradation is critical to establishing signal output and plays a critical role in oncogenic transformation downstream of Met as well as other RTKs.

1.21 RTKs in human cancer

RTK signaling is implicated in a wide variety of roles in human cancer. Several RTKs are recurrently mutated or amplified in specific cancer types, including EGFR in lung cancer, HER2 in breast cancer, and c-Kit in gastrointestinal stromal tumours, and the presence of these genetic lesions generally predispose patients to respond to RTK-directed therapies. In this case, the mutated or amplified RTK is considered to act as a driver oncogene, and inhibition of its signaling output generally is associated with a loss of cell proliferation and an increase in apoptosis. Many additional recurrently mutated cancer genes, including notably the GTPase *KRAS*, the MAPK *BRAF* and the lipid kinase *PIK3CA* and phosphatase *PTEN*, encode activators of the cytoplasmic signaling pathways that are utilized by RTKs. The activation of wild-type RTKs is presumed to be required at some level for cancer-associated signaling through these pathways even upon their oncogenic mutation. Furthermore, RTK activation has been implicated in diverse secondary roles in cancer biology, notably including several of the "hallmarks of cancer": remodeling of the tumour microenvironment; the acquisition of invasive and migratory capacity required for metastasis; the process of neoangiogenesis required for tumour vascularization; the relief of contact

inhibition; the evasion of immune surveillance and induction of pro-tumour inflammatory signaling; and the reprogramming of tumour metabolism to an anabolic state (Hanahan and Weinberg, 2000, 2011; Lemmon and Schlessinger, 2010). While our understanding of cancer continues to evolve, research continues to implicate RTKs in emerging features of cancer biology. Notable recent efforts include the suppression of the antitumour T-cell response in RTK-activated cancers, and the efficacy of checkpoint inhibtion in combination with RTK-directed therapies (Ahn et al., 2019; Hsu et al., 2019; Kasikara et al., 2019; Soo et al., 2018). Alternative mechanisms of activating each of these biological functions that are independent of RTKs are well known in cancer, but the relevance of RTK-dependent signaling processes to cancer biology has made the study of RTKs an important element of cancer research (Du and Lovly, 2018; Yamaoka et al., 2018).

1.22 RTKs as targets of precision medicine in cancer

RTKs and their ligands have been targeted both as oncogenic driver genes as well as in their secondary roles supporting tumour growth. Much of the evidence for the role of RTKs in advanced cancers comes from research in experimentally tractable model systems, in which RTK signaling can be selectively activated or inactivated in controlled settings, or from analysis of the expression of receptor, ligand or downstream signaling intermediates at steady-state in late-stage tumours. This has shown extensive evidence for RTK signaling in promoting tumour cell invasion and migration, and in regulating tumour cell state, including the regulation of epithelial-mesenchymal transition and metabolic reprogramming (Akalu et al., 2017; Appert-Collin et al., 2015; Chen et al., 2015; Graham et al., 2014; Nakada et al., 2020; Valiathan et al., 2012). While therapeutic interventions directed at oncogenic driver alterations in RTKs, as well as the

angiogenic functions of the VEGF ligand and its receptors, have been successfully developed for cancer patients, it remains unclear whether clinical intervention in other secondary functions of RTKs yields benefit for patients. This may be due to cellular plasticity in established cancers, enabling cells to bypass RTK-dependent routes to metastasis or cellular reprogramming; alternatively, the presence of heterogeneity within tumours implies that some cells may be predisposed to non-RTK-dependent induction of these biological functions.

Many of the virally-encoded oncogenes derived from RTKs are activated by recurrent point mutations in cancer. Recurrent mutations in RTK genes are found in the extracellular ligand-binding, transmembrane and juxtamembrane domains of RTKs, as well as within the tyrosine kinase domain in several well-characterized “hotspots” associated with elevated kinase activity (Du and Lovly, 2018; Lahiry et al., 2010). ATP-competitive tyrosine kinase inhibitors (TKIs) blocking kinase enzymatic activity were first approved for use against BCR-Abl in patients with CML positive for the Philadelphia chromosome (2001; Cohen, 2002; Druker and Lydon, 2000). This was the first successful example of an approach to cancer therapy then called rational drug design, and lead to a broad class of drugs targeting both tyrosine and the broader family of serine/threonine kinases (Roskoski, 2019; Woolfrey and Weston, 2002). Small-molecule inhibitors targeting RTKs were first approved for use in lung cancer patients with EGFR kinase domain mutations in 2004 (Lynch et al., 2004). Meanwhile, an alternate track of research and development lead to the FDA approval in 1998 of trastuzumab, an antibody raised against HER2 with activity in HER2-amplified breast cancer (1998). In the years since these landmark achievements at the turn of the century, the repertoire of RTKs as targets in cancer medicine has expanded to include 30 RTKs targeted by 33 FDA-approved small-molecule TKIs, notably including

all four members of the FGFR family, the pro-angiogenic RTKs VEGFR2 (*KDR)* and VEGFR3 (*FLT4*), the PDGFR family, and c-Kit (FDA, 2004b). While many TKIs are designed to be exquisitely selective for a particular target, molecules that efficiently inhibit multiple RTKs have proven effective in clinical trials, and the majority of FDA-approved TKIs can be used to target an array of altered genes (Table 1.1).

Table 1.1: FDA-approved TKIs by kinase. *CTKs and inhibitors that only target CTKs are italicized. Met inhibitors are in bold.*

FDA-approved TKIs	Kinases approved for indication
acalabrutinib	*BTK*
afatinib	EGFR, ERBB2, ERBB4
alectinib	ALK, RET
avapritinib	CSF1R, KIT, PDGFRA, PDGFRB
axitinib	FLT1, FLT4, KDR, PDGFRB
baricitinib	*JAK1, JAK2*
bosutinib	*ABL, HCK, LYN, SRC*
brigantinib	ALK, EGFR, FLT3, ROS1
cabozantinib	AXL, FLT1, FLT3, FLT4, KDR, KIT, **MET**, NTRK2, TIE2
capmatinib	**MET**
ceritinib	ALK, IGF1R, INSR, ROS1
crizotinib	ALK, **MET**, MST1R, ROS1
dacomitinib	EGFR, ERBB2, ERBB4
dasatinib	*ABL*, EGFR, EPHA2, *FYN* , KIT, *LCK*, PDGFRB, *SRC*, *YES*
entrectinib	NTRK1, NTRK2, NTRK3
erdafitinib	FGFR1, FGFR2, FGFR3, FGFR4
erlotinib	EGFR
fedratinib	*JAK2*
fostamatinib	*SYK*
gefitinib	EGFR
gilteritinib	FLT3
ibrutinib	*BTK*
imatinib	*ABL,* KIT, PDGFRA, PDGFRB
lapatinib	EGFR, ERBB2
larotrectinib	NTRK1, NTRK2, NTRK3
lenvatinib	FGFR1, FGFR2, FGFR3, FGFR4, FLT1, FLT4, KDR, KIT, PDGFRA, RET

lorlatinib	ALK
midostaurin	FLT3, KDR, PDGFRA
neratinib	ERBB2
nintendanib	FGFR1, FGFR2, FGFR3, FLT1, FLT3, FLT4, KDR, PDGFRA, PDGFRB
osimertinib	EGFR
pazopanib	FGFR1, FGFR3, FLT1, FLT4, *FMS*, *ITK*, KDR, KIT, *LCK*, PDGFRA, PDGFRB
pexidartinib	CSF1R, KIT
ponatinib	*ABL*, EPH family, FGFR family, FLT1, FLT3, FLT4, KDR, KIT, PDGFRA, PDGFRB, RET, *SRC family*, TIE2
regorafenib	*ABL*, EPHA2, FGFR1, FGFR2, FLT1, FLT4, KDR, KIT, PDGFRA, PDGFRB, RET, TIE2
ruxolitinib	*JAK1, JAK2*
sorafenib	FLT1, FLT3, FLT4, KDR, KIT, PDGFRB, RET
sunitinib	AXL, CSF1R, FLT1, FLT3, FLT4, KDR, KIT, PDGFRA, PDGFRB, RET
toacitinib	*JAK3*
upadacitinib	*JAK1*
vandetanib	EGFR, EPH family, ERBB2, ERBB4, FLT1, FLT4, KDR, PTK2, RET, SRC family, TIE2
zanubrutinib	*BTK*

In contrast to this, the generation of antibodies is inherently receptor-specific, as each antibody is raised to target a unique molecular epitope. In addition to trastuzumab, an antibody targeting EGFR, cetuximab, was approved for use in metastatic colorectal cancer in 2004, for use in all colorectal cancers lacking a *KRAS* activating mutation in 2009, and in metastatic head and neck cancer in 2011. Both EGFR and HER2 have been targeted with antibody-drug conjugates based on cetuximab and trastuzumab, respectively, in order to increase the cytotoxicity of these drugs in targeted cells. In addition to this, antibodies have been approved for use against VEGFR2 and its ligand VEGF-A to block angiogenesis in gastric, colorectal, hepatocellular and non-small-cell lung carcinomas (FDA, 2004a, 2014). An antibody raised against PDGFRA, olaratumab, was approved for use in sarcomas in 2016, but unfortunately was removed from US and European

markets in 2019 due to a lack of efficacy in subsequent clinical trials (FDA, 2016; Lilly, 2019). Intriguingly, burosumab, an antibody targeting the RTK ligand FGF23, was approved for use in pediatric patients with X-linked hypophosphatemia in 2018 (Carpenter et al., 2018). Treatment with burosumab antagonizes elevated FGF23 levels in these patients, relieving a blockade on phosphate reabsorption by the kidneys. As FGF23 overexpression also underlies tumour-induced osteomalacia in patients with phosphaturic mesenchymal tumours, it has been approved for use in patients suffering from this rare side-effect of cancer as well (Day et al., 2020). The diverse mechanisms for targeting aberrant receptor tyrosine kinase activity in cancer reflect the many roles these genes play in regulating the biology of cancer, and clinical trials undertaken to block RTK activity in human cancers number in the thousands (NIH). Ongoing efforts to expand the repertoire of targetable genes, to improve the efficacy of RTK-directed therapies, and to improve patient stratification for maximal efficacy remain key priorities in drug development as of this writing.

1.23 MET alterations in human cancer

The Met RTK has been implicated in human cancers since its discovery as a transforming oncogene upon chromosomal rearrangement (Gonzatti-Haces et al., 1988; Park et al., 1986; Park et al., 1987). Met protein overexpression and constitutive phosphorylation was associated early on with amplification of the *MET* gene in human cancer cell lines, and the early association of *MET* or *HGF* overexpression with poor prognosis in several human cancers indicated that Met-dependent biology was broadly implicated in cancer progression (Di Renzo et al., 1992; Ghoussoub et al., 1998; Hida et al., 1999; Kuniyasu et al., 1992; Oda et al., 2000; Siegfried et al., 1997; Tuck et al., 1996; Yamashita et al., 1994; Yonemura et al., 1997). Elevated *MET* expression

as well as the acquisition of activating mutations was observed in several metastatic cancers, implying that Met activity may be associated with advanced stages of cancer (Di Renzo et al., 1995; Di Renzo et al., 2000). Furthermore, the discovery of germline mutations in *MET* in familial renal carcinoma implicated Met activation in this disease and was a key early indicator of the potential utility of Met for therapeutic intervention (Schmidt et al., 1997). Mutation of the Cbl binding site, which was missing in the oncogenic TPR-Met fusion protein, was shown to be sufficient for Met-dependent transformation of cells *in vitro* (Peschard et al., 2001). Collectively, these observations showed that Met could play important roles in human cancer, including as an oncogenic driver of cell transformation (Peschard and Park, 2003).

Evidence of the role for Met activity in a variety of cancer-associated processes along with its association with poor prognosis and metastatic cancers helped drive the development of Met-directed therapies (Zou et al., 2007). The sensitivity of *MET*-amplified cell lines to Met-selective TKIs was strong evidence for the potential activity of these agents in patients with *MET*-amplified tumours (Smolen et al., 2006). Mutations in Met leading to the deletion of exon 14, encoding the Cbl-binding site, were discovered in lung and gastric cancers and cancer-derived cell lines, indicating that this may be a common mechanism of oncogenic Met activation, as well as one seen in other RTKs (Asaoka et al., 2010; Kong-Beltran et al., 2006; Lee et al., 2000; Peschard and Park, 2003). As these mutations mostly occur in intronic regions and cause exclusion of exon 14 in *MET*, they are not detected in analyses of cancer based on whole exome sequencing, and were long-undercounted in human cancers, even with the advent of modern high-throughput sequencing techniques. Exon 14 mutations frequently co-occur with amplification of *MET*, and are associated with advanced and metastatic cancers as well (Awad et al., 2016).

The systematic molecular profiling of human cancers performed over the past decade as part of the Pan-Cancer Atlas and other international consortia has helped illuminate the prevalence of Met alterations in human cancer. *MET* gene amplification in the absence of other known driver gene alterations was observed in a minor population of both lung (2.2%) and stomach (4%) adenocarcinomas, in line with prior observations (Cancer Genome Atlas Research, 2014a, b) (Figure 1.7). Mutations affecting the splicing of exon 14 have been reported in lung adenocarcinomas as well as fusions with the *RET*, *ROS1* and *ALK* RTK genes, cumulatively affecting 7% of patients (Cancer Genome Atlas Research, 2014b). Additionally, recurrent gene fusions involving *MET*, predominantly with the phosphatase gene *PTPRZ1*, have been reported in up to 10% of pediatric glioblastomas (International Cancer Genome Consortium PedBrain Tumor, 2016). While *MET* copy number gains or amplifications are not predictive of response to Met-directed therapy in glioblastoma, preclinical models show that autocrine secretion of HGF is capable of inducing Met-dependent transformation in the brain (Xie et al., 2012). HGF autocrine secretion has also been proposed as a mechanism of Met-dependent transformation in sarcomas and acute myeloid leukemia, but this has also primarily been shown using *in vitro* assays from explanted or cultured cell lines (Cortner et al., 1995; Kentsis et al., 2012). As a diagnostic test for human patients with HGF autocrine activation has not been established, it is difficult to predict how frequently this mechanism of Met-dependent activation occurs. Gene amplification of *HGF*, which occurs in 2-3% of lung, esophagogastric, liver, ovarian, and bladder carcinomas, as well as in diffuse large B-cell lymphomas, is predicted to predispose patients to autocrine Met activation (Weinstein et al., 2013).

1.24 The Met receptor tyrosine kinase as a target in cancer

The Met receptor tyrosine kinase has been assessed as a target of precision medicine both as monotherapy and in combination with other treatments. Targeting of Met is usually tested as a second- or third-line therapy after prior exposure to chemotherapy and other precision medicine, primarily targeting EGFR in lung cancer, but Met has also been assessed as part of a first-line therapeutic regimen (Table 1.2) (Catenacci et al., 2017). Met has been targeted using both antibodies directed at the extracellular domain and ATP-competitive TKIs. While promising objective responses have been observed in patients with genetic alterations including gene amplification, kinase domain mutation and exon 14 skipping, patients without these predisposing conditions rarely respond to Met inhibition. After setbacks related to poor patient stratification and worrisome developments using monoclonal antibodies, a number of experimental antibody-based therapies are under evaluation, and the Met-specific TKI capmatinib was recently approved for use in patients with exon-14-skipping mutations (Garber, 2014; Recondo et al., 2020b).

Table 1.2: Met-directed therapies in clinical trials (NIH)

Drug	Class	Trials	Single-agent	Combination	Active	Concluded
AMG-208	TKI (multikinase)	2	2	0	0	1
AMG-337	TKI (Met-specific)	6	5	1	1	1
anlotinib	TKI (multikinase)	152	44	108	128	12
Cabozantinib	TKI (multikinase)	144	66	78	106	30
Capmatinib	TKI (Met-specific)	35	12	23	18	9

Crizotinib	Type I TKI (multikinase)	108	48	60	61	32
EMD 1204831	Type I TKI (Met-specific)	1	1	0	0	0
Kanitinib	Type I TKI (multikinase)	2	2	0	2	0
merestinib	Type I TKI (multikinase)	11	5	6	5	6
glesatinib	Type I TKI (multikinase)	7	5	2	1	5
sitravatinib	Type I TKI (multikinase)	12	5	7	12	0
PF-04217903	Type I TKI (Met-specific)	1	1	0	0	0
PBL1001	Type I TKI (Met-specific)	4	4	0	2	2
SAR125844	Type I TKI (Met-specific)	3	3	0	0	3
savolitinib	Type I TKI (Met-specific)	27	13	14	14	11
tepotinib	TKI (Met-specific)	13	4	9	4	9
tivantinib	Allosteric TKI	46	26	20	1	39
TPX-0022	TKI (macrocycle) (multikinase)	1	1	0	1	0
ficlatuzumab	HGF-neutralizing mAb	10	2	8	2	3
JNJ-61186372	Bispecific antibody (EGFR)	2	0	2	2	0
onartuzumab	Met mAb	18	2	16	0	16
rilotumumab	Met mAb	16	4	12	1	10
Sym-015	Met mAb mixture	1	1	0	1	0
telisotuzumab	Met mAb drug conjugate	4	3	1	3	1
JNJ-38877605	Type I TKI (Met-specific)	1	1	0	0	0
REGN5093	Met mAb (bivalent)	1	1	0	1	0

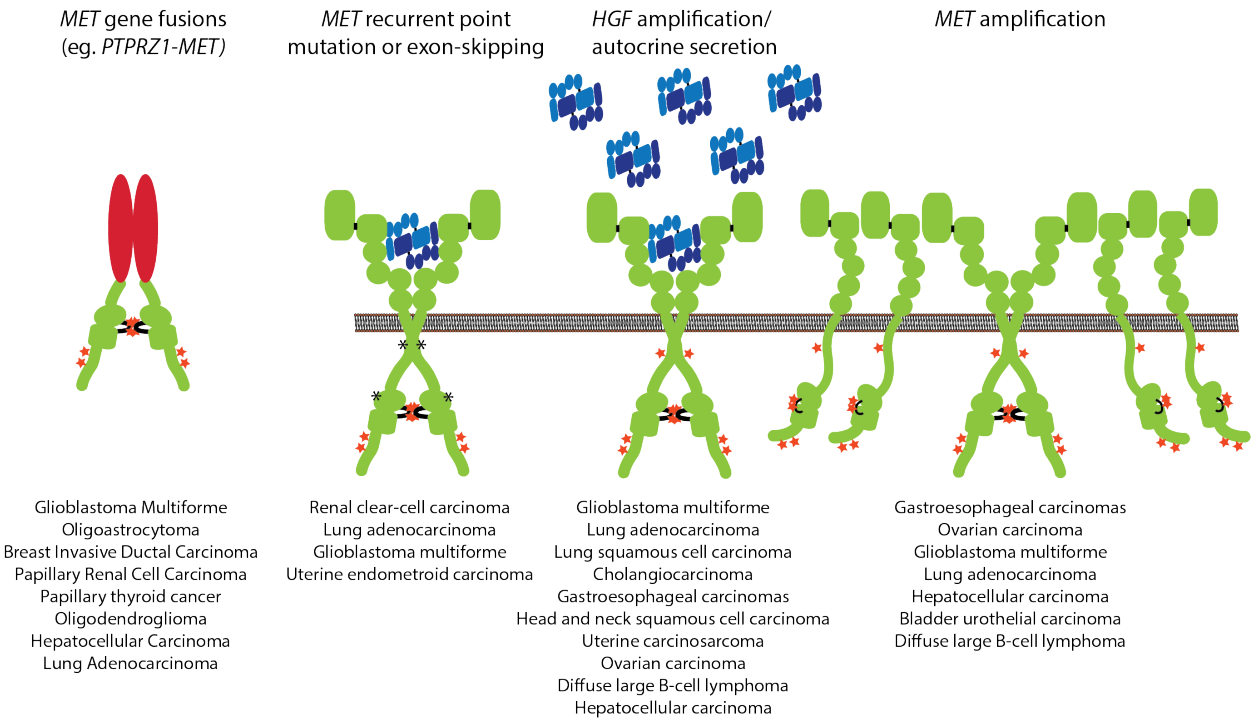


Figure 1.7: Mechanisms of* MET *oncogenic activation in cancer*. Met can be activated by a variety of mechanisms, including gene fusions induced by chromosomal alteration, most frequently with the phosphatase gene* PTPRZ1*; by recurrent point mutations affecting the kinase domain or exon 14 in the juxtamembrane domain; by HGF amplification or autocrine secretion, and finally by amplification of the* MET *gene to overexpress the receptor. While point mutations predispose the receptor to HGF-dependent activation,* MET *gene fusions or amplification can induce HGF-independent signaling and do not require exogenous ligand for oncogenic activity. Cancers in which these modes of Met activation can be found in the Pan-Cancer Atlas are listed.*

1.25 Antibody-based targeting of Met-dependent signaling in cancer

Met has been targeted in clinical trials by antibodies directed at extracellular regions of the receptor or by tyrosine kinase inhibitors selective for the Met kinase domain over the majority of other tyrosine kinases. Antibodies targeting the Met receptor in clinical trials have primarily been directed at the ligand-binding function of the receptor. Initial clinical trials with both antibodies showed reasonable tolerance in patients with advanced cancer, and while a significant benefit was not observed, partial responses were observed in multiple patients during phase II trials with both antibodies (Scagliotti et al., 2013; Spigel et al., 2012). Unfortunately, the development of

both antibodies was halted during phase III clinical trials due to concerning trend towards earlier mortality in patients on antibody-treatment arms, and this method of targeting the Met receptor has largely been abandoned. Retrospective analysis of phase II trials had shown some benefit of ligand-competitive antibodies in patients with *MET* amplification, but this was not borne out in phase III trials with either antibody (Catenacci et al., 2017; Garber, 2014).

Extracellular antibody mixtures have been developed that target the Met receptor at multiple sites, inducing Met internalization in both HGF-dependent and -independent modes of activation (Grandal et al., 2017; Poulsen et al., 2017). This mixture is currently under preliminary assessment for safety and response in humans, but results have not been reported yet (Clinical trial number NCT02648724) (Camidge et al., 2019). An additional approach of targeting the Met pathway led to the development of ficlatuzumab, an HGF-neutralizing antibody (D'Arcangelo and Cappuzzo, 2013). Like antibodies directed at the extracellular ligand-binding domains of the Met receptor, this drug was well-tolerated by patients with advanced cancer in phase I trials, but did not show statistically significant benefit in combination with EGFR-directed TKIs in phase II trials (Mok et al., 2016; Patnaik et al., 2014; Tabernero et al., 2014). Ficlatuzumab is still under investigation in pancreatic and head-and-neck squamous cell carcinomas, but trials involving this drug in other diseases were terminated due to lack of expected response and poor enrollment. A bispecific antibody targeting Met and EGFR, JNJ-61186372, is also under active investigation in clinical trials, although it is being prioritized for patients with *EGFR* exon 20-skipping mutations over patients with *MET* alterations (Table 1.1).

1.26 Tyrosine kinase inhibitors targeting the Met RTK in cancer

Tyrosine kinase inhibitors comprise a much broader class of Met-directed therapeutic agents. While target specificity is central to the design of both therapeutic antibodies and small-molecule ATP-competitive kinase inhibitors, the similarity between ATP binding pockets in kinase domains creates additional challenges in achieving selectivity by this method. For this reason, most TKIs, including FDA-approved kinase inhibitors, show activity against multiple kinases (Bishop et al., 1998; Davis et al., 2011; Knight and Shokat, 2005; Roskoski, 2010). While this is a major challenge in drug development, it does have the advantage of allowing well-tolerated drugs to be repurposed for a wide variety of malignancies. This has proven to be extremely beneficial in the case of Met, which is effectively inhibited by the FDA-approved multikinase inhibitors crizotinib and cabozantinib.

Crizotinib was developed as a Met inhibitor but was discovered to have activity against the ALK and ROS RTKs *in vitro*, and responses to crizotinib in patients with *ALK* gene rearrangements were observed in phase I clinical trials (Camidge et al., 2012; Christensen et al., 2007; Zhang et al., 2010). Clinical trials for crizotinib were initiated to target non-small-cell lung cancer (NSCLC) with ALK, ROS or Met expression by immunohistochemical scoring. Initial trials showed a significant benefit for patients with ALK positivity, most of whom had gene fusions oncogenically activating *ALK* (Camidge et al., 2012). Patients with high Met protein levels were not more likely to respond to treatment, but individuals with *MET* gene amplification were identified as exceptional responders (Landi et al., 2019; Lennerz et al., 2011; Ou et al., 2011). FDA approval was given for crizotinib based on its efficacy in ALK-rearranged patients, but given its recognized activity in *MET*-amplified tumours, Met alterations are included as an on-label indication for this drug

(Camidge et al., 2012; FDA, 2011; Kazandjian et al., 2014; Solomon et al., 2018). Subsequent trials have shown elevated rates of response to crizotinib in patients with high-level amplification of the *MET* gene, consisting of at least a 2.2-fold increase in *MET* gene signal over the chromosome 7 centromere (CEP7) (Camidge et al., 2014). While definitive improvements in overall survival have not been reported in patients with *MET*-amplified tumours treated with crizotinib, its availability as an FDA-approved inhibitor of Met has led to the inclusion of *MET* amplification in many ongoing basket trials for patients with advanced cancers (Table 1.1).

Cabozantinib is another FDA-approved multikinase inhibitor with activity against Met. Unlike crizotinib, which was initially selected for development due to its activity against the Met kinase, cabozantinib has been developed largely as a potent inhibitor of the VEGF receptor 2 (VEGFR2) tyrosine kinase, encoded by the *KDR* gene, which is a major regulator of pathological angiogenesis in multiple human cancers (Shibuya and Claessonwelsh, 2006). Cabozantinib is approved for use in medullary thyroid cancer, metastatic renal clear cell carcinoma and hepatocellular carcinoma previously treated with the VEGFR2-targeting multikinase inhibitor sunitinib (COMETRIQ® and CABOMETYX® product labels). In preclinical models and patients treated off-label with cabozantinib, Met kinase inhibition has been shown to have direct clinical value distinct from its activity against other targets such as VEGFR2 (Wang et al., 2019; Yakes et al., 2011). Cabozantinib has been especially useful as a treatment in the bone metastatic setting, where it has been shown to reduce metastatic burden in patients in a difficult-to-treat disease setting. Importantly, this activity has been linked in preclinical models to Met signaling specifically, demonstrating a benefit of multikinase over single-kinase therapy in disease control (Daudigeos-Dubus et al., 2017; Xiang et al., 2014).

Recently, FDA approval was granted for capmatinib, a first-in-class highly selective Met inhibitor. Capmatinib has some off-target activity *in vitro* like all known TKIs, but its selectivity for Met is sufficient for its use in humans as a selective monokinase inhibitor (Baltschukat et al., 2019; Liu et al., 2011). Capmatinib was approved for use in NSCLC patients with Met exon-14-skipping mutations in May 2020, based on promising results in phase II clinical trials and ongoing phase III trials. While activity in patients with *MET* amplification has been reported, the use of capmatinib for this indication is still under active investigation to determine survival benefit.

1.27 Stratification of likely responders has remained a challenge for Met-directed cancer therapies

The development of Met-directed therapies in cancer has seen repeated setbacks. The failures of onartuzumab and rilotumumab suppressed industrial interest in designing Met-targeted therapies until further evidence of efficacy could be shown. Trials targeting Met continued using the FDA-approved multikinase inhibitors crizotinib and cabozantinib, while several newer Met-targeting TKIs were developed in parallel, including the recently-approved Met inhibitor capmatinib. Unfortunately, these efforts have yet to show statistically significant survival benefit for Met-directed therapies in a randomized controlled trial; nonetheless, *MET* amplification is still considered as a target of precision medicine in ongoing basket trials. Met was initially investigated due to the observation that elevated levels of Met protein correlated with poor prognosis in several cancers, but stratification methods based on immunohistochemistry as a method of detected elevated Met levels have a poor record of successfully predicting patient response to Met-directed therapies (Catenacci et al., 2014; Garber, 2014; Lambros and Uguen, 2019; Mignard et al., 2018). It has been suggested that variability in immunohistochemical

scoring between different batches of samples may contribute to the failure of this method in patient stratification, while other studies have indicated that the dynamic range of Met protein levels associated with Met dependency in cancer are not captured by current immunohistochemical assays (Boyle et al., 2019; Catenacci et al., 2014; Mignard et al., 2018). Retrospective analyses of underperforming clinical trials identified *MET* gene amplification as predictive of response to Met-targeted therapy, leading to the adoption of fluorescence *in-situ* hybridization (FISH) as a primary assay for identifying patients likely to respond to Met inhibition (Lennerz et al., 2011; Ou et al., 2011). *MET* copy gain in the context of chromosome 7 polysomy is generally associated with lower absolute copy number than *MET* focal amplifications involving a small region within chromosome 7, and is also more likely to predict response to Met-directed therapy (Lai et al., 2019). Additionally, patients with low-level *MET* focal amplification respond at lower rates to Met inhibition, and frequently have additional oncogenic drivers that may be better drug targets (Noonan et al., 2016; Tong et al., 2016). Thus, patients with at least a 5-fold increase in signal for the *MET* gene over CEP7 stand to benefit by even modest response rates to Met inhibition, but lower-level copy gains at the *MET* locus are not predictive of response over alternative therapies available (Guo et al., 2019; Noonan et al., 2016; Tong et al., 2016). Thus, while *MET* gene amplification remains an important mechanism of Met-dependent transformation, it remains unclear exactly how this works at a mechanistic level. There has been considerable interest in identifying other clinical indications for Met inhibition.

Mutations affecting the negative regulation of Met are also considered a major mechanism of Met-dependent transformation. Mutations causing exon 14 skipping frequently overlap with *MET* gene amplification as well, but objective responses to the Met TKI crizotinib have been

observed both in exon-14-mutated tumours lacking *MET* amplification and in amplified tumours with no evidence of exon-skipping events (Awad et al., 2016; Caparica et al., 2017; Drilon et al., 2016). Nonetheless, responses to Met inhibitors have been reported in patients lacking gene amplification, clear evidence of receptor overexpression, or known activating or exon 14 mutations, indicating that there may be exceptional responders to Met-targeted therapy that current stratification techniques cannot detect (Zhang et al., 2015). Thus, new methods of identifying patients likely to respond to Met-targeted therapy and a clearer understanding of the mechanisms of Met-dependent transformation could lead to better patient stratification and an improvement in clinical care.

1.28 MET gene amplification is a well-studied model of Met-dependent cancer

While several genetic alterations in *MET* have been linked to Met-dependent oncogenicity and thus sensitivity to Met inhibition, *in vitro* models of Met-dependent cancers have long been shown to involve overexpression of *MET*, often through focal genomic amplification. The initial human cancer cell lines in which full-length Met was observed to be phosphorylated all possessed focal amplification of the *MET* gene, resulting in overexpression and constitutive phosphorylation of the receptor (Giordano et al., 1988; Giordano et al., 1989; Rodrigues et al., 1991). Spontaneous amplification of the endogenous murine *MET* gene was reported to transform NIH-3T3 fibroblasts (Cooper et al., 1986). Moreover, in cells transduced with cDNA encoding Met, multiple copies of the transgene were found in all transformed cells, implying that transformation was accompanied by expansion of the Met copy number (Iyer et al., 1990). In subcutaneous tumours grown from NIH-3T3 cells transduced with either wild-type Met or kinase domain mutants observed in familial kidney cancers, all resultant tumours showed overexpression of Met

compared to the injected cells, indicating that Met kinase domain mutations alone are insufficient to oncogenically activate the Met RTK (Jeffers et al., 1997).

While the above evidence had long implicated gene amplification in the oncogenic activation of Met, the observation that patients with *MET* gene amplification were more likely to respond to Met-directed therapy in clinical trials renewed interest in *MET*-amplified cancer cell lines as a model (Garber, 2014; Spigel et al., 2017). *MET* amplification had already been shown to confer sensitivity to Met-directed TKIs in tissue culture and *in vivo* in comparison to non-amplified cell lines (Lutterbach et al., 2007; Smolen et al., 2006). *MET*-amplified cell lines have thus been used to characterize TKI and antibody-based inhibitors of Met, and in particular as a model system to study the development of acquired resistance to Met-directed therapy both in cell culture and in xenograft mouse models (Apicella et al., 2018; Cepero et al., 2010; Goltsov et al., 2018; Kim et al., 2015; Kim et al., 2019; Pennacchietti et al., 2014; Yamaoka et al., 2016).

In addition to this, *MET* amplification had been shown as a bypass mechanism of acquired resistance to the EGFR TKI gefitinib in *EGFR*-mutated NSCLC cell lines (Engelman et al., 2007). *MET* amplification has since been demonstrated to be a mechanism of acquired resistance to EGFR-directed TKIs in human patients, and recent clinical trials have shown a recurrent benefit of combining Met- and EGFR-directed TKIs in NSCLC patients who have acquired resistance to EGFR inhibition (Gainor et al., 2016; Sequist et al., 2020). While acquired resistance to first- and second-generation EGFR TKIs usually arises through secondary EGFR mutations, more recent third-generation EGFR TKIs can maintain growth inhibition in the presence of these mutations. The occurrence of *MET* amplification as a mechanism of acquired resistance is more common in patients treated with these newer EGFR TKIs (Roper et al., 2020). Thus, despite its low incidence

as a *de novo* driver alteration, *MET* amplification remains an actionable genetic alteration with an increasingly important role in human cancers.

1.29 RTK crosstalk in the MET-*amplified setting*

One key difference observed between *MET*-amplified and non-amplified cell lines is the Met-dependent phosphorylation of EGFR-family RTKs. *MET*-amplified cell lines have frequently been shown to exhibit Met-dependent activation of EGFR, HER2 and HER3, and co-inhibition of these RTKs has been shown to potentiate the effect of Met inhibition both in cell culture and xenograft mouse models of *MET*-amplified cancer (Lutterbach et al., 2007; Moores et al., 2016; Xu et al., 2012; Zhang et al., 2013). The activation of EGFR or HER3 by their ligands has been shown to rescue proliferation in *MET*-amplified cells under growth arrest due to Met kinase inhibition or siRNA-mediated depletion (Bachleitner-Hofmann et al., 2008). While experimental models have frequently identified Met kinase domain mutations, stromal HGF secretion or further amplification of *MET* as major mechanisms of acquired resistance to Met-directed TKIs, both cell-culture and patient-derived organoid models have identified EGFR signaling as an important additional bypass mechanism to Met inhibition (Kim et al., 2019). Thus, there is evidence that signaling downstream of EGFR is important in mediating the response to Met inhibition in the *MET*-amplified context.

Efforts to clinically target crosstalk between Met and EGFR itself have not shown therapeutic benefit in clinical trials in patients with *de novo* mutations in *EGFR* or amplification of *MET* (Spigel et al., 2013). Nonetheless, *MET* amplification is recognized as a mechanism of resistance following small-molecule inhibitor targeting of EGFR mutant lung cancer, and conversely, EGFR

and HER3 amplification or mutation have been observed as resistance mechanisms to Met inhibition in human cancers (Engelman et al., 2007; Gainor et al., 2016; Recondo et al., 2020a). Recently, the combination of Met inhibition and EGFR inhibition has shown significant clinical benefit in patients with acquired resistance to the third-generation EGFR inhibitor osimertinib, as this can effectively inhibit the common secondary EGFR mutations often acquired in patients treated with gefitinib or erlotinib (Roper et al., 2020; Sequist et al., 2020). In addition, ligand-mediated activation of EGFR-family RTKs, including HER3, restores cell proliferation in *MET*-amplified cells treated with a small-molecule Met inhibitor (Bachleitner-Hofmann et al., 2008).

Additional instances of crosstalk with other RTKs has been reported in *MET*-amplified cells (recently reviewed in Paul and Hristova, 2019). The closely-related RTK Ron can heterodimerize with Met to promote receptor *trans*-phosphorylation and oncogenic signaling (Benvenuti et al., 2011; Follenzi et al., 2000). One key study identified crosstalk with the Ret RTK as well as EGFR, HER2 and HER3 in *MET*-amplified cell lines, and proposed differential roles for each receptor in mediating signal output downstream of Met (Tanizaki et al., 2011b). Ligand-mediated activation of FGFR family RTKs have been implicated in resistance to Met inhibition *in vitro*. A recent study by our group reported that Met and FGFR1 cooperate to promote tumour initiating capacity in a genetically-engineered mouse model with spontaneous *MET* amplification (Kim et al., 2015; Sung, 2019; Sung et al., In press). Thus, it remains critical to understand the contribution of RTK crosstalk to oncogenic signaling and the emergence of acquired resistance cancer patients with *MET*-amplified tumours.

1.30 Rationale

While Met signaling in the context of HGF-dependent activation has been extensively characterized using hypothesis-driven as well as empirical observational research, the signaling output required for a proliferative and anti-apoptotic response underlying sensitivity to Met inhibition is poorly understood. Many of the pathways shown to contribute to Met-dependent proliferative signaling in the context of *MET* amplification or mutation in cancer are known to be involved in the invasive growth program initiated by HGF-dependent signaling seen in most normal cell lines, where little HGF-dependent proliferation is observed (Johnson et al., 2013; Lai et al., 2014; Stoker et al., 1987).

Crosstalk between Met and the EGFR family of RTKs, frequently involved in cancer-associated proliferative signaling, has been reported in many *MET*-amplified cancer cell lines. We hypothesized that Met-EGFR family crosstalk may be involved in rewiring Met-dependent signaling from an invasive toward a proliferative output, rendering *MET*-amplified cells sensitive to Met inhibition. RTK crosstalk between Met and the EGFR family would thus underlie the sensitivity of *MET*-amplified tumours to Met-directed therapies. To test this hypothesis, we characterized a panel of *MET*-amplified cell lines for crosstalk with the EGFR family of RTKs. We then employed cell lines from this panel as a model system to delineate the contribution of Met-dependent crosstalk to proliferative signaling in the amplified setting. We further identified novel Met-dependent phosphoproteins in the amplified setting using mass spectrometry and developed an ATP analogue-sensitive variant of Met to be used for substrate-labeling experiments. As better stratification of patients likely to respond to Met-directed therapies remains a critical challenge in effective intervention in human cancer, a clear understanding of

the mechanism underpinning Met-dependent cell transformation will improve our precision in targeting this pathway in cancer.

CHAPTER 2: RESULTS

2.1 Crosstalk between Met and EGFR family RTKs in *MET*-amplified cancer cell lines

In order to study crosstalk between Met and EGFR-family RTKs in Met-dependent cancers, we first assembled a panel of *MET*-amplified cancer cell lines that maintain high protein levels of Met and display constitutive activation and tyrosine phosphorylation of Met. These cells would be predicted to show Met-dependent proliferation and promotion of cell survival signaling, and could serve as model systems for Met-dependent cancers (Lai et al., 2014; Smolen et al., 2006). For this we collected cell lines already validated as Met-dependent through previous experiments conducted by our group as well as other reports in the literature (Abella et al., 2010a; Grandal et al., 2017; Lai et al., 2014; Smolen et al., 2006; Tanizaki et al., 2011a; Zhang et al., 2013). We also included cell lines that were annotated to have copy number gains at the MET genomic locus by the Cancer Cell Line Encyclopedia (CCLE) or the Catalogue of Somatic Mutations in Cancer (COSMIC) (Malme-3M, HCC70, HCC1395). We included HeLa cells as a well-characterized control cell line that maintains Met protein levels but does not exhibit Met activation or phosphorylation in the absence of exogenous treatment with the Met ligand, HGF (Abella et al., 2010a; Parachoniak et al., 2011) (Table 2.1).

2.2 Building a panel of MET*-amplified cell lines with constitutive Met phosphorylation*

To establish a panel of Met-dependent cancers, we assessed Met expression and tyrosine phosphorylation, as well as overall tyrosine phosphorylation, in our set of cell lines with *MET* copy gains or amplification. We found that high levels of Met protein were only observed in cell lines already established as Met-dependent by studies in our group and other laboratories, while the

Table 2.1:* MET-*elevated and -amplified cell lines. *Characteristics of cell lines used in figure 2.1 and for* MET-*amplified cell line panel*

Cell line	Tissue	Morphology	Log_2[*MET*] (CCLE)	Met copy number (COSMIC)	Reference
HeLa	cervix	epithelial			(Abella et al., 2010a)
Malme-3M	skin	fibroblast	1.02	7	(Rohrbeck et al., 2016)
HCC70	breast	epithelial	0.41		(Sameni et al., 2016)
HCC1395	breast	epithelial	1.35		(Sohn et al., 2014)
OE33	oesophagus	epithelial	2.11	14	(Grandal et al., 2017)
Snu5	stomach	suspension	3.45	14	(Lai et al., 2014)
Okajima	stomach	suspension			(Lai et al., 2014)
Kato II	stomach	suspension			(Lai et al., 2014)
MKN45	stomach	epithelial	2.63	9	(Lai et al., 2014)
EBC1	lung	epithelial	3.5		(Zhang et al., 2013)
H1993	lung	epithelial		10	(Tanizaki et al., 2011b)
SkBr3	breast	epithelial	1.6		(Brockhoff et al., 2001)

additional cell lines with copy gains at the *MET* locus did not express elevated levels of Met protein or show constitutive Met phosphorylation (Figure 2.1A). All previously-characterized Met-dependent cell lines (OE33, Snu5, Okajima, KatoII, MKN45, EBC1 and H1993) displayed constitutive phosphorylation of Met, and tyrosine phosphorylation of several other proteins as observed by pan-phosphotyrosine Western blotting (Figure 2.1A). The constitutive phosphorylation of Met in the absence of exogenous HGF at high protein levels is consistent with a simple mass-action-based mechanism of Met activation, whereby increased Met concentrations within the plasma membrane promote its ligand-independent association and kinase activation (Figure 2.1B). This was associated with elevated levels of Met protein

normalized to total cellular protein (Figure 2.1C). We also performed cell proliferation assays in the presence of the Met inhibitor PHA-665752 (PHA) (0.5 μM) and the EGFR inhibitor gefitinib (1 μM) to confirm the sensitivity of *MET*-amplified cell lines to Met inhibition (Figure 2.1D) (Christensen et al., 2003; Davis et al., 2011). While PHA treatment reduced cell proliferation in all *MET*-amplified cell lines tested, it had no effect on an *ERBB2*-amplified breast cancer cell line, SkBr3, where HER2-driven activation of EGFR and HER3 is important for proliferation (Brockhoff et al., 2001). Instead, SkBr3 cell proliferation was sensitive to gefitinib inhibition (Figure 2.1D). By contrast, 5 out of 7 *MET*-amplified cell lines tested showed no statistically significant impact on proliferation when treated with gefitinib. Mkn45 gastric cancer cells were more sensitive to PHA but showed partial sensitivity to gefitinib as well (8 vs. 69% of control, respectively; Ordinary one-way ANOVA, $p<0.0001$ and $p=0.0009$, respectively). OE33 cells, which are both *MET*- and *ERBB2*-amplified, show comparable sensitivity to both PHA and gefitinib, though both of these effects are below the cutoff we used for statistical significance. We proceeded to use the seven *MET*-amplified cell lines in our study of the role of crosstalk with the EGFR family in Met-dependent cancer.

2.3 Characterization of Met-EGFR family crosstalk in MET*-amplified cell lines*

To better understand whether constitutive Met activation promoted heterotypic signaling that would contribute to Met-dependent proliferation, we first assessed the phosphorylation of the EGFR family RTKs EGFR, HER2 and HER3 in our panel of *MET*-amplified cell lines. We included SkBr3 cells as a control in which *ERBB2* amplification drives overexpression and constitutive phosphorylation of HER2, as well as its homologues EGFR and HER3 (Brockhoff et al., 2001). We assessed levels of total and tyrosine phosphorylated HER3, EGFR and HER2 using site-specific

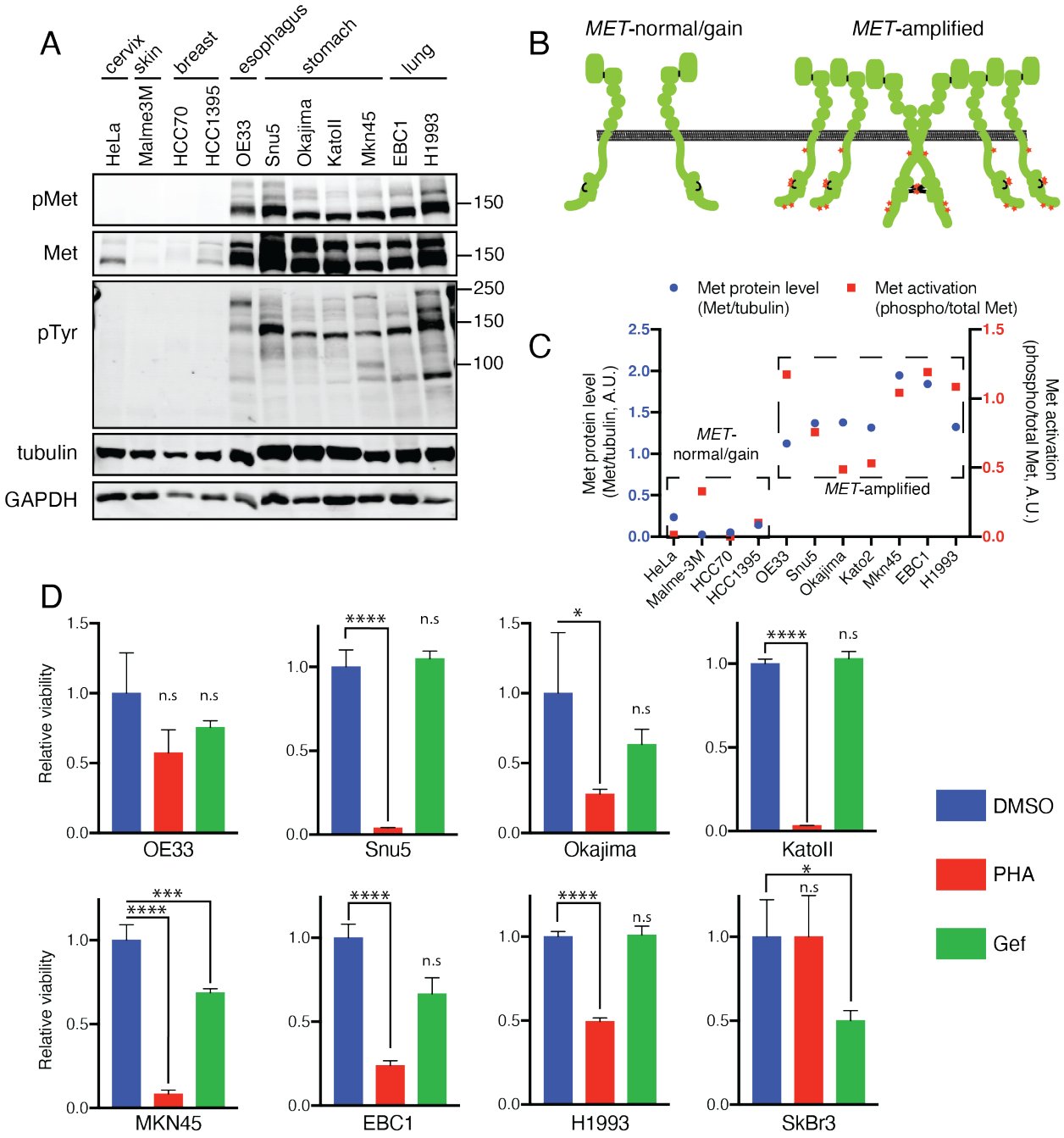


Figure 2.1: Elevated Met protein predicts constitutive tyrosine phosphorylation in* MET*-amplified cells. *(A) Western blot of Met activation loop phosphorylation (Tyr 1234/35) (pMet), total Met protein, and general tyrosine phosphorylation (pTyr). Tubulin and GAPDH levels are shown as loading controls (n=1). (B) Autoinhibitory mechanisms in Met that keep the unliganded receptors inactive in normal cells (left) may be overcome at high concentrations of receptor in the plasma membrane, as seen in* MET*-amplified cells (right). (C) Quantification of total Met protein levels relative to tubulin (left) and phospho-Met relative to total (right) from (A) show that high protein levels are associated with the induction of Met phosphorylation. (D)* MET*-amplified cells are sensitive to PHA-665752 (PHA) in proliferation assays, while these cell lines are mostly insensitive to the EGFR inhibitor gefitinib. In contrast,* ERBB2*-amplified SkBr3 cells are sensitive to gefitinib but not PHA treatment. Ordinary one-way ANOVA: * p<0.05; *** p<0.001; **** p<0.0001; n=1.*

phosphotyrosine antibodies directed at residues in the RTK C-tails. All seven *MET*-amplified cell lines expressed HER3, EGFR and HER2 at detectable levels and showed basal tyrosine phosphorylation using steady-state conditions, as was also observed in SkBr3 cells (Figure 2.2A). To evaluate dependence of EGFR-family RTK phosphorylation on Met activity, the cell line panel was treated for one hour with the PHA-665752 (0.5 μM) or with DMSO as control. Met inhibition reduced phosphorylation of HER3 in all 7 *MET*-amplified cell lines tested (Figure 2.2B). EGFR phosphorylation was dependent on Met activity in 4 out of 7 cell lines (OE33, MKN45, EBC1 and H1993) whereas HER2 phosphorylation was dependent on Met activity only in MKN45 and H1993 cells. This indicated that crosstalk between Met and the EGFR family of RTKs primarily involves HER3 in *MET*-amplified cells, with convergent selection for a Met-HER3 phosphorylation axis across seven independently derived cell lines.

2.4 Met-dependent EGFR family phosphorylation is not dependent on robust RTK heterointeraction

In EBC1 and H1993 cells, Met inhibition diminishes not only tyrosine phosphorylation of HER3, but also EGFR and HER2, suggesting that Met may engage directly with all three EGFR family RTKs in these cell lines (Figure 2.2). Previous studies have reported co-immunoprecipitation of Met with HER3, EGFR and HER2, as well as the RET RTK (Tanizaki et al., 2011b). We failed to detect co-immunoprecipitation of Met with HER3 and EGFR over control IgG antibody under comparable conditions, indicating that any protein-protein interaction between Met and these

proteins in these cell lines is labile and not easily amenable to structure-function based analysis (Figure 2.3 A and B).

HER2 similarly did not robustly co-immunoprecipitate with Met in these cell lines (Figure 2.3 C). Supporting this, co-overexpression of Met with EGFR did not promote their co-immunoprecipitation in HEK293 cells, whether or not kinase activity was impaired by the Lys1110Ala substitution in the kinase domain (K1110A) (Figure 2.3 D). When Met and HER3 were co-overexpressed in the related HEK293T cell line, we similarly did not detect their co-immunoprecipitation (Figure 2.3 E). Collectively, these observations indicate that the Met-dependent phosphorylation of these receptors is not accompanied by the formation of a stable complex that can be detected by immunoprecipitation.

2.5 Canonical substrate binding sites in Met and HER3 are not required for HER3 phosphorylation

As an alternative to co-immunoprecipitation to detect protein-protein interactions, we have developed a *trans*-phosphorylation assay to identify the structural requirements of HER3 and Met for transphosphorylation. As HER3 lacks intrinsic kinase activity, expression of HER3 in HEK293T cells does not induce HER3 autophosphorylation, unlike what is observed for EGFR or HER2. Using lentiviral transduction, we stably expressed full-length HER3 in HEK293T cells under the constitutively-activated EF1α promoter, and transiently transfected constructs expressing a panel of Met mutants or the wild-type Met RTK from a vector combining the adeno-major late promoter with the SV40 origin of replication.

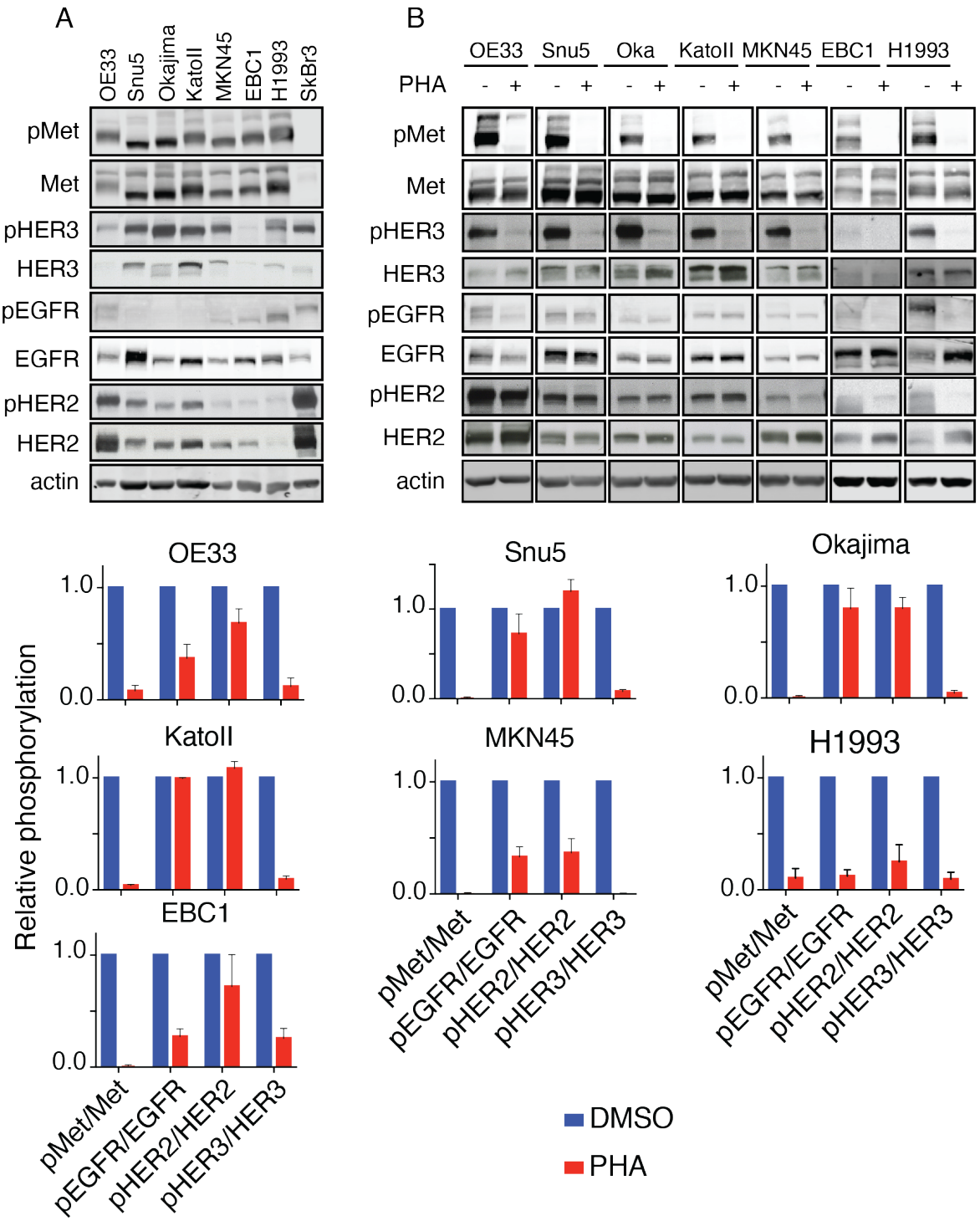


Figure 2.2: Met-dependent phosphorylation of HER3, EGFR and HER2 in* MET*-amplified cell lines. *(A)* MET*-amplified cell lines co-express HER3, EGFR and HER2 and maintain basal phosphorylation of these RTKs (pHER3: Tyr1289; pHER2: Tyr1221/22; pEGFR: Tyr1173) similar to the basal phosphorylation of HER3 and EGFR in the* ERBB2*-amplified SkBr3 cell line (n=3). (B) Treatment of* MET*-amplified cell lines with the Met-selective TKI PHA-665752 (PHA) inhibits HER3 phosphorylation in all cell lines tested (n=3). EGFR and HER2 phosphorylation is dependent on Met activity in a subset of* MET*-amplified cell lines (quantified below Western blot panels, n=3).*

This facilitated episomal amplification of the Met-expressing construct, driving high-level overexpression as in the amplified setting (Figure 2.4A). Tyrosine-to-phenylalanine substitution of both Tyr1349 and Tyr1356 in the substrate-binding Met C-tail abolishes canonical downstream signaling through Grb2, Shc and Gab1 (Fixman et al., 1996; Fixman et al., 1997; Fixman et al., 1995; Nguyen et al., 1997; Zhu et al., 1994). The loss of exon 14 (Δ14) is predicted to prevent efficient ubiquitination of Met by the Cbl E3 ligase, while the combined substitution of the Cbl-binding Tyr1003 with phenylalanine and uncoupling from Grb2 through an Asn1358His substitution (ΔGrb2/Cbl) abolishes Cbl recruitment to Met (Fournier et al., 1996; Peschard et al., 2001). Collectively, these mutants substitute or delete the three major tyrosine residues involved in substrate recruitment downstream of Met, and abolish the signaling required for cell scattering, tubulogenesis or transformation (Fixman et al., 1997; Fournier et al., 1996; Zhu et al., 1994). All of these mutants were capable of inducing HER3 tyrosine phosphorylation upon transient overexpression in HEK293T cells stably expressing HER3, and only a kinase-dead Met mutant (K1110A) lost the ability to induce HER3 phosphorylation (Figure 2.4B). To test whether phosphorylation of HER3 by Met requires the formation of heterodimeric kinase-kinase interactions as observed for its dimerization with EGFR or HER2, we generated mutant HER3 proteins to perform a structure-function analysis. Mutations within a conserved sequence in the N-lobe of EGFR and HER2 can prevent these kinases from occupying the “receiver” position an asymmetric active kinase dimer (I682Q in EGFR), while mutations in the C-lobe can prevent these kinases from occupying the “activator” position (V924R in EGFR) (Zhang et al., 2006). We introduced mutations at corresponding positions in HER3 (I684Q and V926R) and tested whether these mutants could be phosphorylated by Met (Figure 2.4C-D). In addition to this, we introduced

deletions in the C-tail, either eliminating the majority (5/6) of the well-characterized p85α binding sites in the distal portion of the C-tail ("Short tail" mutant, truncated after position T1141), or the entirety of the C-tail ("No tail" mutant, truncated after position F965) (Figure 2.4C).

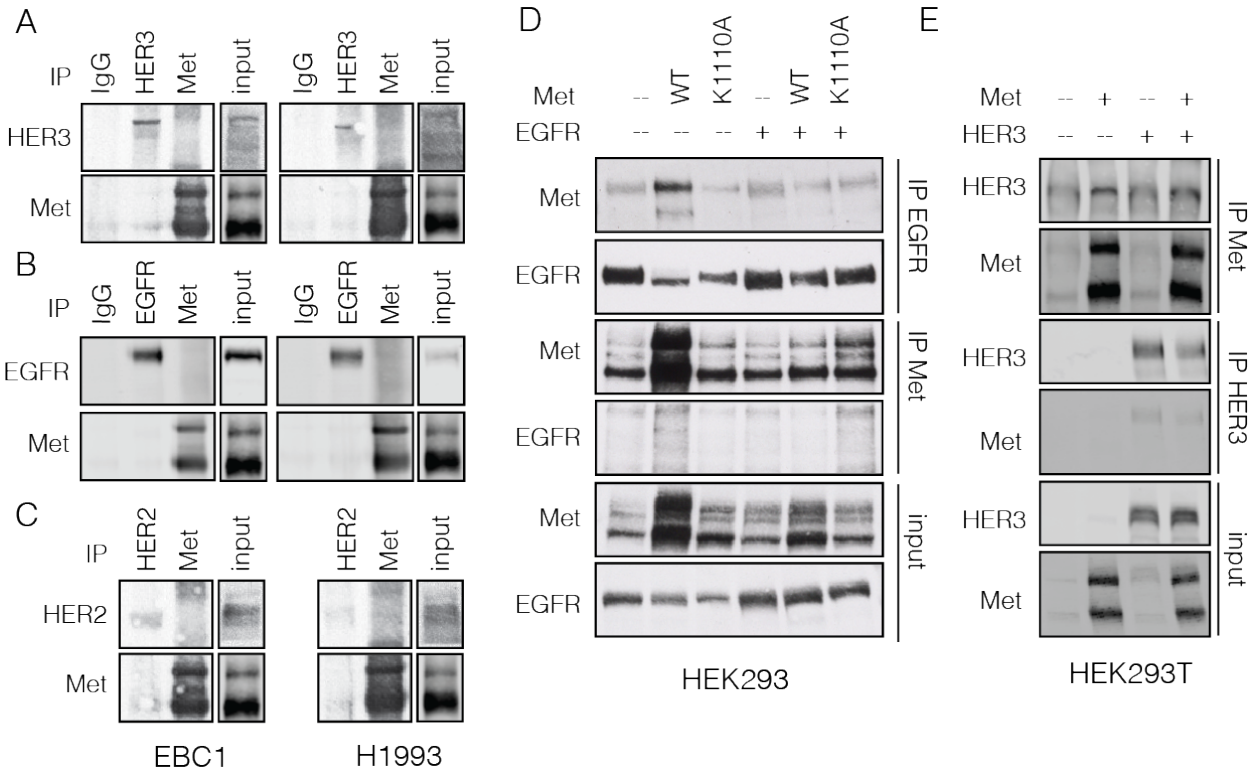


Figure 2.3: Met-dependent EGFR family phosphorylation does not require robust RTK heterointeraction. *(A) HER3 does not co-immunoprecipitate with Met in EBC1 or H1993 cells above the level observed with control antibodies (IgG) (n=1). (B) EGFR similar does not co-immunoprecipitate with Met in these cell lines (n=1). (C) HER2 does not appreciably co-immunoprecipitate with Met in EBC1 or H1993 cells (n=1). (D) Co-expression of wild-type (WT) or kinase dead (K1110A) Met with EGFR does not increase the co-immunoprecipitation of these RTKs above background in HEK293 cells (compare lanes 2 and 5 or 3 and 6) (n=3). (E) Co-expression of Met and HER3 similarly does not increase their co-immunoprecipitation in HEK293T cells (n=1).*

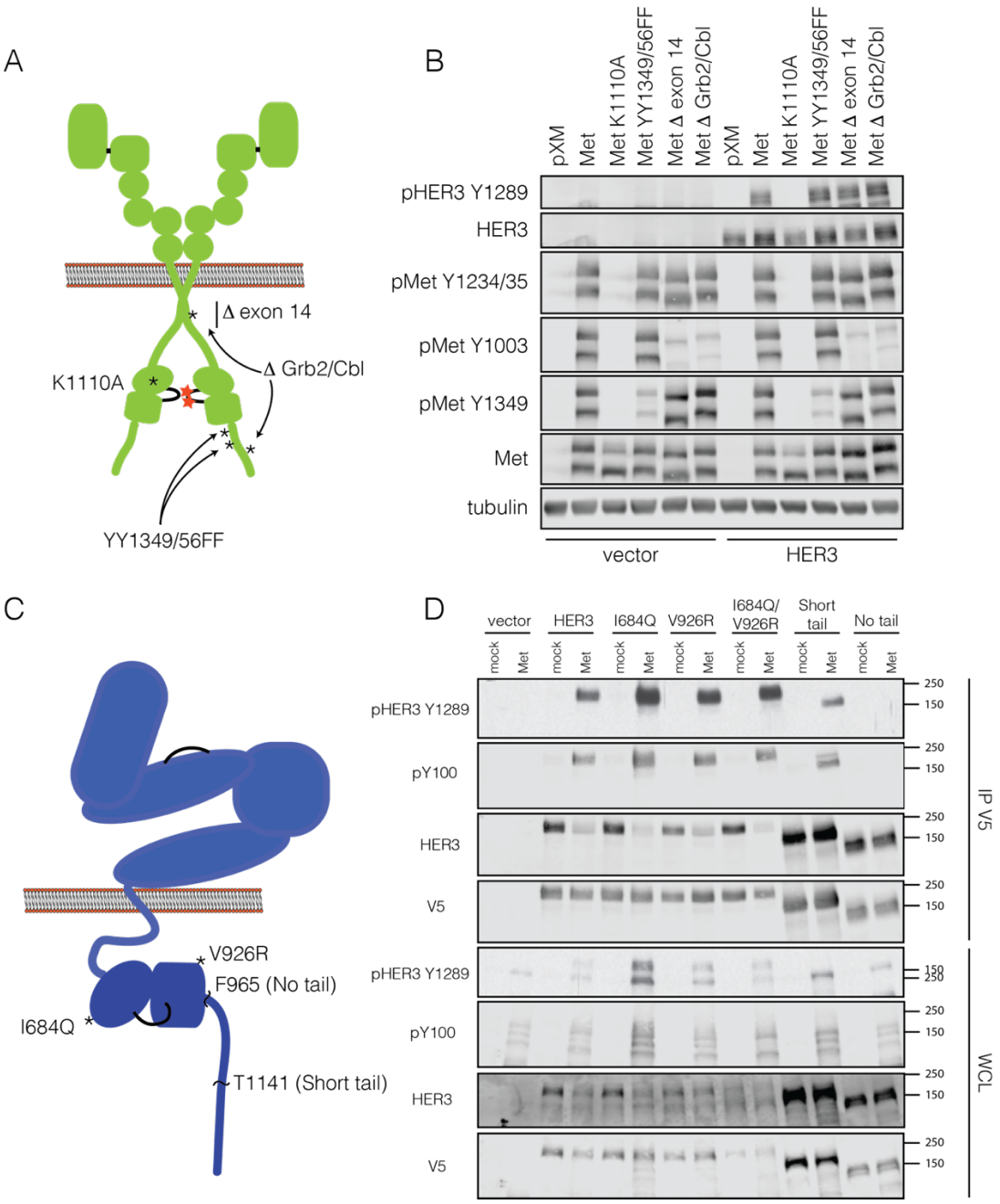


Figure 2.4: Canonical substrate binding sites in Met and HER3 are not required for HER3 phosphorylation. *(A) Mutation of the major SH2-domain binding sites to phenylalanine (YY1349/56FF) and the Cbl binding sites (Δexon 14, ΔCbl) prevent the recruitment of known downstream interactors of the Met RTK, while mutation of Lys1110 in the kinase domain (K1110A) impairs nucleotide binding and inactivates Met kinase activity. (B) While kinase activity in Met is required for HER3 phosphorylation, mutation of the SH2-domain recruitment sites in Met does not affect HER3 phosphorylation (n=3). (C) Mutations in the N-lobe (I684Q) and C-lobe (V924R) in the HER3 kinase domain prevent kinase-kinase interactions formed among EGFR family RTK heterodimers. Deletions within the distal HER3 C-tail (short tail) remove the majority (5/6) of the p85 binding sites in HER3, as well as 11/14 tyrosine residues in the C-tail, while removal of the entire C-tail (no tail) removes 14 tyrosines and leaves only the kinase and juxtamembrane domains in the cytoplasmic regions of HER3. (D) Mutation of the N- and C-lobe binding sites in the kinase domain does not affect Met-dependent phosphorylation of HER3, while deletion of the distal C-tail still results in phosphorylation of remaining tyrosines within the HER3 intracellular regions (n=4). Only deletion of the entire C-tail abolishes Met-dependent HER3 phosphorylation, likely by deletion of all tyrosines available for Met-dependent phosphorylation.*

Surprisingly, mutations in both the HER3 N- and C-lobes were tolerated and still led to Met-dependent tyrosine phosphorylation, as well as an N-lobe/C-lobe double mutant (Figure 2.4D). Deletion of the distal C-tail, comprising the majority of cytoplasmic surface-accessible tyrosines in HER3, similarly resulted in phosphorylation of the short-tail HER3 mutant in the presence of Met overexpression (Figure 2.4D). Only complete removal of the C-tail from HER3 prevented Met-dependent phosphorylation. Thus, tyrosines involved in substrate recruitment in both Met and HER3 are dispensable for Met-dependent HER3 phosphorylation. Collectively, these results provide evidence that HER3 is not recruited to Met upon kinase activation as part of a phosphotyrosine-dependent complex. Similarly, the observation that mutations in the HER3 kinase domain do not uncouple HER3 from tyrosine phosphorylation demonstrate that canonical heterodimers of Met and HER3, comparable to interactions formed between EGFR or HER2 and HER3, are not involved in Met-dependent HER3 phosphorylation. Thus, Met-dependent HER3 tyrosine phosphorylation does not involve major substrate-binding sites in either protein and does not proceed via the formation of asymmetric kinase dimers as seen within the EGFR family.

2.6 Kinases canonically upstream of HER3 are dispensable for its activation

While it is possible that the Met kinase domain directly phosphorylates tyrosines in the HER3 C-tail, our experiments with Met and HER3 mutants do not rule out the involvement of other RTKs or CTKs. Mutation of the C-lobe of the HER3 kinase domain (V926R) abolishes its heterodimerization with EGFR and HER2 upon stimulation with the HER3 ligand neuregulin, but EGF-mediated activation of EGFR has been shown to induce phosphorylation of the HER3 V926R mutant. This is presumed to be due to EGFR homodimeric activation, and the recruitment of HER3 as a substrate to the activated EGFR homodimer (see Figure 1.3D and 1.4B) (Van Lengerich

et al., 2017). We thus reasoned that the phosphorylation of HER3 may involve the activation of the EGFR or HER2 kinase domain downstream of Met, and that this could be blocked by the use of the EGFR TKI gefitinib or the HER2/EGFR TKI lapatinib (Figure 2.5B and C). Furthermore, as Src has been shown to potentiate HER3 activation through HER2 and to potentiate EGFR activity through kinase domain phosphorylation, we reasoned that Src or related CTKs may mediate phosphorylation of HER3 downstream of Met via a cytoplasmic shuttling mechanism (Figure 1.3C). This could be inhibited by the broad-spectrum TKI dasatinib, which has activity against several Src- and Abl-family CTKs in addition to the RTKs c-Kit and PDGFRα (Figure 2.5D). As predicted, treatment with PHA-665752 reduced HER3 phosphorylation in all seven *MET*-amplified cell lines in our panel, while none of the other inhibitors listed above impacted HER3 phosphorylation in these cells (Figure 2.5E). Notably, HER2 phosphorylation was frequently regulated by lapatinib but not by PHA (OE33, Snu5, Okajima, KatoII), indicating that HER2 phosphorylation is regulated independently of the Met-HER3 signaling axis in this context. As seen in Figure 2.2B, EGFR phosphorylation is Met-dependent in a subset of *MET*-amplified cell lines, but in contrast to HER2 it is not impacted by inhibition of EGFR or HER2 kinase activity using gefitinib or lapatinib. This stands in contrast to our observation of HER2-dependent EGFR and HER3 phosphorylation in *ERBB2*-amplified SkBr3 cells (Figure 2.5E, right-most panels). Src phosphorylation is either not impacted or slightly increased by Met inhibition using PHA-665752, but is inhibited by dasatinib, indicating that Src acts independently of Met and the EGFR family of RTKs in *MET*-amplified cells. Thus, the activity EGFR, HER2 and Src, kinases canonically involved in HER3 phosphorylation, are not required for Met-dependent HER3 phosphorylation.

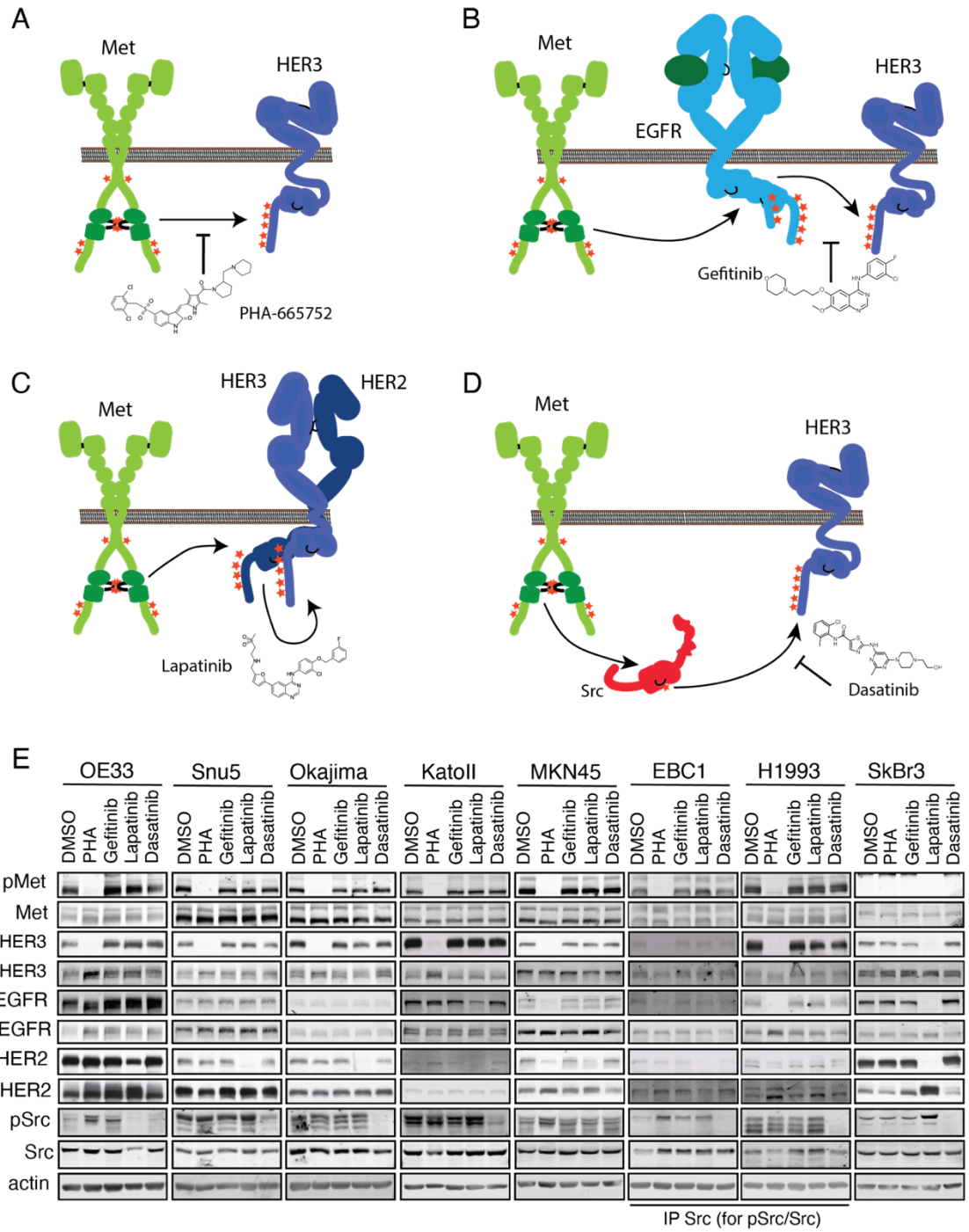


Figure 2.5: EGFR, HER2 and Src activity are not required for HER3 phosphorylation in* MET*-amplified cells. *Potential mechanisms for HER3-dependent phosphorylation downstream of Met could include the direct phosphorylation of HER3 as a substrate of Met (A), the ligand-mediated activation of EGFR homodimerization (B) or EGFR/HER2 heterodimerization with HER3 (C), or through a cytoplasmic shuttling mechanism involving Src-family CTKs (D), which are known to associate with and phosphorylate EGFR-family RTKs. (E) Treatment with PHA-665752 (PHA) (A), gefitinib (B), lapatinib (C) or dasatinib (D) could distinguish these mechanisms by inhibiting kinase activity downstream from Met (n=3). Only PHA treatment inhibits HER3 phosphorylation downstream from Met, implying that (A) is possible as a mechanism for Met-HER3 crosstalk, but ruling out the other canonical models for HER3 phosphorylation.*

2.7 Depletion of HER3 by shRNA impairs proliferation of MET*-amplified cells*

As our experiments using Met inhibitors identified a recurrent role for a Met-HER3 crosstalk axis over one between Met and EGFR or HER2, we proceeded to test the role of HER3 in the biology of *MET*-amplified cells by depletion of HER3 using RNAi. Vectors encoding shRNA hairpins targeting the HER3 mRNA, or an empty-vector control, were introduced into *MET*-amplified EBC1, H1993 and KatoII cell lines, and stable integration of the shRNA cassette was selected using puromycin. All three cell lines showed potent depletion of HER3 at the protein level by Western blot (Figure 2.6A). Cells with HER3-targeting shRNAs expanded more slowly in culture than control cells, indicating that proliferation may underlie HER3-dependent signaling in *MET*-amplified cell lines. To test this, we performed proliferation assays by live-cell microscopy using an IncuCyte imaging system (Essen Biosciences) to measure relative cell proliferation as a function of cell confluence (Figure 2.6B). All three cell lines showed impaired proliferation following knockdown of HER3 using both shRNA hairpins, confirming that a Met-HER3 crosstalk axis regulates cell proliferation in *MET*-amplified cancer cell lines. In addition to proliferation assays, we assessed the ability of the KatoII *MET*-amplified cell lines to form colonies in soft agar. KatoII *MET*-amplified cells grow partially in suspension and do not form colonies in two-dimensional cell culture, so we tested their colony-forming capacity by 3-dimensional soft agar assay. In addition to cell proliferation, this assay tests for anoikis resistance, as single cells plated in agar lack contact with one another and with extracellular biologically-reactive substrates. Depletion of HER3 impairs colony formation in this assay as well (Figure 2.6C). Hence, HER3 is required for proliferation of *MET*-amplified cells, and depletion of HER3 furthermore impairs the ability to form and grow colonies from single cells.

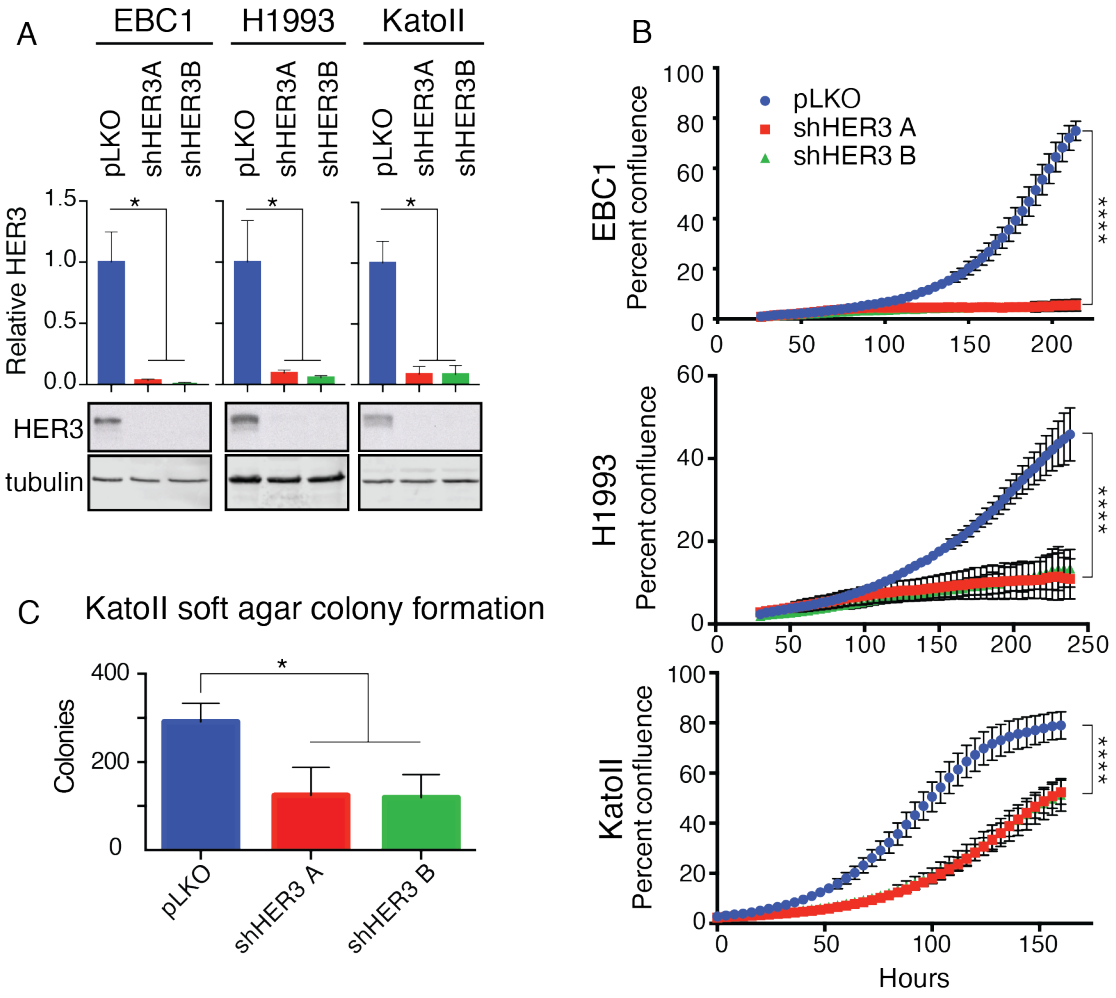


Figure 2.6: HER3 depletion impairs proliferation in* MET*-amplified cell lines. *(A) HER3 depletion with either of two shRNA hairpins effectively reduces HER3 protein levels by Western blot in comparison to empty-vector-transduced control cells (pLKO) (n=3). (B) IncuCyte time-lapse microscopy of HER3-depleted EBC1, H1993 and KatoII* MET*-amplified cells shows impaired proliferation in comparison to controls (representative replicates shown; n=3). (C) HER3 depletion also impairs KatoII colony formation in soft agar (n=4).*

2.8 HER3 depletion delays tumour outgrowth in vivo

Having stablished a crucial role for HER in MET-amplified cell proliferation *in vitro*, we next tested whether this could have an impact *in vivo.* We injected immune-deficient NOD.Cg-Prkdc^{scid}Il2rg$^{tm1Wjl/SzJ}$ mice (Jackson) subcutaneously with H1993 and KatoII cells stably selected for HER3-targeting shRNAs or an empty-vector control. Tumours formed rapidly with 100% penetrance in mice injected with KatoII cells. Tumours grown from HER3-depleted cells were

smaller than control tumours when first measured (Figure 2.7A). Xenografted KatoII cells grew rapidly, and mice were sacrificed after the second measurement due to ethical considerations. At both timepoints, control tumours were larger than HER3-depleted tumours (Figure 2.7C). H1993 cells formed tumours with variable and longer latency. Like tumours grown from KatoII cells, HER3-depleted H1993 tumours were smaller when initially measured (Figure 2.7B). Once established, however, knockdown tumours grew at a similar rate as controls (Figure 2.7D). Thus, HER3 knockdown delays early tumour growth kinetics in multiple *MET*-amplified cell lines, reflecting our observations in colony-forming assays. We collected KatoII tumours at the experimental endpoint did not observe re-expression of HER3 by Western blot, indicating that shRNA-mediated knockdown of HER3 remained stable through the experiment (Figure 2.7E). Collectively, these results support a role for HER3-dependent proliferative signaling early in *MET*-amplified tumour expansion.

2.9 A cell line established from a metastatic lung cancer with constitutive Met phosphorylation exhibits Met-HER3 crosstalk

These experiments using *MET*-amplified cancer cell lines demonstrate a conserved role in cancer cell proliferation for a Met-HER3 crosstalk axis. To our knowledge, Met-dependent HER3 phosphorylation has neither been demonstrated in patient-derived xenograft (PDX) tumours grown in mice, nor in other patient-derived models. Thus, a key question in addressing the relevance of Met-HER3 crosstalk in cancer biology is whether this is observed in clinically-relevant samples. To address this, we used a cell line generated from a xenograft established directly from a brain metastasis of NSCLC.

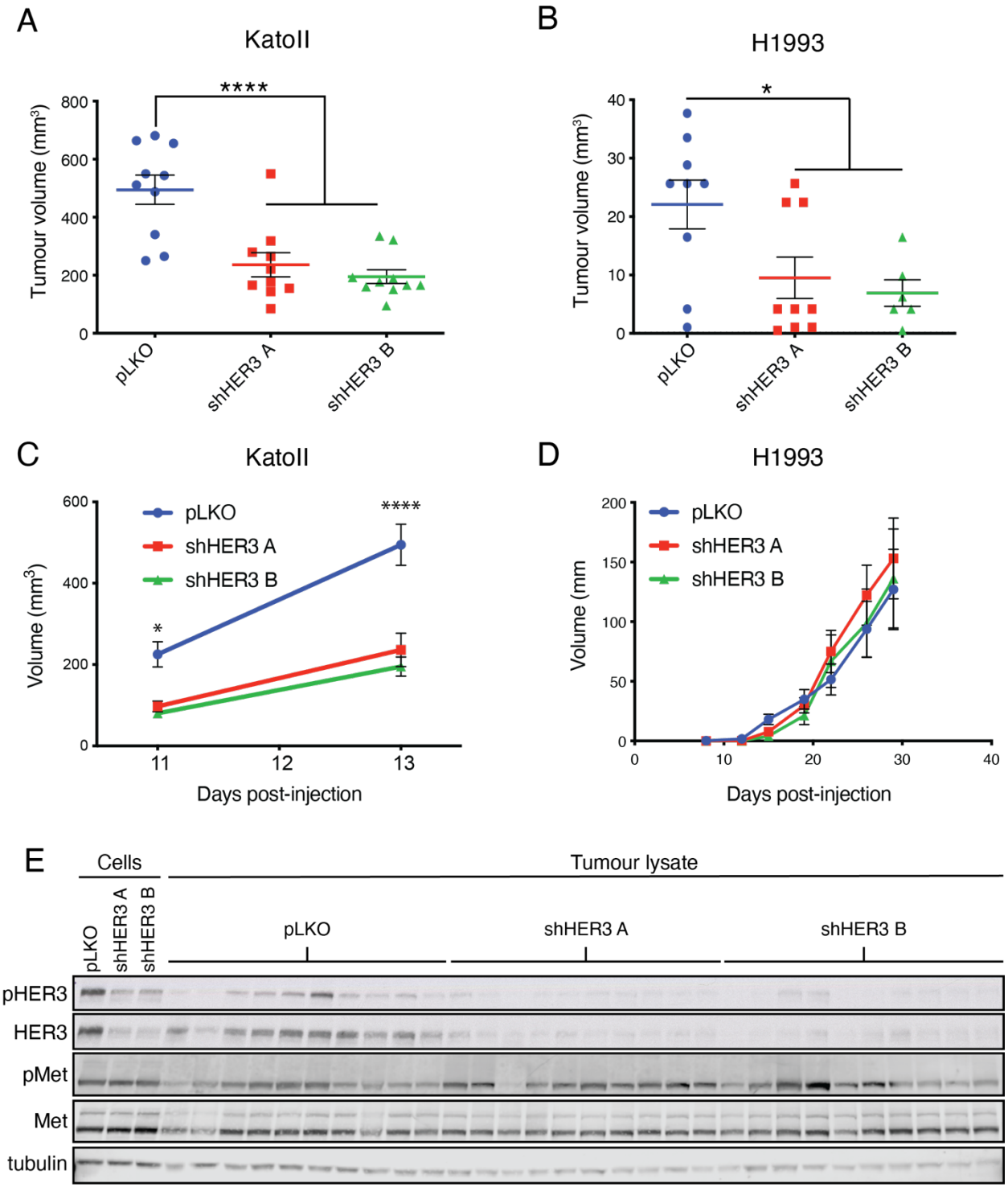


***Figure 2.7: HER3 depletion impairs the outgrowth of* MET*-amplified cells* in vivo.** *(A) KatoII cells depleted for HER3 form smaller tumours than controls (day 15 shown) (n=10 tumours). (B) H1993 cells depleted for HER3 form smaller tumours than controls at early timepoints (day 15 shown) (n=8 tumours). (C) Tumours formed from HER3-depleted KatoII cells grow at a slower rate than control tumours. (D) HER3-depleted tumours grown from H1993 eventually begin to grow at a comparable rate to control tumours and are indistinguishable in size and take rate after day 15. (E) Western blot analysis of KatoII-derived tumours at endpoint show*

that HER3 is not re-expressed in tumours that do form, and that HER3 depletion does not impact Met expression or phosphorylation in vivo *(all samples shown).*

This tumour was surgically resected by Dr. Jonathan Spicer at the McGill University Health Centre, in collaboration with the Park laboratory biobank. A patient-derived xenograft (PDX), GCRC1994, was expanded subcutaneously in immune-deficient mice by Dr. Peter Siegel and his student Matthew Dankner as part of a collaboration to study brain metastases. We analyzed PDX lysates from a panel of samples established from lung cancers that gave rise to brain metastases for Met expression and phosphorylation and observed Met phosphorylation in PDX GCRC1994 (personal communication, T. Golenar). Consistent with this, Met was overexpressed and constitutively phosphorylated in a cell line derived from PDX GCRC1994, and was sensitive to inhibition with crizotinib, an FDA-approved Met inhibitor currently in clinical use (Figure 2.8A). To evaluate the dependency on Met we quantified proliferation by live-cell microscopy. PDX GCRC1994-derived cells grown in the presence of crizotinib at 500 nM or higher displayed reduced proliferation, indicating that Met activity promotes cell proliferation in this sample (Figure 2.8B).

To evaluate Met-dependent crosstalk, we analyzed EGFR-family RTK phosphorylation in PDX GCRC1994 cells. Crizotinib treatment strongly inhibited HER3 phosphorylation within 1 hour, with a concurrent increase in HER3 protein levels. This is consistent with our observations in *MET*-amplified cell lines, as well as reports in the literature (Figure 2.2B) (Lai et al., 2014). Cells treated with crizotinib for 24 hours showed persistent Met inhibition and elevated HER3 levels, while HER3 phosphorylation was reduced compared to DMSO-treated cells. EGFR and HER2 phosphorylation was also reduced in cells treated with crizotinib for 24 hours, although we did not observe a change in their phosphorylation in cells treated for one hour (Figure 2.8A). Thus, Met-dependent crosstalk with the EGFR family in a Met-dependent, patient-derived sample

reflects our observations in *MET*-amplified cells. HER3 phosphorylation is acutely dependent on Met activity in these cells, while the delayed impact on EGFR and HER2 may reflect adaptive feedback on these receptors.

2.10 MPZL3, a novel HER3 interactor, contributes to *MET*-amplified cell proliferation

Our observations in *MET*-amplified cells collectively support an important role for Met-dependent crosstalk with HER3. Increased proliferation, colony-forming capacity and early tumour outgrowth provide a selective advantage for Met-dependent HER3 phosphorylation. Selective kinase inhibition and structure-function analysis demonstrate that this does not require EGFR or HER2 kinase activity, or the integrity of the HER3 asymmetric dimerization interface in the C-lobe. We also do not observe robust co-association of Met and HER3.

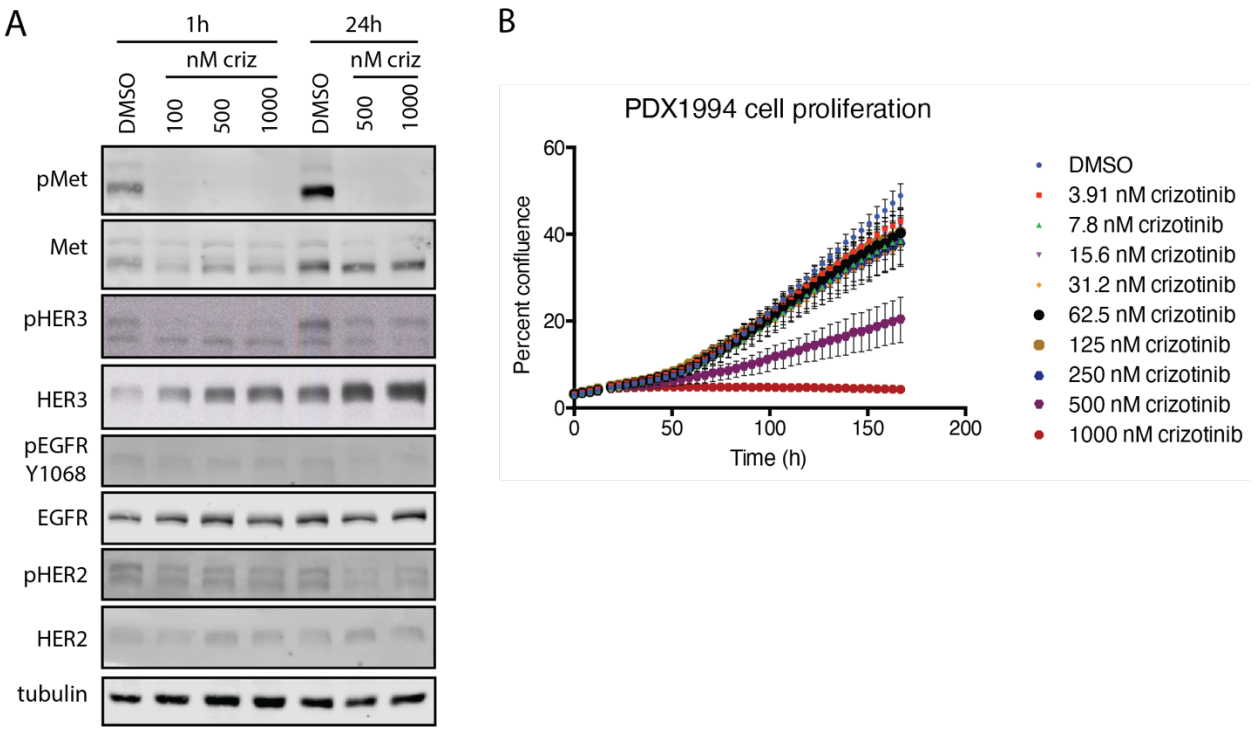


Figure 2.8: A patient-derived tumour model shows Met-dependent HER3 phosphorylation. *(A) Crizotinib treatment of a PDX-derived cell line induces an acute decrease in HER3 phosphorylation but delayed inhibition of EGFR or HER2 phosphorylation (n=1). (B) Crizotinib inhibits cell proliferation, measured as a function of cell confluence by live-cell microscopy (n=1).*

Thus, our observations suggest that HER3 plays a role as a scaffold downstream of Met. We employed hypothesis-driven and discovery-based approaches to identify the consequences of this heterotypic RTK signaling. Our results support a role for MPZL3, a previously-unknown HER3 interactor, in promoting *MET*-amplified cancer cell proliferation.

2.11 Signaling pathways that promote oncogenic Met-dependent proliferation are not impacted by HER3 depletion

We assessed the role of HER3 in Met-dependent signaling using EBC1, H1993 and KatoII *MET*-amplified cell lines. HER3 activates the PI3K-Akt pathway by recruitment of the p85 regulatory subunit to the activated RTK complex (Hellyer et al., 1998; Hellyer et al., 2001). Met signaling in HGF-dependent contexts is characterized by sustained activation of PI3K-Akt signaling through Gab1 as a tyrosine-phosphorylated scaffold (Maroun et al., 1999a). This has been hypothesized to promote cell survival in oncogenic contexts, and it is possible that HER3 contributes to Akt activation in a manner similar to Gab1 (Figure 2.9A) (Tanizaki et al., 2011b). Additionally, Met activates the Erk and STAT3 pathways in *MET*-amplified gastric cancer cell lines (Lai et al., 2014). To examine the consequences of HER3 depletion we assessed the phosphorylation of Akt, Erk, and STAT3 in HER3-depleted EBC1, H1993 and KatoII cells by Western blot (Figure 2.9B). No decrease in the phosphorylation of these intracellular signaling molecules was observed, indicating that HER3 does not significantly contribute to their activation in *MET*-amplified cells.

HER3 phosphorylation and consequent PI3K-Akt signaling are thought to suppress apoptotic signaling through cell survival pathways in HER2-dependent cancer cells (Junttila et al., 2009). In HCC827 cells with acquired resistance to gefitinib through *MET* amplification, HER3 is also

required for Akt phosphorylation and the suppression of apoptosis. We used flow cytometry to determine the proportion of HER3-depleted *MET*-amplified cells undergoing apoptosis by annexin-V positivity (Figure 2.9D). Consistent with our observation of sustained Akt phosphorylation, we did not observe a statistically significant increase in apoptosis in HER3-depleted cells. Collectively, we were unable to detect an increase in canonical apoptosis in HER3-depleted cells and did not observe decreased activation of the pathways known to regulate cell survival and proliferation downstream of Met and HER3. Thus, we used a discovery-based approach to understand the consequence of Met-HER3 crosstalk in *MET*-amplified cells.

2.12 Gene expression profiling identifies MPZL3 as a novel HER3-dependent transcript

The output of sustained RTK signaling is mediated by the maintenance of RTK-dependent gene expression (Herschman, 1991; Woodgett, 1989). Differential gene expression due to ligand stimulation, kinase inhibition, RNA interference or other methods has thus been used to characterize RTK-dependent pathways in cancer and development (Amit et al., 2007; Lai et al., 2014; Lapin et al., 2014; Leatherbarrow and Halfon, 2009; Sweeney et al., 2001). To identify HER3-regulated genes in *MET*-amplified cells we sequenced RNA extracted from stably HER3-depleted KatoII cells. Seven genes were identified with significantly altered transcripts in HER3-depleted versus control cells (FDR < 0.05, $\log_2$FC ≥ |1|). Genes with a decrease in abundance upon HER3 knockdown included *ERBB3*, encoding HER3 itself, as well as *LGR6*, *MPZL3* and *NYNRIN*, while genes elevated in expression ($\log_2$FC > 1, qval < 0.05) included *PPM1K*, *RAMP1*, and *SPNS2* (Figure 2.10A and B). We expanded our analysis to include BHLHE41, a transcription factor involved in mammalian circadian regulation and a repressor of differentiation slightly below our cutoff ($\log_2$FC =-0.91, q-value = $8.42*10^{-4}$) (Ow et al., 2014).

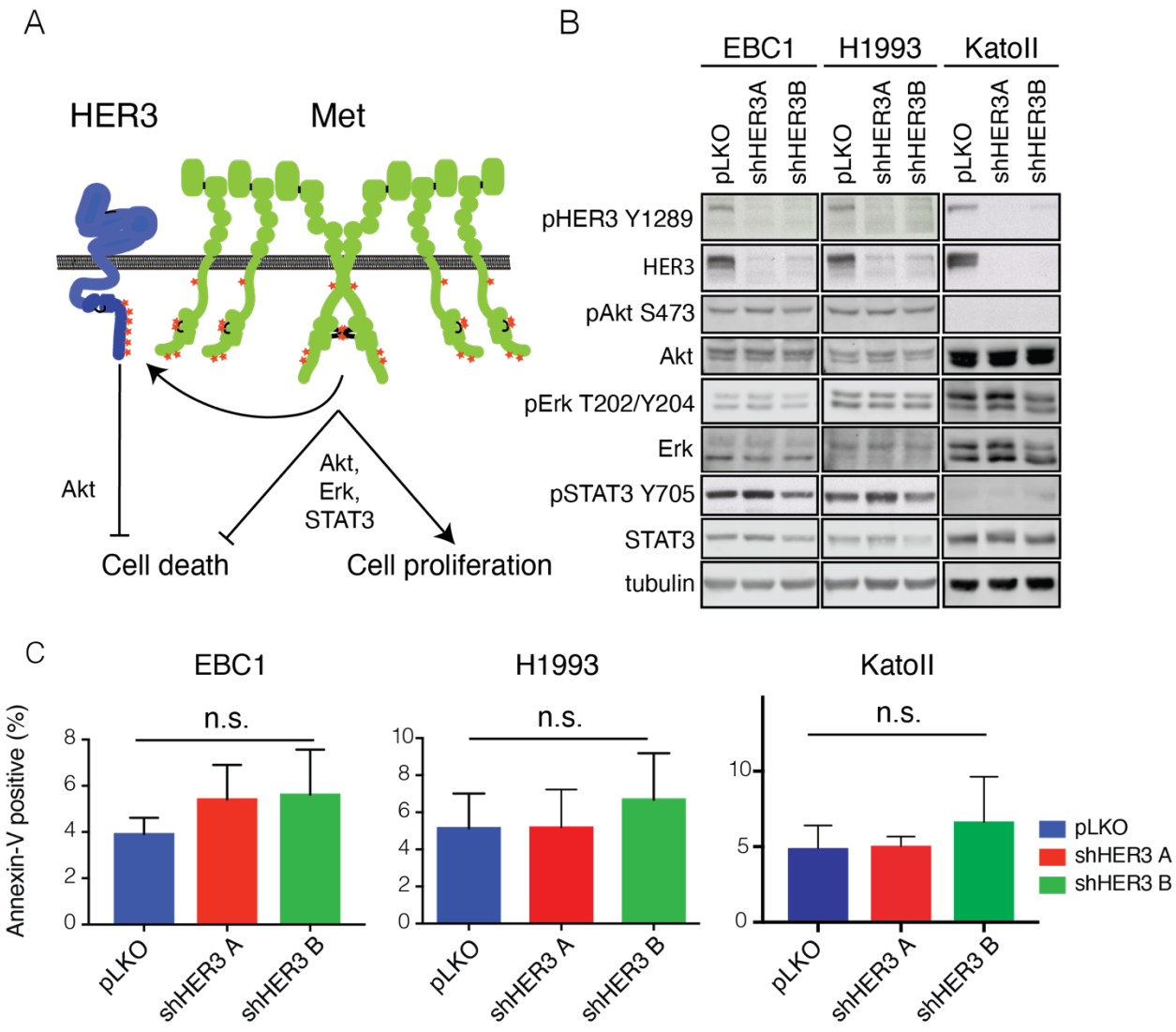


Figure 2.9: Canonical Met- and HER3-dependent signaling is intact in HER3-depleted* MET*-amplified cells. *(A) Met and HER3 activate overlapping signaling pathways and are known to activate both proliferative and anti-apoptotic signaling through the Akt, Erk, STAT3 and Src pathways in cancer cells (n=3). (B) Phosphorylation of Akt (Ser473), Erk (Thr202/Tyr204), and STAT3 (Tyr705) is not impacted by loss of total and phospho-HER3 (Tyr1289) (n=3). (D) We did not observe a statistically significant increase in annexin-V positivity measured by flow cytometry in HER3-depleted cells (n=3).*

To validate which transcripts were recurrently dependent on HER3 in a *MET*-amplified setting, we measured the expression of genes downregulated in KatoII cells by RT-qPCR in *MET*-amplified EBC1 and H1993 cells following HER3 knockdown. Genes that increased in expression upon HER3 knockdown in KatoII cells (*PPM1K, RAMP1, SPNS2*) did not show comparable regulation in EBC1 and H1993 cells (Figure 2.10C and D). Of the genes tested, *ERBB3*, *MPZL3* and *NYNRIN* were

reproducibly decreased in expression in all HER3-depleted cells tested (EBC1, KatoII and H1993) (figure 2.10E and F). Other genes identified in our RNA sequencing may be regulated by HER3 in a more cell type-specific manner. NYNRIN encodes a poorly characterized gene with predicted RNA-binding functions. Germline truncating mutations in *NYNRIN* have been reported to predispose carriers to pediatric Wilms tumours, and it is upregulated upon mechanical stimulus in bladder smooth muscle cells (Mahamdallie et al., 2019; Peng and Luo, 2018). MPZL3 encodes a predicted adhesion receptor with a role in epidermal differentiation but no previously-characterized role downstream of RTKs (Cao et al., 2007; Wikramanayake et al., 2017). NYNRIN, which was detected at lower levels than MPZL3 in our RNA sequencing and qRT-PCR data, was not characterized in the literature at the time of our experiment, and no reagents for studying it were available. We pursued *MPZL3* as a convergently HER3-regulated gene in *MET*-amplified cells and hypothesized that it may play a role in Met-dependent proliferation downstream of HER3.

2.13 MPZL3 contributes to HER3-dependent proliferation in MET*-amplified cells*

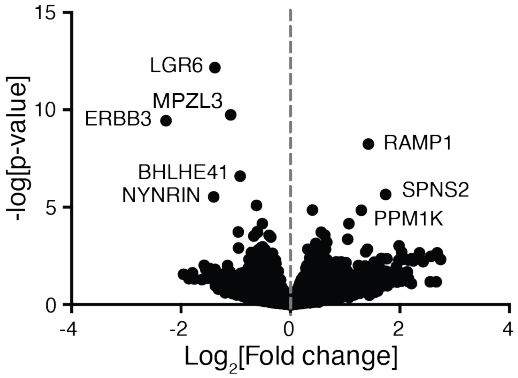
To assess whether MPZL3 is involved in HER3-dependent proliferation, we overexpressed MPZL3-V5, corresponding to the full-length protein, in EBC1 cells. We selected for stable expression using puromycin and transfected these EBC1-MPZL3 cells with siRNA targeting HER3 or control duplexes. The ectopically expressed MPZL3 protein was not sensitive to HER3-targeted siRNA, while HER3 levels were comparably reduced in both empty-vector control (pLKO) and MPZL3-overexpressing cells (Figure 2.11A). As expected, HER3 depletion impaired cell proliferation in control cells, but this effect was attenuated by MPZL3 overexpression (44 vs. 72% of siCtl-transfected cells at 65% confluence, respectively) (Figure 2.11B). To test whether MPZL3 was required for colony formation in *MET*-amplified cells, we transduced KatoII cells with shRNA

targeting MPZL3 or a control non-targeting hairpin and the corresponding empty vector and established stable cell lines (Figure 2.11C). MPZL3-depleted KatoII cells formed colonies in 3-dimensional soft agar culture at a lower rate than control cells (Figure 2.11D). HER3 protein levels and phosphorylation were not impacted by MPZL3 shRNA (Figure 2.11E). MPZL3 depletion induces comparable phenotypes to HER3 depletion in *MET*-amplified cells, while MPZL3 overexpression partially rescues that phenotype.

2.14 MPZL3 and HER3 interact in a Met-dependent manner in MET*-amplified cells*

To begin an analysis of how MPZL3-V5 rescues HER3-dependent proliferation, we used co-immunoprecipitation assays and a structure-function approach to assess whether it interacts at the protein level with phosphorylated RTKs. MPZL3 encodes a transmembrane protein with an extracellular Ig-like domain and a short cytoplasmic tail with no predicted secondary or tertiary structure (Racz et al., 2009). To test whether MPZL3-V5 interacts with Met or HER3, we stably expressed MPZL3-V5, GFP-V5 or an empty-vector control (pLKO) in *MET*-amplified KatoII cells. We immunoprecipitated MPZL3-V5 from the lysates of PHA- or DMSO- treated cells (0.5 μM, 1 hour) and assessed Met and HER3 co-immunoprecipitation by Western blot (Figure 2.12A). While we did not observe any interaction between Met and MPZL3, HER3 co-immunoprecipitated with MPZL3 in a Met-dependent manner (Figure 2.12B) (53% of control, p=0.04). We did not observe any co-immunoprecipitation between Met or HER3 and GFP-V5 or in our empty-vector controls, indicating that the interaction between HER3 and MPZL3 was not due to nonspecific binding. Thus, we observe an association between MPZL3 and HER3 in *MET*-amplified cells, and this is reduced upon Met kinase inhibition.

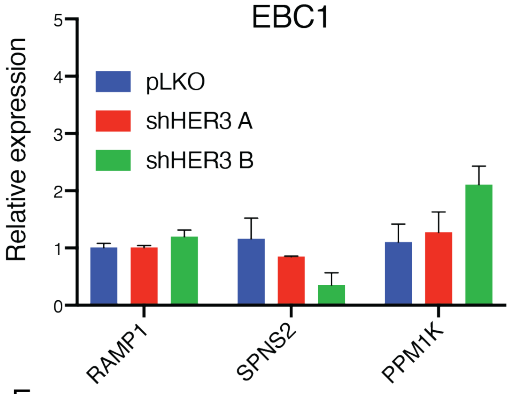
A



B

Gene name	Log_2[fold change]	q-value
ERBB3	-2.27	$2.03x10^{-6}$
LGR6	-1.38	$1.13x10^{-8}$
MPZL3	-1.09	$1.49x10^{-6}$
BHLHE41	-0.92	$8.42x10^{-4}$
NYNRIN	-1.40	$7.01x10^{-3}$
SPNS2	1.74	$6.06x10^{-3}$
RAMP1	1.43	$2.40x10^{-5}$
PPM1K	1.30	$2.42x10^{-2}$

C



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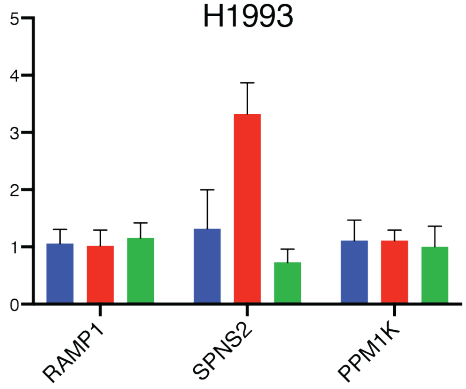

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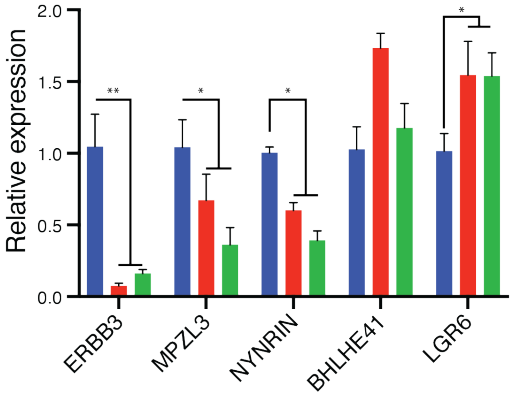

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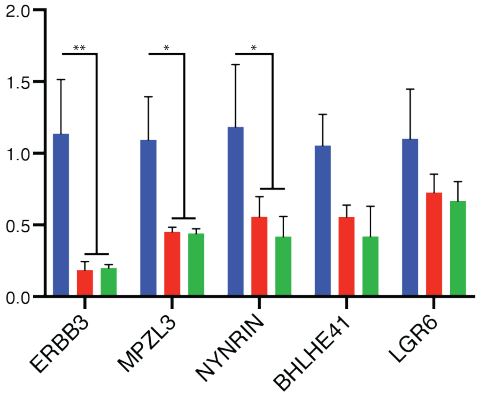


Figure 2.10: MPZL3 is a HER3-regulated transcript in multiple* MET*-amplified cell lines. *(A) RNA sequencing of HER3-depleted KatoII cells identifies a small number of significantly regulated transcripts in comparison to control cells (n=3). (B) Significance and fold change of altered genes identified by RNA sequencing. (C-F) Expression of altered genes identified by RNA sequencing in EBC1 and H1993* MET*-amplified cell lines (n=3). (C, D) Expression of genes upregulated upon HER3 depletion in KatoII cells. (E, F) Expression of genes downregulated upon HER3 depletion in KatoII cells.*

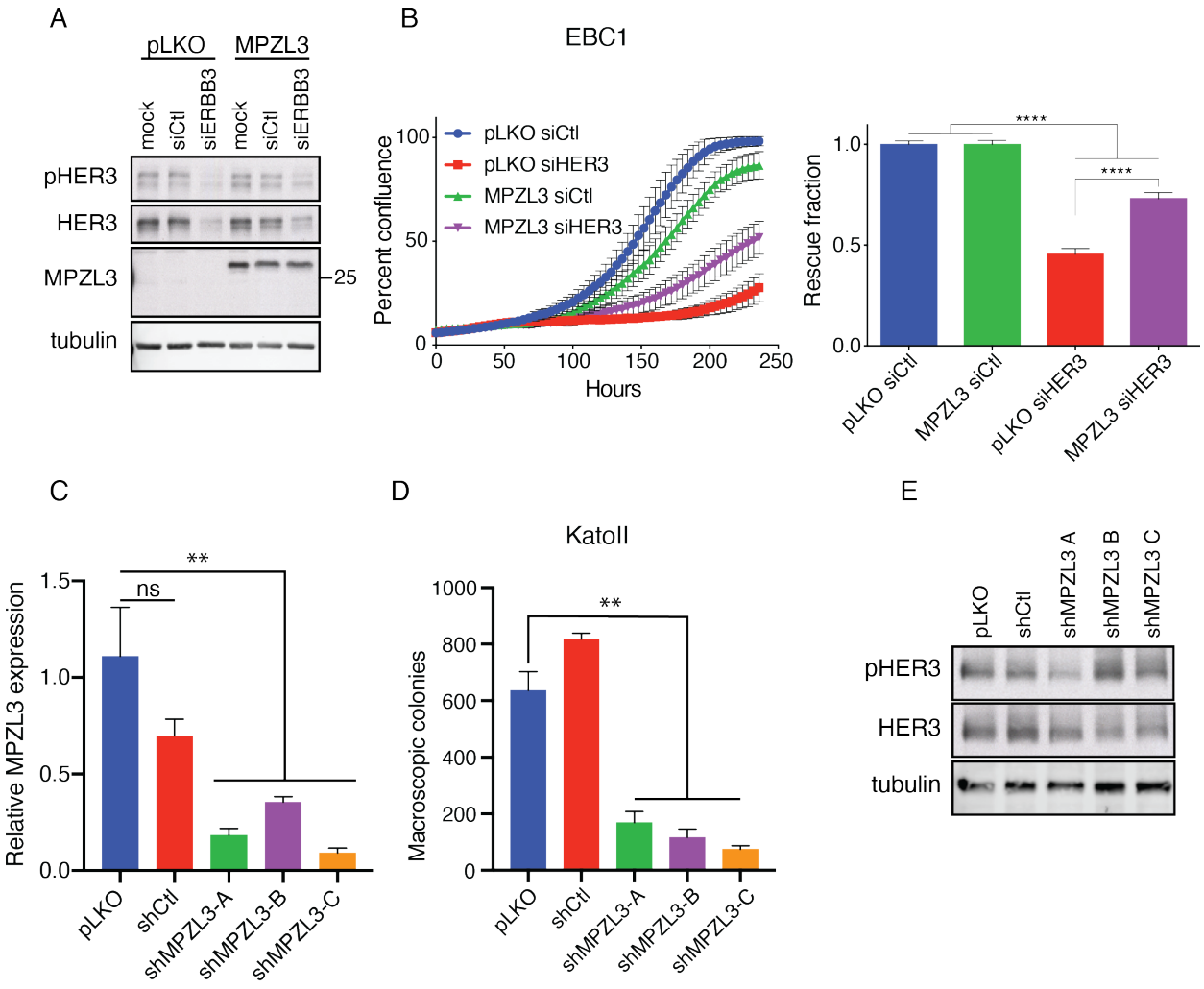


Figure 2.11: MPZL3 contributes to HER3-dependent proliferation. *(A) Overexpression of MPZL3-V5 is stable in EBC1 cells and is resistant to HER3-targeted siRNA (n=3). (B) Overexpression of MPZL3-V5 partially rescues proliferation in the presence of siRNA targeting HER3. Left, percent confluence measured by live-cell microscopy (representative). Right, percent confluence relative to siCtl-treated cells (average of n=3 proliferation assays). (C) MPZL3 expression measured by qRT-PCR in control and MZPL3-depleted KatoII cells (n=4). (D) KatoII colony formation in soft agar is impaired by MPZL3 depletion (n=4). (E) HER3 phosphorylation and protein levels do not correlate with MPZL3 expression in MPZL3-depleted cells (n=4).*

While the association between HER3 and MPZL3 is promoted by Met activity in KatoII cells, we sought to determine whether Met was required for HER3 and MPZL3 to interact in a simpler system. To test this, we transiently expressed MPZL3-V5 in HEK293T cells stably expressing HER3 or the short-tail and no-tail truncation mutants used previously (Figure 2.4C and D). In the absence of Met overexpression, HER3 phosphorylation is not observed in this system (Figure 2.4B and D). All three HER3 mutants co-immunoprecipitated with MPZL3, demonstrating that the

HER3-MPZL3 interaction could be reconstituted in the absence of Met overexpression, and that the C-tail of HER3 is not involved in the interaction with MPZL3 (Figure 2.12B). Thus, HER3 and MPZL3 can interact in a Met-independent manner, but their association is regulated by Met activity in the *MET*-amplified setting.

2.15 A new protein-protein interaction assay suggests a direct interaction between HER3 and MPZL3

We further sought to determine whether MPZL3 and HER3 may interact in a direct manner. We used a newly developed assay for protein-protein interactions called the split intein-mediated protein ligation (SIMPL) assay (Yao et al., 2020). In this assay, the N- and C-segments of an intein domain are fused to bait and prey proteins, respectively. Reconstitution of the intein domain upon direct interaction of the bait and prey protein induces its self-excision from the flanking polypeptide sequences. As the N-terminal fragment of the intein domain is fused to the bait protein using a V5-tagged linker, and the C-intein domain is flanked by a FLAG tag, intein reconstitution induces the transfer of the FLAG tag from the prey to the bait protein (Figure 2.13A). Co-expression of bait HER3 with prey MPZL3 induced transfer of the FLAG tag to HER3 (~190 kDa), while the inverse caused transfer of the tag to MPZL3 (~30 kDa) (Figure 2.13B).

MPZL3 was initially discovered by mapping the *rough coat* (*rc*) genotype, a spontaneous variant of C57BL/6J mice discovered in Jackson Laboratory, to a point mutation in the exon encoding the Ig-like domain of the gene. This caused the substitution of Arg100 (Arg99 in human MPZL3) to Gln, and is presumed to impair the function of the domain as an adhesion receptor (Cao et al., 2007).

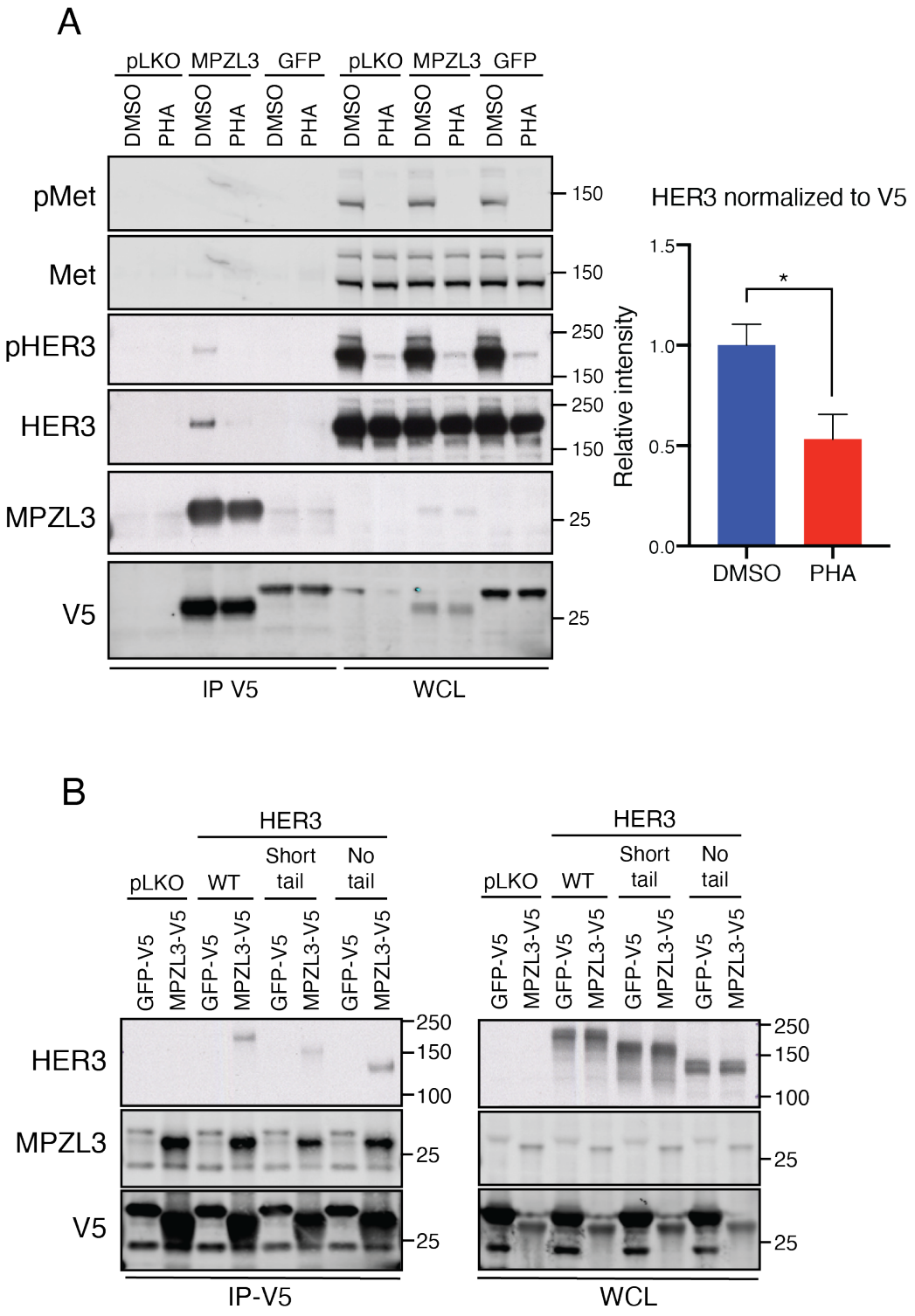


Figure 2.12: MPZL3 and HER3 interact at the protein level. *(A) HER3 co-immunoprecipitates with MPZL3-V5 in a Met-dependent manner in KatoII cells, while empty-vector (pLKO) or GFP-V5-expressing cells show no HER3 enrichment. Met inhibition with PHA-665752 (PHA) reduces the level of co-immunoprecipitated HER3 to 53% of that observed in DMSO-treated cells (p=0.04, n=5). (B) Full-length HER3 as well as truncated mutants lacking the distal C-tail (short tail) or the entire C-tail (no tail) co-immunoprecipitate with MPZL3-V5 in HEK293T cells (n=3).*

We introduced the Arg99Gln mutation into our MPZL3 SIMPL assay constructs to test whether it uncoupled MPZL3 from HER3. As Ig-like domains are also known to depend on the formation of disulfide bonds between conserved cysteine residues, first demonstrated using the homologous myelin protein zero (MYP0) Ig-like domain, we also mutated one of the conserved cysteine residues (Cys52 in human MPZL3) to alanine to impair the binding function of the Ig-like domain. We co-expressed mutant MPZL3 SIMPL constructs with corresponding HER3 SIMPL constructs and found that both the Arg99Gln and Cys52Ala mutants were capable of interacting with HER3 SIMPL constructs (Figure 2.13C). Thus, mutations predicted to impair the function of the MPZL3 extracellular domain do not affect its ability to bind HER3.

2.16 MPZL3-V5 does not accumulate at the plasma membrane in MET*-amplified cells*

In order to better understand how the interaction between HER3 and MPZL3 is involved in proliferative signaling, we next assessed the subcellular localization of our HER3-interacting MPZL3-V5 construct. MPZL3 has been observed in mitochondrial networks, but has not been demonstrated to localize to the plasma membrane, using an MPZL3-V5 overexpression construct comparable to the one used in our rescue experiments (Figure 2.11A and B) (Bhaduri et al., 2015; Racz et al., 2009). We assessed cell surface levels of MPZL3-V5 in EBC1 and KatoII *MET*-amplified cell lines using a biochemical approach. We labeled cell-surface proteins with biotin and used streptavidin-coated beads to isolate surface-exposed biotinylated proteins from the ensuing lysates of EBC1 and KatoII cells overexpressing MPZL3 (Figure 2.14A and B). Met and HER3 were detected among the streptavidin-precipitated proteins in a biotin-dependent manner in both cell lines. In contrast to this, MPZL3-V5 was only detected in the input lysates and was not observed among the biotin-dependent cell-surface-exposed proteins.

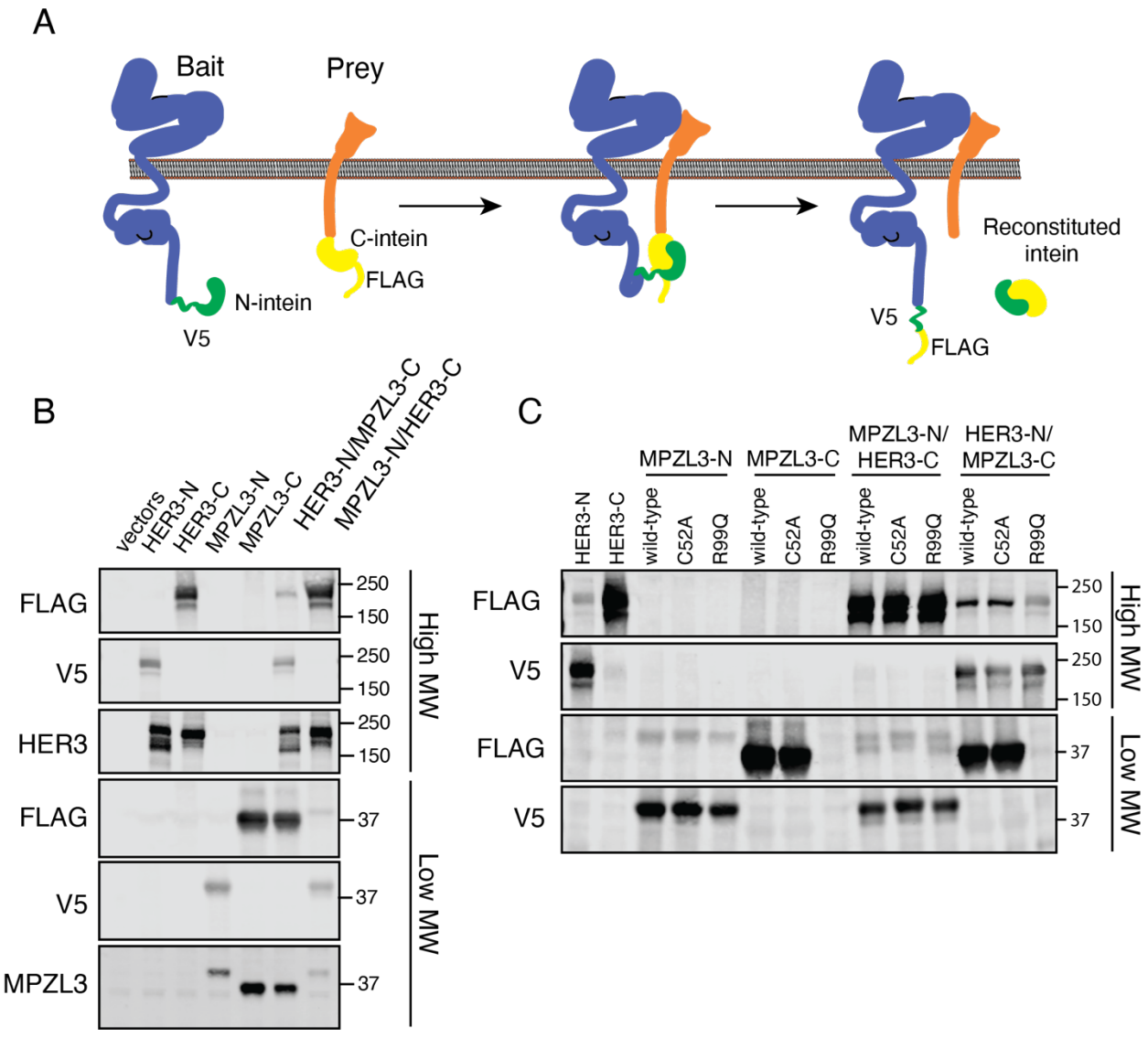


Figure 2.13: The SIMPL assay suggests a direct HER3-MPZL3 interaction. *(A) The SIMPL assay measures protein-protein interaction by detecting the transfer of a FLAG peptide tag from the C-intein-tagged prey protein to the N-intein tagged bait protein. (B) Co-expression of bait-HER3 with prey-MPZL3 induces FLAG tag transfer to HER3 (~190 kDa), while the inverse induces transfer to MPZL3 (~30 kDa) (n=3). (C) Point mutations in the extracellular domain of MPZL3 predicted to impair its function by blocking disulfide bond formation (C52A) or by blocking its cell adhesion function (R99Q) do not affect the ability of HER3 and MPZL3 to interact (n=3).*

We also analyzed MPZL3-V5 localization by indirect immunofluorescence in adherent EBC1 cells. As expected, Met was found at the plasma membrane, cell-cell contacts and at intracellular puncta irrespective of MPZL3-V5 expression. MPZL3-V5, by contrast, was only observed in

intracellular compartments, including punctate structures, and was not observed at the plasma membrane (Figure 2.14C). These experiments show that the MPZL3-V5 construct is a predominantly intracellular protein and is not detected at the plasma membrane. Thus, both biochemical and immunofluorescence-based approaches suggest that the proliferative function exhibited by MPZL3-V5 may proceed via functions that do not require MPZL3 at the plasma membrane.

2.17 Expression of MPZL3 is elevated in RTK-amplified cancer cell lines and RTK-associated cancers

Our experiments in HER3-depleted *MET*-amplified cells collectively show a key role for MPZL3 downstream of HER3 in facilitating efficient cell proliferation. We hypothesized that the HER3-MPZL3 axis involved in cancer cell proliferation may not be restricted to cells with *MET* amplification specifically and may instead represent a novel signaling axis downstream from multiple RTKs. We profiled cell lines in the CCLE for RTK copy number and labeled cell lines with >5-fold copy number gain of any RTK relative to diploid cells as amplified for that receptor. MPZL3 expression is higher in *MET*-amplified cell lines than in cell lines with no RTK amplification (median TPM of 2.8 [*MET*-amplified] vs. 1.7 [no RTK amplified], p=0.03 [Mann-Whitney test]) (Figure 2.15A). When we looked across all recurrently amplified RTKs in the CCLE, we found that *EGFR* and *ERBB2* amplification both predicted significantly higher MPZL3 expression than in non-RTK-amplified cells (Figure 2.15B). While not statistically significant in this dataset, the RTK genes *EPHA10*, *EPHB4*, *DDR2*, *FGFR1*, *FGFR2*, *IGF1R*, *LMTK2* and *MET* all trended towards higher median MPZL3 expression than non-RTK-amplified cell lines using the more rigorous ANOVA test.

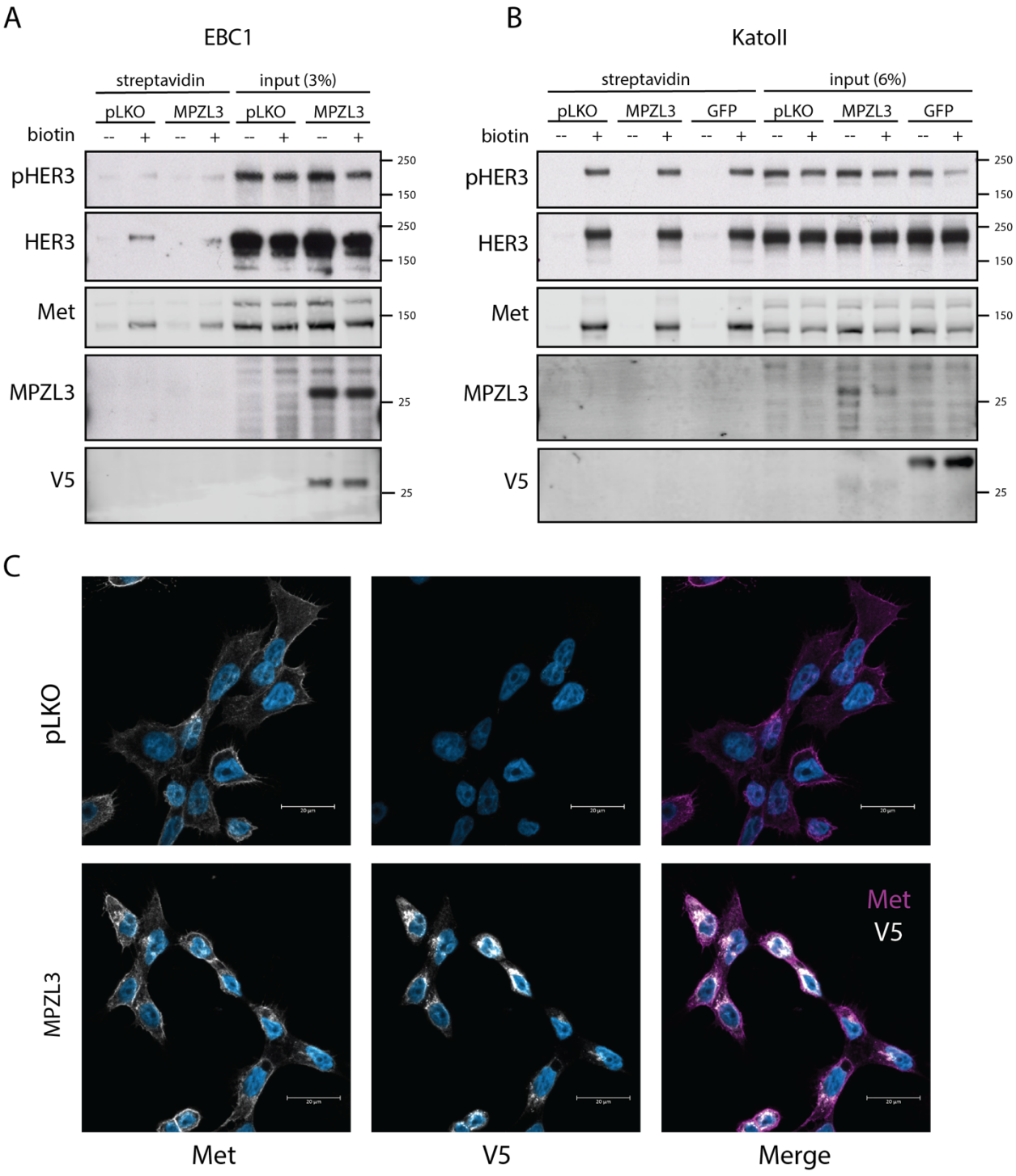


Figure 2.14: MPZL3-V5 does not localize to the cell surface in* MET*-amplified cells. *(A) Cell-surface biotinylation of EBC1-MPZL3 or control (pLKO) cells is shown by their precipitation using streptavidin-coated beads. MPZL3-V5 is not detected in the surface-biotinylated fraction in EBC1 cells, although it can be detected by both MPZL3 and V5 antibody in the input lysate (n=3). (B) Met and HER3 are similarly biotinylated at the surface of KatoII cells, but MPZL3-V5 and the cytoplasmic GFP-V5 are not detected in the biotinylated fraction (n=3). (C) MPZL3-V5 is localized to cytoplasmic puncta and the perinuclear regions of EBC1-MPZL3 cells, and does not overlap with membrane proteins like Met. Detection of MPZL3-V5 is specific using the V5 antibody, as no signal is detected in control (pLKO) cells (n=1).*

As *EGFR*- and *ERBB2*-amplified cell lines are more heavily represented than all others in this dataset, this may be due to the low frequency of amplification of these RTKs within the CCLE dataset. To establish if elevated MPZL3 is observed in human cancers, we examined MPZL3 expression levels across cancer types using publicly available data from the Pan-Cancer Atlas (Figure 2.15C). Cancers with the highest proportion of MPZL3 overexpression included ovarian, colon, kidney and lung carcinomas, all of which are diseases in which RTK amplification has been postulated to represent important driver events.

Our data support a role for MPZL3 in *MET*-amplified cell proliferation downstream of HER3. MPZL3 overexpression partially rescues proliferation in HER3-depleted EBC1 cells, and MPZL3 depletion phenocopies the loss of colony formation seen in HER3-depleted KatoII cells. MPZL3 interacts with HER3, and in *MET*-amplified cells Met activity promotes this interaction. MPZL3 is not required to localize to the plasma membrane to interact with HER3 or rescue HER3-dependent proliferation, indicating that MPZL3-dependent functions occur in intracellular membrane-bound compartments. We observe elevated MPZL3 expression in *MET*-amplified cell lines compared to non-RTK-amplified cell lines, and a similar effect in *EGFR*- and *ERBB2*-amplified cell lines. Thus, a MPZL3 may contribute to proliferation in several cancers that are associated with HER3-dependent signaling and represents an important novel HER3 interactor in cancer.

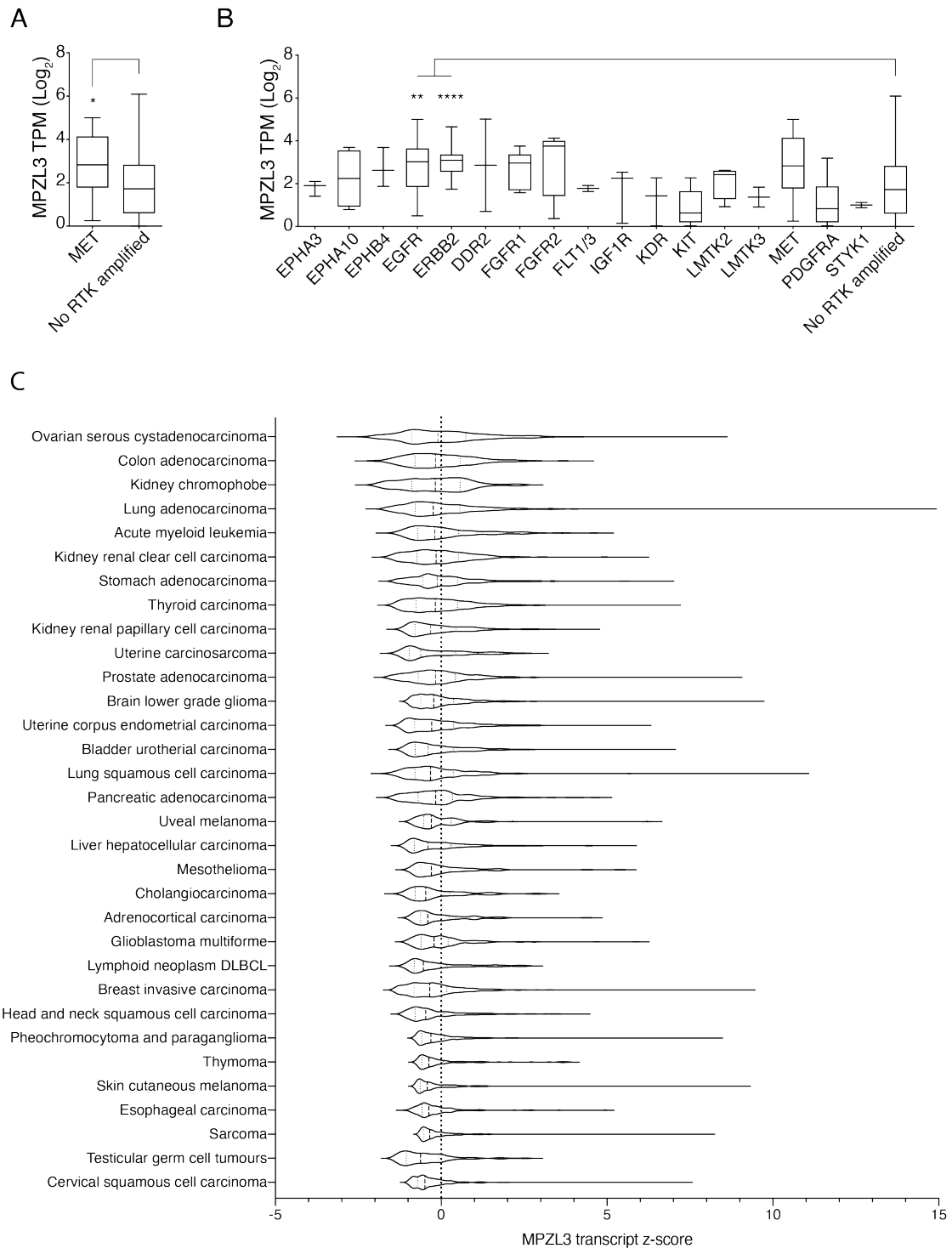


Figure 2.15: MPZL3 expression is elevated in cancer cell lines with RTK amplification and cancers with recurrent RTK amplifications. *(A) Cell lines in the CCLE database were scored for RTK amplification. MPZL3 levels in* MET*-amplified cell lines are elevated compared to cell lines without any RTK amplification (Mann-Whitney test, p=0.03). (B) Across RTKs amplified in more than one cell line,* EGFR *and* ERBB2 *amplification predicted significantly higher MPZL3 expression than cell lines with no RTK amplified (ANOVA, p<0.0001). (C) MPZL3 transcript Z-scores in the Pan-Cancer Atlas project. Cancers with recurrent RTK amplifications show the highest incidence of MPZL3 overexpression.*

2.18 Expanded Met-dependent phosphorylation is observed in proliferative *MET*-amplified cells

Our results support an important role for heterotypic signaling through HER3 in *MET*-amplified cells. In our cell proliferation, colony formation and tumour xenograft experiments, HER3-depleted cells are ultimately able to adapt and proliferate without the re-expression of HER3 at the protein level (Figure 2.7E). This suggests that additional pathways downstream of the amplified Met receptor contribute to cell proliferation under normal or adaptive conditions. In order to gain a better understanding of how signal rewiring in the amplified setting could contribute to proliferation in the oncogenic state, we used global phosphoproteomics to identify additional Met-dependent phosphorylation sites in *MET*-amplified cells. We also show that HGF accelerates tumour outgrowth in *MET*-amplified cells without changing the level of Met phosphorylation, indicating that some HGF-dependent pathways are not activated by the constitutively phosphorylated Met receptor, but can still contribute to oncogenic tumour growth downstream of Met.

2.19 MET-*amplified KatoII cells show high sensitivity to multiple Met-directed TKIs*

Met-dependent tyrosine signaling has been extensively characterized in HGF-dependent contexts using both structure-function and phosphoproteomic approaches, but HGF-dependent signaling frequently induces a cell-dispersal or migratory response in the cell types studied in these contexts (Fournier et al., 1996; Johnson et al., 2013; Lamorte et al., 2000; Maroun et al., 1999b; Maroun et al., 2000; Nguyen et al., 1997; Paliouras et al., 2009; Pasdar et al., 1997; Peschard et al., 2001; Peschard et al., 2004; Rajadurai et al., 2012; Royal and Park, 1995; Zhu et

al., 1994). Meanwhile, *MET*-amplified cell lines have been shown to harbour exquisite sensitivity to Met inhibition through their impact on proliferation, with a subset of cells also showing an induction of apoptosis upon Met inhibition (Ali et al., 2014; Lai et al., 2014; Smolen et al., 2006). In order to better understand how Met signaling promotes oncogenic proliferative and survival outcomes in the amplified setting, we sought to identify additional components of Met-dependent proliferative signaling using a phosphoproteomic approach using cell lines that primarily exhibit a proliferative response to Met signaling.

To validate the KatoII *MET*-amplified cell line as a model for proliferative Met-dependent signaling, we tested their sensitivity to Met inhibition using crizotinib and PHA-665752. These Met-directed TKIs both bind to other kinases with comparable affinity to Met, but the inhibitors have differential binding affinity for other kinases (Davis et al., 2011). Both inhibitors blocked proliferation at nanomolar concentrations and induced sustained inhibition of tyrosine phosphorylation, including tyrosine phosphorylation of Met at the activation loop (Figure 2.16A). We serum-starved the cells to reduce the impact of ligand-dependent signaling activated by growth serum, and surprisingly observed an increase in Met phosphorylation as well as an increase in the concentration of inhibitor required to fully inhibit Met phosphorylation to 500 nM (Figure 2.16B). Thus, serum starvation raised the enzymatic activity of the Met kinase, indicating that factors in serum partially suppress Met signaling. In contrast to this, KatoII proliferation in serum-containing media was inhibited by both drugs at concentrations as low as 30 nM (Figure 2.16 C). Under serum-starved conditions, treatment of *MET*-amplified cells with either inhibitor at 500 nM rapidly and sustainably inhibited Met inhibition over the course of 2 hours (Figure 2.16 D).

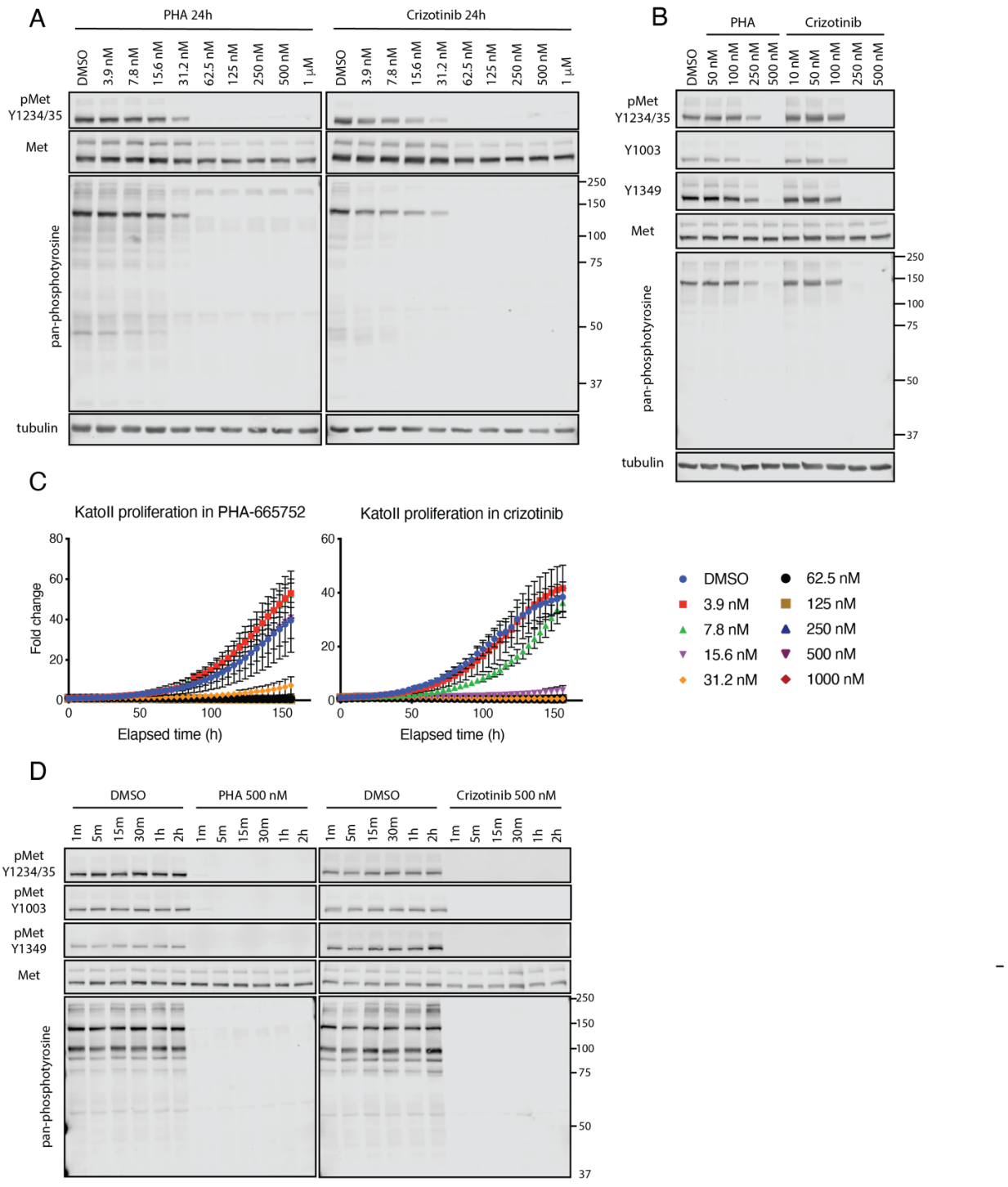


Figure 2.16: PHA-665752 and crizotinib inhibit cell proliferation in* MET*-amplified KatoII cells. *(A) Treatment of KatoII cells in serum-containing media such as that used in proliferation assays shows inhibition of Met activity at low nanomolar concentrations with each drug (n=3). (B) Under serum-starved conditions, Met phosphorylation is more robust and requires higher levels of inhibitor for full receptor dephosphorylation at the activation loop as well as the juxtamembrane and C-tail tyrosines (n=3). (C) KatoII cell proliferation is inhbited by low nanomolar concentrations of both PHA and crizotinib, mirroring receptor phosphorylation under serum-fed conditions (n=3). (D) Treatment with Met inhbitors results in rapid and sustained receptor dephosphorylation, with full receptor inhibition in serum-free media within 1 minute of drug treatment at 500 nM (n=3).*

2.20 SILAC phosphoproteomics identifies a Met-dependent tyrosine phosphorylation network

To discover novel phosphosites associated with proliferative Met signaling, we aimed to identify Met-dependent tyrosine phosphorylation sites in using mass spectrometry. We performed stable isotope labeling by amino acids in cell culture (SILAC) by growing KatoII cells in media containing light, medium or heavy isotopes of lysine and arginine. SILAC has been validated as a quantitative method of global phosphoproteomic profiling and is among the most accurate techniques available for this purpose (Hogrebe et al., 2018; Ong et al., 2002; Zhang and Neubert, 2009). We first confirmed that KatoII cells grew with similar kinetics in SILAC and regular media by measuring cell doubling time across multiple passages (Table 2.2). We then mixed equal numbers of high-, medium- and low-mass SILAC-labeled cells and were able to recover comparable levels of peptides from all three conditions (Table 2.3).

Table 2.2: Doubling time in regular (RPMI), light, medium and heavy SILAC media

Media	**Doubling time (days)**	
	P2	**P4**
RPMI	1.98	2.02
Light	1.92	2.12
Medium	1.78	2.19
Heavy	1.79	2.03

Table 2.3: Isotopic ratios in equal numbers of mixed, SILAC-labeled cells

Passage	**Amino acid**	**Isotopic ratios**		
		Medium:low	**High:low**	**High:medium**
P2	Lys	0.99	1.05	1.06
	Arg	0.96	0.94	0.99
P4	Lys	1.07	1.12	1.05
	Arg	1.04	1.03	0.99

We treated serum-starved, SILAC-labeled cells with either PHA or crizotinib at 500 nM or DMSO as a control. Cell pellets were frozen and tyrosine-phosphorylated peptides were isolated by immunoaffinity after tryptic digestion by our collaborator at the Université de Montréal, Dr. Pierre Thibault. Both phosphorylated and non-phosphorylated peptides were identified at comparable average intensities after enrichment by phosphotyrosine immunoaffinity, although the majority of peptides identified by this approach were not phosphorylated (Figure 2.17 A and B). PHA and crizotinib both induced a decrease in tyrosine phosphorylation, with a high proportion of overlap between the two drugs (Figure 2.17C-F). Phosphoproteins were also enriched by affinity for titanium dioxide (TiO_2), which does not discriminate between tyrosine, serine and threonine phosphorylation. This method resulted in higher average intensity for phosphorylated peptides over non-phosphorylated peptides, and the majority of peptides identified were phosphorylated (Figure 2.17 G and H). We observed smaller changes in serine and threonine than tyrosine phosphorylation, but these also displayed reduced overall phosphorylation with high overlap between PHA- and crizotinbi-treated samples (Figure 2.17I-L). We identified 53 peptides in which tyrosine phosphorylation decreased at least 2-fold above a cutoff of p=0.05 (Table 2.4). Many of these (CRK, CRKL, NCK2, HGS, STAM, IQGAP1, IQGAP2, ITSN2, STAT3) are known to be phosphorylated in a Met-dependent manner. We also identified one site in AFAPL12 in which phosphorylation increased more than 2-fold. In addition, we identified 23 serine and 2 threonine phosphorylation sites that decreased at least 2-fold in the presence of both PHA and crizotinib, and one serine phosphorylation site in BAP1 that was increased upon treatment (Table 2.5).

Table 2.4: Phosphosites identified by anti-pTyr immunoaffinity (novel Met-dependent proteins or phosphosites in bold)

Gene	Site	Log[PHA/DMSO]	Log[Crizotinib/DMSO]	p-value PHA	p-value criz
AAK1	**Y34**	-2.084266663	-2.182134946	2.75E-02	5.86E-05
ABI1	**Y305**	-1.333045959	-1.66950345	9.31E-03	9.75E-03
ACTG1	**Y53**	-2.995290518	-3.222054402	1.86E-03	5.28E-04
ACTG1	**Y198**	-2.037072102	-2.195942442	9.34E-04	3.18E-02
AFAP1L2	**Y54**	3.311836243	2.62051487	2.88E-02	3.79E-02
ANXA2	Y30	-3.76748999	-4.112846613	2.47E-02	1.61E-02
ANXA2	Y188	-3.693778594	-3.926996787	2.01E-02	6.30E-03
ARHGAP12	**Y63**	-1.401375055	-1.635012229	2.37E-02	1.30E-02
C11orf52	Y103	-1.774192691	-2.091362874	3.22E-02	1.20E-02
CALB2	**Y35**	-3.857764482	-4.757815997	1.03E-02	1.34E-02
CALM1	Y100	-1.184326907	-1.2622811	4.34E-02	3.75E-02
CFL1	**Y140**	-0.645972192	-0.840972245	2.45E-02	3.36E-02
CRK	Y136	-1.875021497	-2.171492378	3.59E-03	1.14E-02
CRK	Y108	-1.275001168	-1.558441877	1.35E-02	1.85E-03
CRKL	Y132	-1.466108918	-1.632022699	1.08E-02	1.62E-02
CRKL	Y92	-0.543795675	-0.860862056	9.74E-03	4.08E-03
DLG3	**Y222**	-3.656048616	-4.035151243	5.36E-03	1.77E-04
DNAJC7	**Y38**	-1.536472321	-2.048834324	4.06E-02	2.05E-02
DSC2	**Y839**	-2.04699254	-2.188006481	9.11E-03	5.34E-03
DSC2	**Y853**	-1.103513837	-1.117429892	1.75E-02	1.86E-03
DSP	**Y56**	-1.46371762	-1.523801684	2.46E-02	6.49E-03
EHBP1	**Y1030**	-1.286638101	-1.287701329	3.97E-02	4.01E-03
EIF5A	**Y69**	-1.819562117	-2.158278624	1.74E-03	8.21E-03
EPS8	**Y774**	-0.340141197	-0.490522484	3.65E-02	3.35E-02
FARP2	**Y914**	-1.709568103	-2.140901764	2.91E-02	1.63E-02
GAREM	**Y453**	-1.113882919	-1.104308069	1.09E-02	1.61E-02
GOLGA4	**Y2141**	-2.103821556	-2.498226086	1.37E-02	1.36E-03
GTF2F2	**Y196**	-1.743444165	-1.627690077	1.39E-02	1.55E-02
HGS	**Y286**	-1.374862115	-1.618986487	1.01E-02	3.45E-02
IQGAP1	Y855	-3.116945267	-3.164519866	7.50E-05	2.73E-02
IQGAP2	**Y770**	-2.521232605	-2.843430758	2.70E-03	5.08E-03
IRS2	Y675	-1.163646777	-1.477989237	3.11E-03	2.09E-02
IRS2	**Y742**	-0.833061477	-1.058556537	3.85E-02	3.07E-02
ITSN2	**Y553**	-2.709106763	-2.812017043	6.37E-03	7.85E-03
JUP	**Y550**	-2.177651286	-2.031506896	4.26E-02	4.90E-03
JUP	**Y660**	-2.055226604	-2.188106855	6.66E-03	2.40E-03

KRT18	Y13	-1.853592436	-2.005078634	2.29E-02	2.31E-02
LDHA	**Y10**	-1.396791975	-1.569298764	1.81E-02	4.14E-02
LYN	**Y439**	-1.801339904	-1.84475402	2.38E-02	3.05E-02
MARVELD2	**Y434**	-1.222085019	-1.497353355	2.90E-02	9.04E-03
MET	Y1234	-1.417537928	-3.59433953	1.27E-02	1.64E-02
MPZL1	Y139	-1.877271096	-2.432922284	3.79E-02	6.11E-04
MYO6	Y1121	-2.756419897	-3.127667189	7.87E-03	2.79E-04
NCK2	Y50	-1.571972768	-1.969473124	4.84E-04	1.50E-02
PABPC1	**Y364**	-0.914104521	-1.157581051	1.28E-02	3.35E-02
PABPC4	**Y364**	-0.879832566	-1.10917598	1.15E-02	3.58E-02
PARD3	**Y445**	-1.62216874	-1.852607091	1.74E-02	9.69E-03
PDCD5	Y80	-4.081906239	-4.61727651	1.09E-03	3.95E-03
PELO	**Y99**	-1.4628582	-1.737507701	9.95E-04	1.54E-02
PI4KA	Y528	-4.194211404	-4.794254462	3.47E-03	1.54E-02
PKP2	**Y166**	-1.725590785	-1.792298079	2.21E-02	1.93E-03
PPP2R5D	Y580	-1.688085794	-1.868969003	3.99E-03	4.39E-02
PTBP3	Y127	-0.68253239	-1.040612141	3.55E-02	8.33E-04
RAI14	Y219	-3.098894755	-3.525143623	3.05E-02	6.84E-04
RAN	Y39	-2.589286407	-2.819080194	2.72E-03	2.37E-02
SHB	Y268	-1.97929585	-2.400721312	4.03E-02	4.53E-03
SLC9A1	Y659	-2.672631264	-3.188575029	8.75E-03	6.95E-03
STAM	Y384	-1.415977319	-1.682652791	2.24E-02	5.66E-03
STAT3	Y704	-1.663842082	-2.016520103	3.91E-02	4.15E-03
STAT5A	Y694	-3.322531621	-3.317712466	1.72E-02	2.33E-03
TAOK1	**Y309**	-2.129639228	-2.48613143	2.67E-03	4.57E-03
TM9SF3	**Y272**	-0.445730964	-0.659926514	7.01E-03	7.14E-03

Table 2.5: Phosphorylation sites identified by Ti_2O enrichment

Gene	Site	Log[PHA/DMSO]	Log[Crizotinib/DMSO]	p-value PHA	p-value criz
AHNAK	S5110	-2.294333935	-2.153045932	1.87E-04	6.31E-03
ARAF	S186	-1.589220126	-1.739450216	7.10E-03	4.67E-02
ATR	S435	-1.175109287	-1.253415187	2.64E-02	7.02E-04
ATXN2L	S449	-1.033993423	-1.059450289	3.42E-03	3.44E-02
BAP1	S513	1.788571199	1.886518757	3.99E-04	7.58E-03
C11orf52	Y103	-1.535871585	-1.990348577	2.69E-02	1.56E-02
C2orf49	S193	-1.059058428	-1.064339896	3.77E-03	5.56E-03
CCNL1	S335	-1.175547024	-1.051414589	1.05E-02	3.69E-02
CCNL1	S341	-1.167451859	-1.043826203	1.20E-02	3.91E-02

DDX17	S492	-1.186786095	-1.086758812	2.05E-02	1.66E-02
FAM114A1	S196	-1.008671939	-1.43883733	1.82E-02	1.10E-02
FAM129B	S678	-1.09332184	-1.050354342	1.08E-02	4.00E-02
FAM129B	S683	-1.032318493	-1.061173459	4.90E-03	2.89E-02
HDGFRP2	S454	-2.149864435	-2.179578503	1.61E-03	2.17E-02
IRS2	S1174	-1.86322693	-2.201768756	9.68E-03	3.18E-02
IRS2	S365	-1.348524729	-1.522416552	3.23E-02	2.41E-02
NCBP1	S22	-1.166416109	-1.262507021	2.61E-02	3.59E-02
POF1B	S123	-1.14039129	-1.255757709	7.13E-03	1.94E-02
PXN	S106	-1.468628565	-1.711836775	1.24E-02	2.51E-02
RIMS2	T866	-1.491510252	-1.595626354	4.09E-02	4.28E-02
RPS6	S236	-3.18710653	-3.291802327	2.10E-02	3.55E-02
RPS6	S240	-3.179575761	-3.246497114	2.04E-02	4.13E-02
RPS6KA3	S369	-1.109682222	-1.124490837	4.31E-02	6.04E-03
RPS6KA3	T365	-1.109682222	-1.124490837	4.31E-02	6.04E-03
STMN1	S25	-1.537544092	-1.5085	3.61E-02	4.91E-02
TACC2	S437	-1.1065678	-1.215180159	3.25E-03	2.99E-02
TRIM33	S1102	-1.711613735	-1.696247896	3.49E-03	1.07E-02
ZNF609	S413	-1.28180627	-1.37930874	3.22E-03	1.31E-03

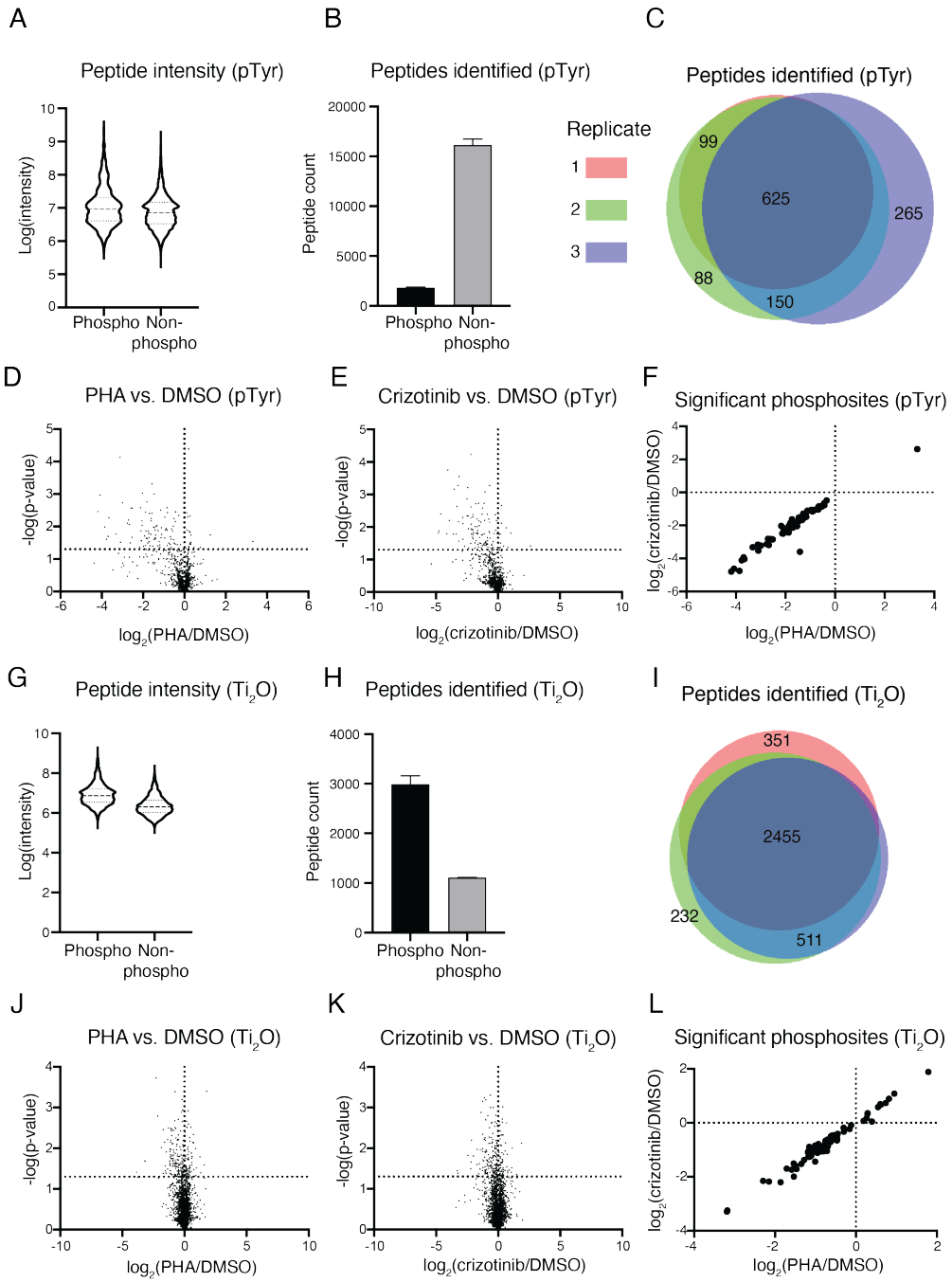


Figure 2.17: Enrichment of phosphopeptides by anti-pTyr immunoaffinity and TiO_2. *(A) Tyrosine-phosphorylated peptides were detected with slightly higher average intensity than non-phosphorylated peptides after pTyr immunoaffinity enrichment. (B) A minority of peptides identified after pTyr immunoaffinity enrichment were phosphorylated (10.1%). (C) Venn diagram of peptides identified by biological replicate after pTyr immunoaffinity enrichment. (D, E) Volcano plot showing log_2-transformed ratio of phosphorylated peptides identified in PHA- (D) or crizotinib- (E) vs DMSO-treated cells. (F) Significantly changed phosphopeptides were coordinately regulated by PHA and crizotinib. (G) Phosphorylated peptides were detected with higher average intensity than non-phosphorylated peptides after TiO_2 enrichment. (H) The majority of peptides identified after TiO_2 enrichment were phosphorylated (72.9%). (I) Venn diagram of peptides identified by biological replicate after TiO_2 enrichment. (J, K) Volcano plot showing log_2-transformed ratio of phosphorylated peptides identified in PHA- (J) or crizotinib- (K) vs. DMSO-treated cells. (F) Significantly-changed phosphopeptides were coordinately regulated by PHA and crizotinib.*

To identify known networks within the phosphorylation sites identified in our experiment we used the STRING database (Figure 2.18A) (https://string-db.org/; Szklarczyk et al., 2019b). The phosphosites identified by our approach include well-characterized substrates and interactors of Met as well as in proteins known to be associated with Met activity in an oncogenic context such as the calmodulin-dependent signaling pathway and proteins involved in ribosomal protein S6 (RPS6) phosphorylation, which is tightly associated with proliferative signaling. Of particular interest to us was the scaffold protein IRS2, a large multisubstrate protein with homology to Gab1 that is associated with insulin-dependent signaling. We observed a decrease in both tyrosine and serine phosphorylation of IRS2, including on Tyr675, a conserved site required for IRS2-dependent PI3K activation (Landis and Shaw, 2014).

In addition to this, we identified a number of sites not known to be associated with Met-dependent activity. Some of these, such as the adaptor GAREM, an adaptor upstream of the Erk MAPK pathway, are known to be involved in RTK-dependent activation of canonical downstream signals. Other novel Met-dependent phosphosites were found in a number of intermediate-filament cytoskeletal and adhesion proteins, such as cytokeratin-18 (KRT18), desmocollin-2 (DSC2), desmoplakin (DSP), and junctional plakoglobin (JUP), and plakophilin-2 (PKP2). While related intermediate-filament junctional proteins such as plakophilin-3 have been reported as Met-dependent phosphorylation sites by mass spectrometry before, our discovery of several novel tyrosine-phosphorylated proteins in these complexes expands the repertoire of intermediate filament junctional proteins that may be regulated by Met-dependent signaling in a proliferative context.

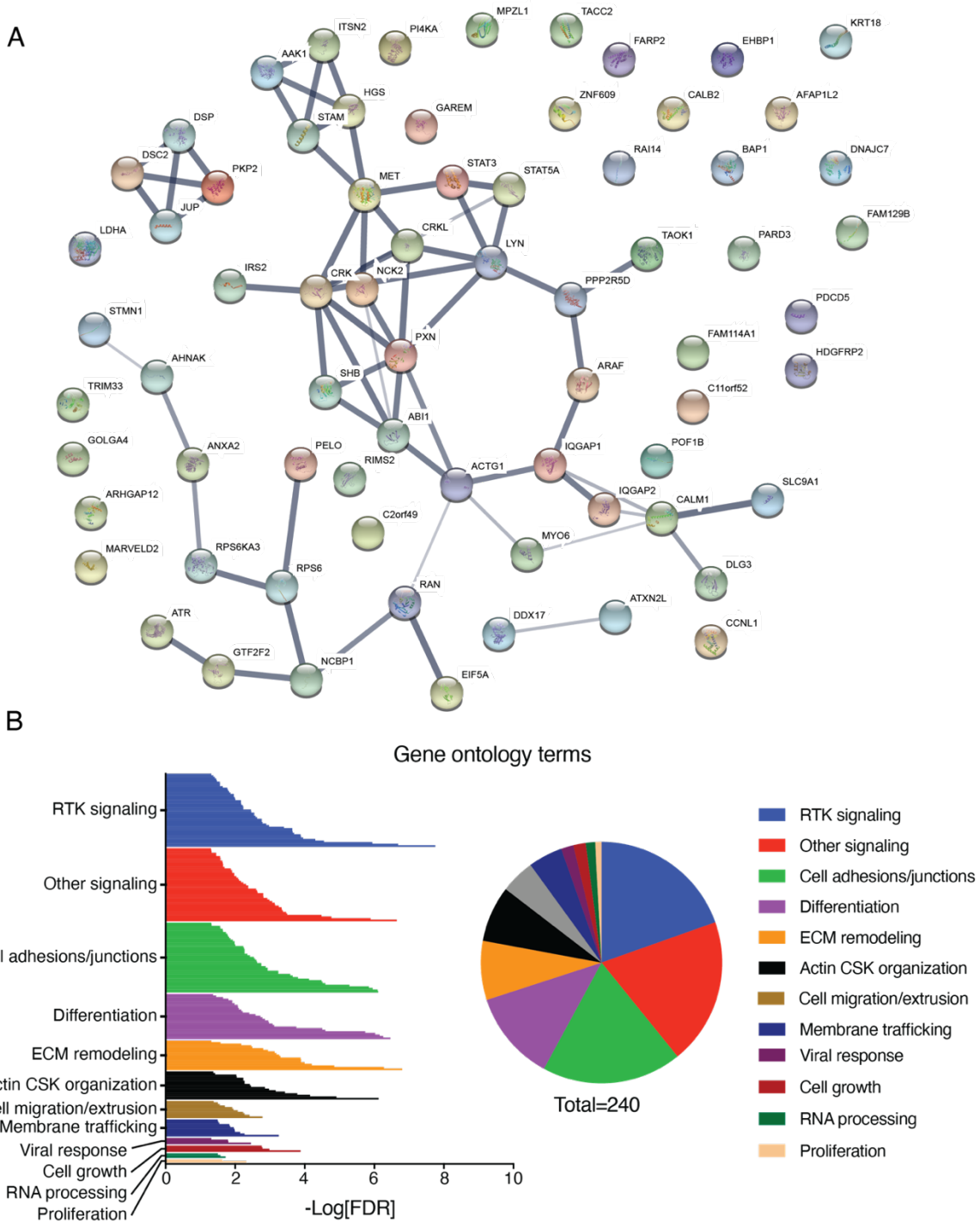


Figure 2.18: Met-dependent phosphosites cluster in signaling and adhesion networks. *(A) STRING network of known protein-protein interactors among crizotinib- and PHA-sensitive phosphosites. Edge thickness indicates confidence. (B) More than 70% of genes associated with crizotinib- and PHA-sensitive phosphosites are enriched in gene ontology terms associated with known RTK and downstream signaling processes (20%), other known signaling pathways (20%), functions involved in the regulation of cell adhesions and junctions (19%) or differentiation (12%). Additional processes are involved in ECM remodeling, actin cytoskeletal organization, cell migration or extrusion through endothelial tissue, with few pathways associated with proliferation or cell growth.*

2.21 MPZL1 is phosphorylated upon co-expression with Met

We also observed Met-dependent tyrosine phosphorylation of a site (Y139) in MPZL1. This has been reported in mass spectrometry-based datasets, but MPZL1 phosphorylation not been validated downstream of Met. MPZL1 is a homologue of MPZL3 with a similar domain organization and high homology in the extracellular Ig-like domain (Cao et al., 2007; Racz et al., 2009). Both MPZL1 and MPZL3 contain tyrosine residues in their cytoplasmic C-tails (Figure 2.19A). Phosphorylation of MPZL1, also known as protein-zero related (PZR), in the distal C-tail has been shown to mediate recruitment of the tyrosine phosphatase SHP2 (Zhao and Zhao, 2000; Zhao and Zhao, 1998). We co-expressed Met with MPZL1-V5, MPZL3-V5, or GFP-V5 as a control in HEK293T cells and performed immunoprecipitations against both Met and V5 to test whether MPZL1 or MPZL3 co-immunoprecipitated with Met or showed Met-dependent phosphorylation (Figure 2.19B). A band at 170 kDa corresponding to the unprocessed pro-Met protein co-immunoprecipitated with MPZL3. This band was recognized by antibodies raised against both the phosphorylated activation loop tyrosines (Tyr1234/35) and the total Met protein, but no co-immunoprecipitation was observed between the 145 kDa mature beta-chain of Met and MPZL3. In contrast to this, MPZL1-V5 was phosphorylated upon Met overexpression, as shown by pan-phosphotyrosine antibody upon V5 immunoprecipitation (~36 kDa). MPZL3-V5 and GFP-V5 did not show any increase in pan-phosphotyrosine reactivity upon Met overexpression. Thus, MPZL3 can co-immunoprecipitate with pro-Met upon co-overexpression but is not phosphorylated by Met in this context, while MPZL1 is phosphorylated upon Met overexpression, indicating that both adhesion receptors may play a role downstream of Met.

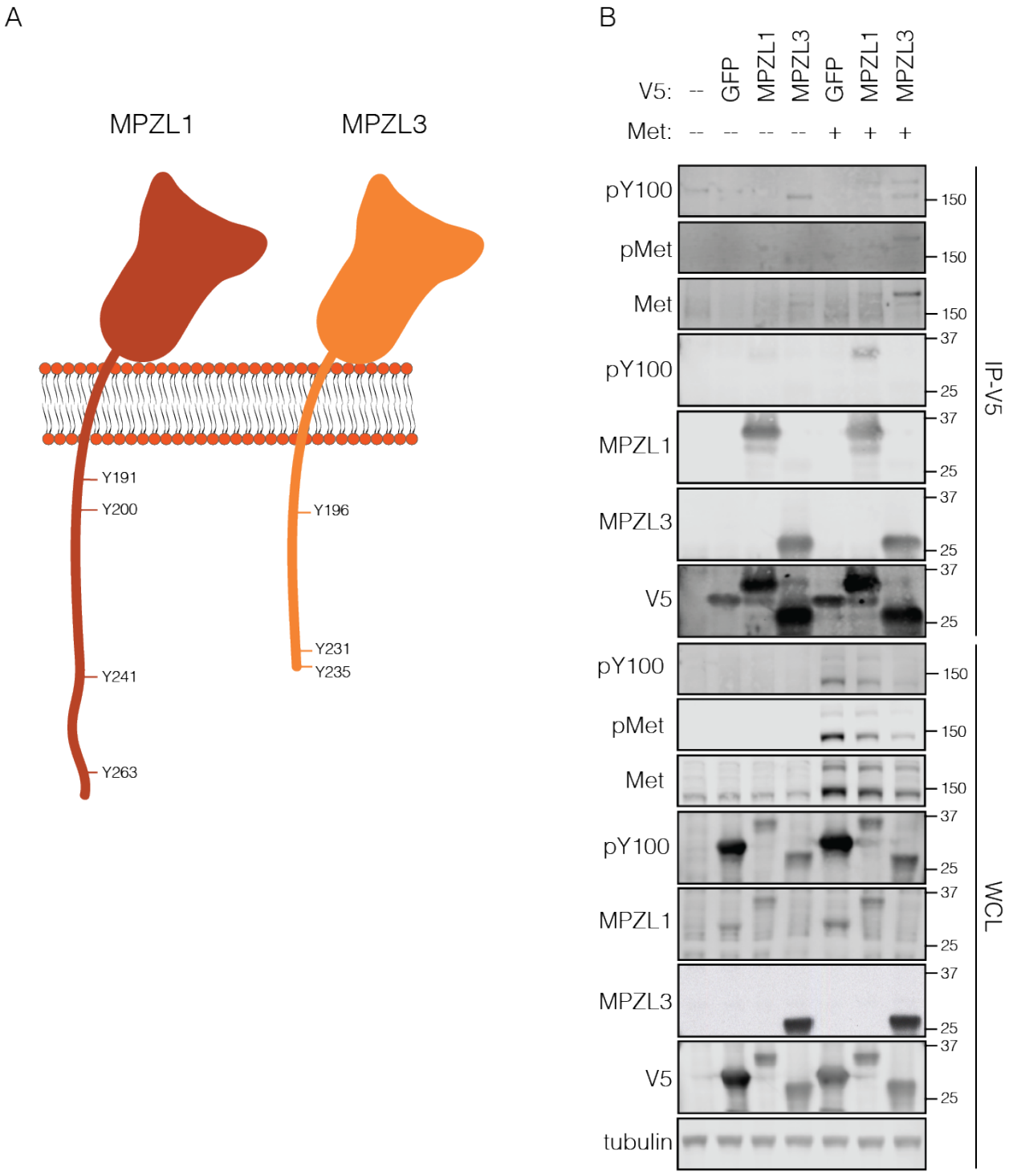


Figure 2.19: MPZL1 is phosphorylated upon Met overexpression and MPZL3 co-immunoprecipitates with pro-Met. *(A) MPZL1 and MPZL3 share domain organization and both contain several cytoplasmic tyrosine residues that may be phosphorylated by cytoplasmic kinases. (B) Immunoprecipitation of V5 and Met in HEK293T cells overexpressing Met, MPZL1 and MPZL3 (n=1). Co-overexpression of Met with MPZL1-V5 induces MPZL1 phosphorylation, as shown by pan-phosphotyrosine reactivity (pY100). Co-overexpression of Met with MPZL3-V5 leads to the co-immunoprecipitation of phosphorylated pro-Met with MPZL3.*

2.22 HGF accelerates tumour outgrowth in MET*-amplified cells through additional mechanisms*

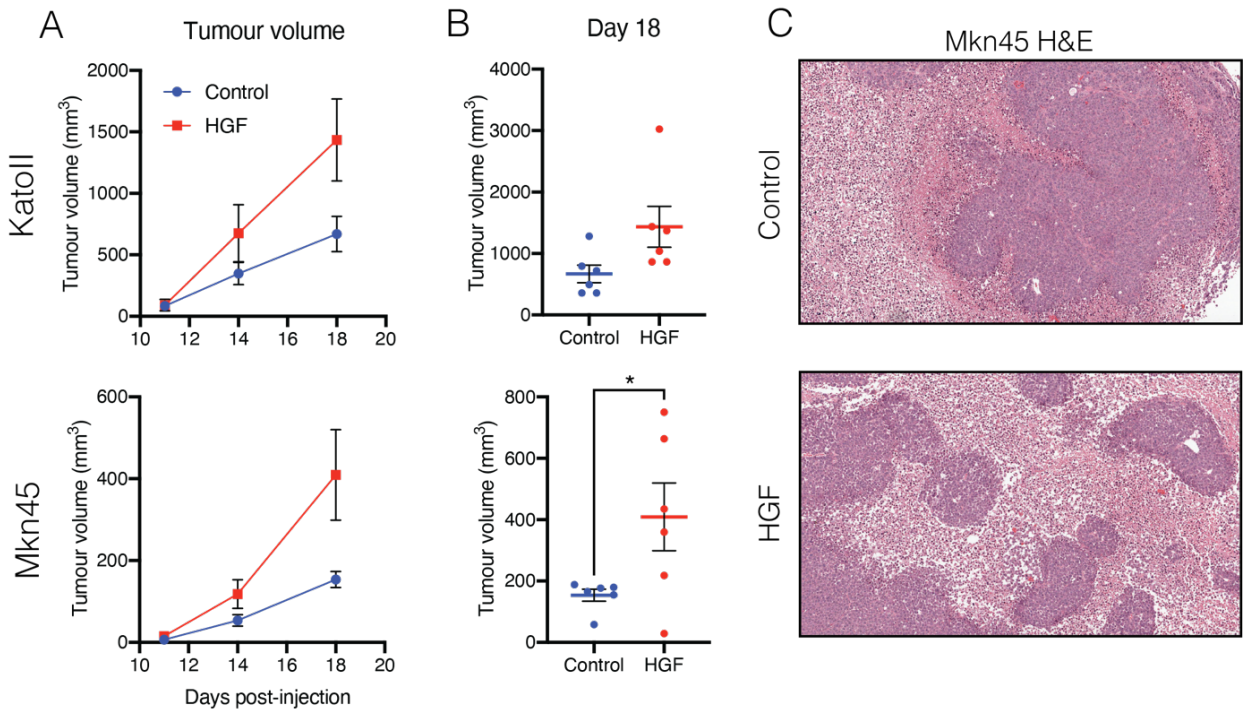
MET-amplified cancer cell lines exhibit constitutive Met phosphorylation in the absence of exogenous HGF, even under conditions of serum starvation or after changing media (Figures 2.1A, 2.16D). This indicates that Met autophosphorylation is not dependent on HGF autocrine secretion for maintenance and is consistent with a mass-action-based model for constitutive Met activation at high receptor concentration within the plasma membrane. Nonetheless, growth of *MET*-amplified tumours in mice engineered to express the human *HGF* gene have been reported to show increased resistance to Met-directed TKI treatment, and exogenous HGF treatment has been reported to attenuate the effects of Met inhibitors in cell culture as well (Ahn et al., 2017; Pennacchietti et al., 2014; Zhang et al., 2005). We similarly observed an increase in the effective concentration of inhibitor required to inactivate the Met receptor upon serum starvation, suggesting that HGF may act in a comparable manner by more fully activating the Met kinase and increasing Met autophosphorylation (Figure 2.16D). These experiments also showed an increase in the kinetics of tumour outgrowth in mice expressing human HGF. We sought to test whether growth of *MET*-amplified cell lines in *HGF*-expressing mice accelerated tumour growth by increasing Met phosphorylation or the downstream phosphorylation of proteins involved in Met-dependent oncogenic signalling such as HER3, Akt, STAT3 and Erk.

We injected KatoII and MKN45 *MET*-amplified cells subcutaneously into immune-compromised mice with (HGF) or without (control) expression of the human *HGF* gene. KatoII cells formed tumours rapidly and had to be sacrificed at day 18 due to ethical considerations. KatoII tumours grown in HGF mice were larger on average at this point, but the difference did not reach statistical

significance (Figure 2.20A and B). MKN45 tumours were smaller and were resected at the same time to monitor for the development of metastases. MKN45 tumours grown in HGF mice were larger than those grown in control mice at the point of resection (Figure 2.20A and B).

We did not observe the growth of metastatic tumours over the course of the experimental follow-up in resected animals. MKN45 tumours resected from HGF-mice displayed multifocal lesions with small islands of cancer cells surviving in necrotic zones, while this was largely absent from tumours grown in control mice (Figure 2.20C). Thus, we saw evidence of an additive effect of HGF expression on early tumour outgrowth in cell lines with constitutively activated Met.

We next treated H1993, KatoII and MKN45 cells in culture with HGF to test whether the action of HGF in promoting early tumour outgrowth arose due to an increase in Met activation or phosphorylation of Met-dependent proteins associated with proliferation. We probed lysates of cells treated with HGF over a course of 3 days for phosphorylation of Met, HER3, STAT3, Akt and Erk phosphorylation (Figure 2.20D). All five proteins showed a loss of phosphorylation upon treatment with PHA, but exogenous HGF treatment did not increase phosphorylation of any sites tested. Untreated cells left in culture over the course of the HGF treatment showed comparable phosphorylation to cells treated with HGF for 3 days, demonstrating that any changes in phosphorylation at this point were due to increased cell confluence or other independent factors, and were not induced by HGF. In contrast to this, HGF treatment of non-*MET*-amplified HeLa cells induced the phosphorylation of 3 phosphosites in Met, as well as activation of Erk and Akt signaling, while STAT3 phosphorylation was induced only after 72h of treatment (Figure 2.20D).


A
Tumour volume
KatoII
Mkn45
Control
HGF
Tumour volume (mm3)
Days post-injection
B
Day 18
Control
HGF
*
C
Mkn45 H&E
Control
HGF

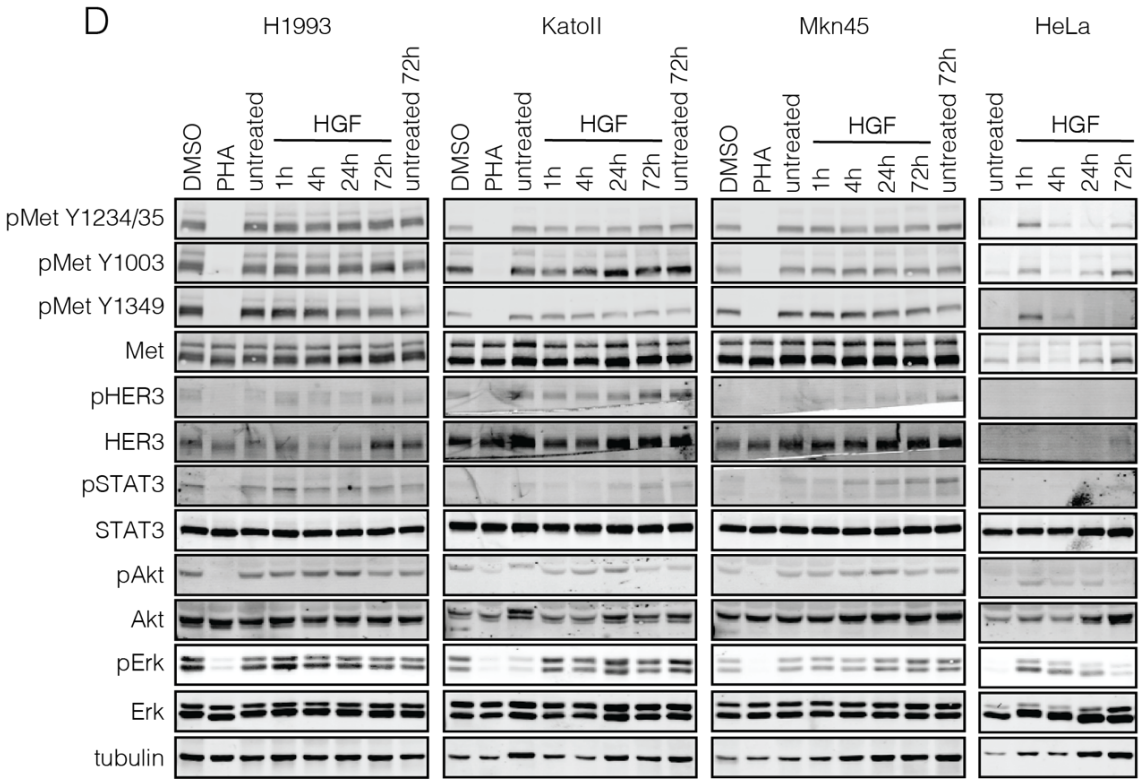

D
H1993
KatoII
Mkn45
HeLa
DMSO
PHA
untreated
HGF
1h
4h
24h
72h
untreated 72h
pMet Y1234/35
pMet Y1003
pMet Y1349
Met
pHER3
HER3
pSTAT3
STAT3
pAkt
Akt
pErk
Erk
tubulin

Figure 2.20: HGF accelerates tumour outgrowth in* MET-*amplified cells. *(A)* MET-*amplified KatoII and MKN45 cells injected in control or human-*HGF-*expressing mice were measured by palpation over the course of 18 days (n=10 tumours). (B) Tumour volume of control or HGF-exposed tumours at endpoint. (C) H&E staining of MKN45 tumours shows islands of surviving tumour cells in necrotic zones in HGF mice, indicative of multifocal tumours. (D) Treatment of* MET-*amplified H1993, KatoII and MKN45 cells with HGF does not show an HGF-dependent increase in Met phosphorylation, or the phosphorylation of known downstream species associated with oncogenic signaling including HER3, STAT3, Akt or Erk.* MET-*normal HeLa cells, by contrast, show HGF-induced Met, STAT3, Akt and Erk phosphorylation, but no induction of HER3 phosphorylation (n=3).*

HER3 expression was increased slightly 72h after addition of HGF to HeLa cells, but it was not detectably phosphorylated under these conditions. Thus, *MET*-amplified cell lines do not show major increases in known Met-dependent phosphorylation upon treatment with exogenous HGF, but alternative mechanisms contribute to the early outgrowth of human *MET*-amplified tumours in the presence of human HGF.

2.23 A bio-orthogonal approach to validating Met substrates and Met-dependent phosphosites

Our experiments in *MET*-amplified cell lines suggest an important role for a number of cell adhesion proteins in Met-dependent proliferation and phosphotyrosine signaling. These include the crosstalk observed with HER3, with its role supporting expression of MPZL3 and their Met-dependent interaction at the protein level, the abundance of adhesion and junctional proteins identified by our phosphoproteomic experiments, and the MPZL3 homologue MPZL1, which is phosphorylated upon co-overexpression with Met. Moreover, in the case of Met-HER3 signaling, the use of EGFR-, HER2- and Src-family-directed TKIs indicate that these kinases do not play a role in Met-dependent HER3 phosphorylation. Given that our experiments were conducted in cells with exceptionally high levels of Met protein and constitutive Met activation, it is reasonable to hypothesize that Met directly phosphorylates the majority of tyrosine phosphosites detected in

our experiments, including HER3. We developed a Met mutant capable of selectively utilizing substrate-labeling ATP analogues in order to test whether Met can directly phosphorylate substrates such as HER3, MPZL1 and the other putative Met-dependent tyrosine phosphorylation sites identified by phosphoproteomics.

2.24 Mutation of gatekeeper residues in the Met kinase domain facilitate ATP analogue binding

Seminal work by Kevan Shokat pioneered a method of developing substrate-labeling kinases by mutating residues in the ATP-binding pocket of the kinase, thereby expanding the pocket and facilitating the binding of ATP analogues containing bulky alkyl adducts at the N6 position (Liu et al., 1998; Shah et al., 1997). We aligned the Met amino acid sequence to several kinases that had been mutated in this manner and successfully utilized in substrate-labeling experiments (Figure 2.21A). This showed that the position Leu1157 in the Met kinase domain aligned with the "gatekeeper" residue in v-Src. We aligned a crystal structure of analogue-sensitized c-Src bound to N6-benzyl-ADP (Thr338Gly; PDB ID: 1KSW) to the crystal structure of the activated Met kinase domain (4IWD) (Northrup et al., 2013; Witucki et al., 2002). This showed that N6-benzyl-ADP docked in the Met ATP binding pocket with the N6 benzyl moiety juxtaposed to Leu1157 (Figure 2.21B). We introduced point mutations substituting Leu1157 for alanine or glycine and expressed the Met Leu1157Ala and Leu1157Gly mutants in HEK293T cells (Figure 2.21C). Met Leu1157Ala expressed at levels comparable to wild-type Met and was similarly constitutively phosphorylated upon high-level overexpression, although the accompanying tyrosine phosphorylation of other proteins was partially attenuated. Met Leu1157Gly was less efficiently autophosphorylated and failed to induce tyrosine phosphorylation of other species. Mutation of Leu1157 to alanine removed a steric clash between the leucine sidechain and the benzyl moiety added to the N6

position of N6-benzyl ADP (Figure 2.21D). Thus, we hypothesized that the Met Leu1157Ala mutant, which is capable of autophosphorylation as well as the tyrosine phosphorylation of downstream proteins, would be capable of utilizing N6-modified ATPγS analogues.

2.25 Met Leu1157Ala is capable of selective autolabeling in vitro *using N6-modified ATPγS*

In order to validate the enzymatic activity of Met Leu1157Ala and Gly mutants with substrate-labeling ATP analogues, we next prepared an *in vitro* kinase reaction. By substituting the gamma phosphate group with a thiophosphate (ATPγS), substrates of a phosphorylation reaction utilizing this nucleotide become thiophosphorylated. Direct substrates of a kinase utilizing ATPγS can be distinguished from other phosphates by conversion to thiophosphoesters in the presence of alkylating agents such as *p*-nitrobenzyl mesylate (PNBM) and can be subsequently detected using a selective thiophosphoester-directed antibody, 51-8 (Figure 2.22A). We expressed Met Leu1175Ala and Met Leu1157Gly in HEK293T cells with wild-type or kinase dead Met constructs as controls (Figure 2.22B). Met protein was immunoprecipitated from cell lysates and split between 4 separate reactions per condition. Wild-type Met and Met Leu1157Ala induced detectable autothiophosphorylation in a manner dependent on both ATPγS and PNBM (Figure 2.22B). This demonstrated that both the wild-type and mutant kinase domain were catalytically active using ATPγS as a nucleotide substrate. We proceeded to test the selectivity of Met Leu1157Ala over wild-type Met for a panel of N6-modified ATPγS analogues (Figure 2.22C). Wild-type and Leu1157Ala constructs were expressed in HEK293T cells for *in vitro* kinase reactions as before (Figure 2.22D).

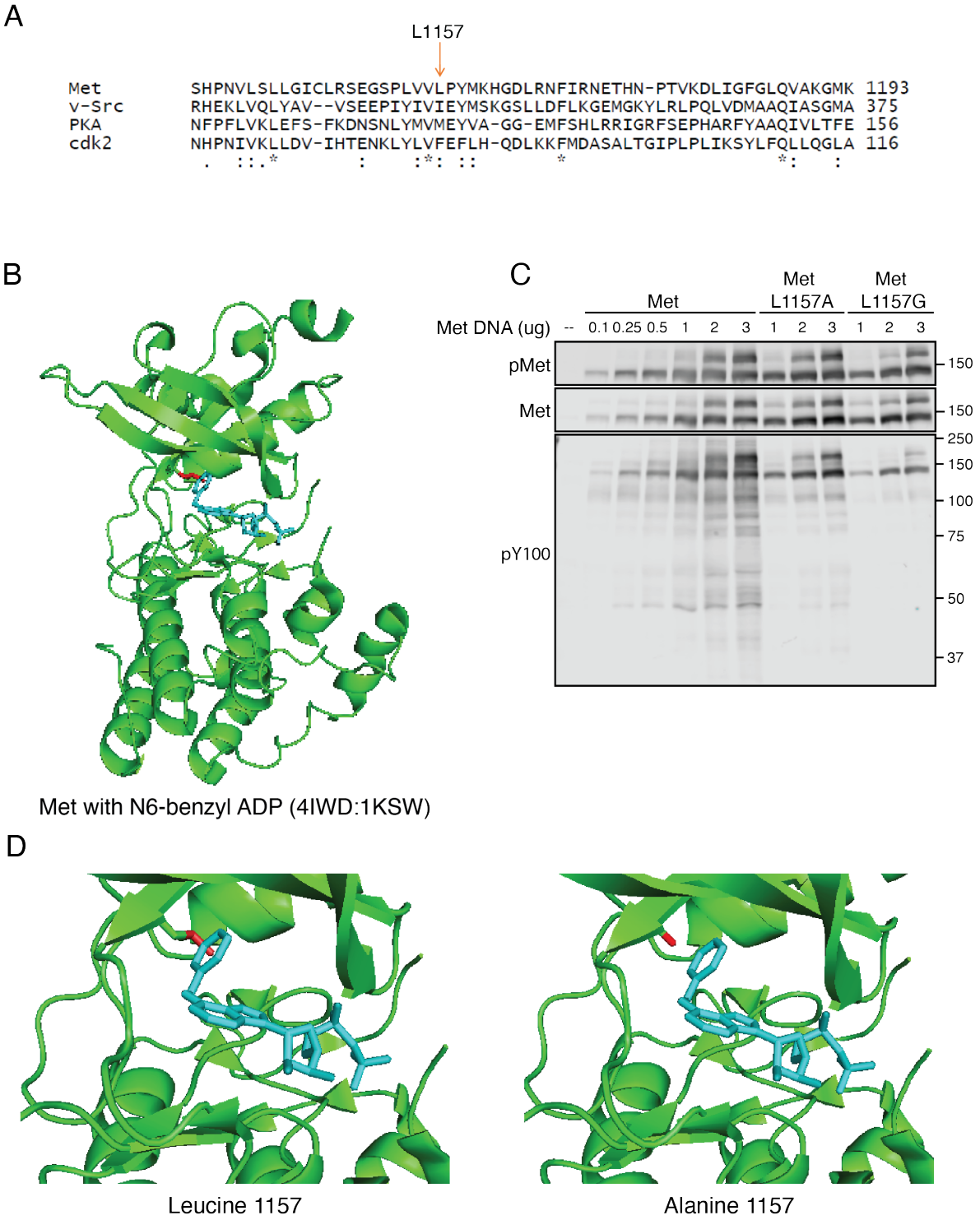


Figure 2.21: Mutation of the Leu1157 gatekeeper residue in Met expands the ATP binding pocket. *(A) ClustalW alignment of Met, v-Src, protein kinase A (PKA) and cyclin-dependent kinase 2 (cdk2) shows that Leu1157 aligns with the gatekeeper residue in these kinases. (B) Structural alignment of activated Met kinase domain (4IWD) to analogue-sensitive c-Src Thr338Gly mutant bound to N6-benzyl ADP (1KSW). (C) Expression and phosphorylation of wild-type, Leu1157Ala (L1157A) and Leu1157Gly (L1157G) Met constructs in HEK293T cells (n=1). Tyrosine phosphorylation of other proteins is monitored by pan-phosphotyrosine antidbody (pY100). (D) Mutation of Leu1157 to alanine removes steric hindrance between the side chain of Leu1157 and the benzyl ring of N6-benzyl ADP.*

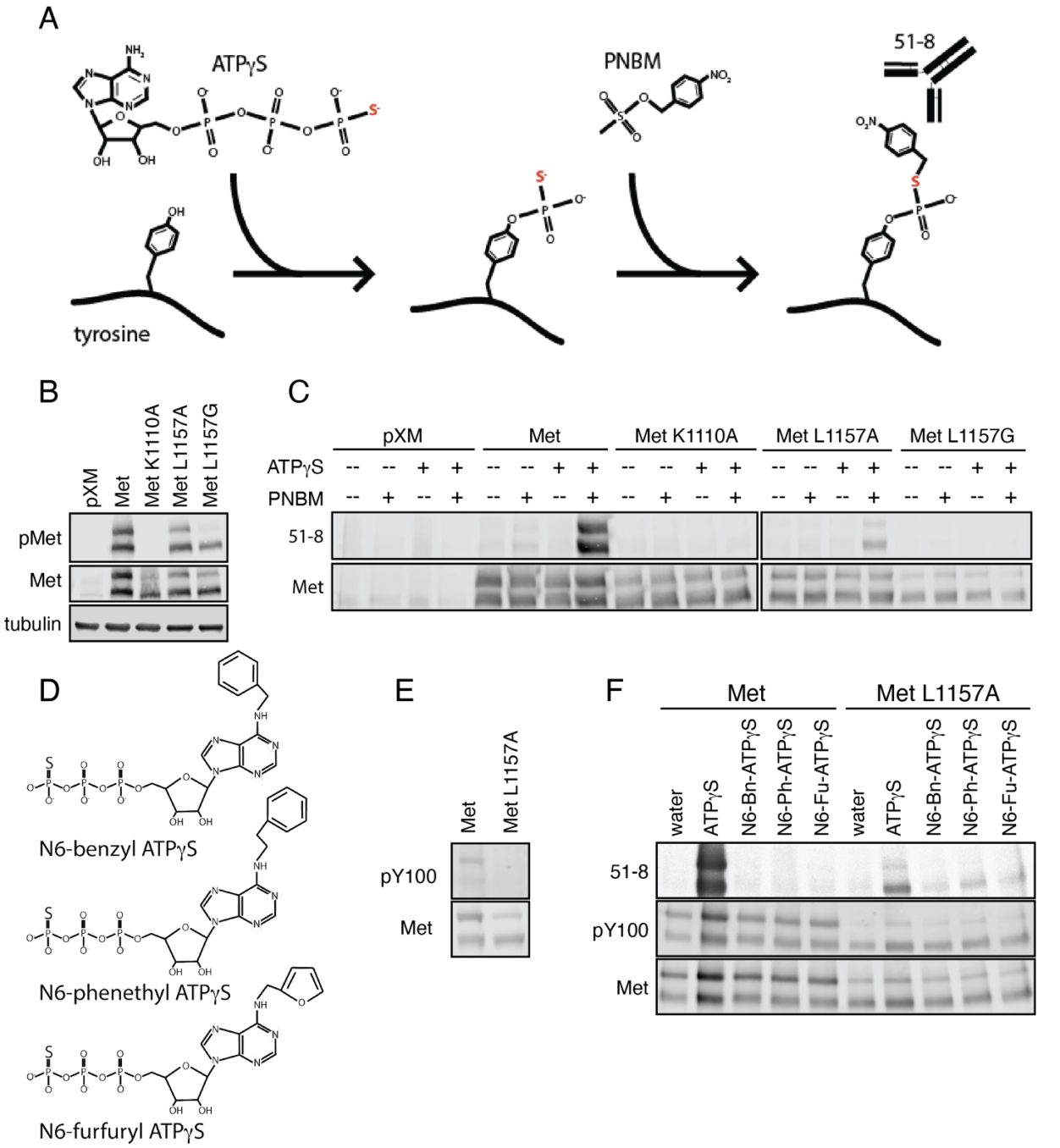


Figure 2.22: Met Leu1157Ala selectively utilizes N6-modified ATPγS analogues in substrate-labeling reactions. *(A) Schematic for thiophosphoester-based substrate recognition using ATPγS.* p-nitorbenzyl mesylate (PNBM) *is used to alkylate thiophosphorylation sites to thiophosphoesters.An antibody against thiophosphoesters (51-8) is used to detect alkylated substrates. (B) Met construct expression and activation loop phosphorylation (Tyr1234/35) in HEK293T cells. Kinase-impaired (Lys1110Ala; K1110A) and gatekeeper residue (Met1157Ala/Gly; MetL1157A/G) are compared to wild-type Met (n=3). (C)* In vitro *kinase reaction using immunoprecipitated Met protein from cells in (B) (n=4). Lysates were supplemented with ATP or ATPγS and alkylated with PNBM.(D) Schematic of N6-substituted ATPγS analogues. (E) Met and Met L1157A expression and overall phosphorylation in HEK293T cells (pan-phosphotyrosine antibody; pY100) (n=3). (F)* In vitro *kinase reactions using N6-substituted ATPγS analogues (n=3). Met L1157A is capable of utilizing N6-Ph-ATPγS and N6-Fu-ATPγS while wild-type of Met can only utilize unsubstituted ATPγS.*

Met Leu1157Ala was able to induce autothiophosphorylation in the presence of all three N6-modified ATPγS analogues tested, while wild-type Met was only capable of utilizing unmodified ATPγS (Figure 2.22E). Met Leu1157Ala is thus an analogue-sensitive mutant of the Met RTK and is biochemically capable of selective substrate-labeling *in vitro*.

CHAPTER 3: DISCUSSION

3.1 Summary

The Met RTK has been identified as a proto-oncogene since its discovery as a component of the fusion oncoprotein, TPR-Met, in experimentally-transformed murine NIH3T3 fibroblasts (Gonzatti-Haces et al., 1988; Park et al., 1986; Park et al., 1987). Early interest in the development of therapeutic agents to target oncogenic RTKs included Met. After the clinical successes of imatinib, gefitinib and trastuzumab in the late 1990's and early 2000's, clinical-grade therapeutic agents targeting the Met kinase were advanced in clinical trials to treat patients overexpressing Met protein in the hopes of replicating patient response success seen by targeting EGFR and HER2. Clinical experience in the subsequent two decades has shown that only a small proportion of patients respond to Met-directed therapy, and that this is generally accompanied by either mutations affecting the negative regulation of Met, or gene amplification (Lennerz et al., 2011; Noonan et al., 2016). Activating kinase domain mutations found in papillary renal carcinomas similarly predispose patients to respond to Met inhibitors, while the recent discovery of recurrent Met gene fusions in glioblastomas heralds additional cases in which mutational activation of Met can be targeted clinically (International Cancer Genome Consortium PedBrain Tumor, 2016; Rhoades Smith and Bilen, 2019). Nonetheless, high expression of Met protein alone has not succeeded as a method in identifying Met-dependent cancers. This may be due to a discrepancy between the threshold for Met positivity by immunohistochemical staining, used historically to identify Met high tumors, and the level of expression required for oncogenic transformation of cells by Met (Guo et al., 2019). Thus, unique mechanisms that accompany the constitutive activation of Met, such as the induction of heterotypic signaling with the EGFR family, may be required for Met to act as a driver oncogene in cancer.

Genomic amplification of *MET* was identified in human cancers in the wake of landmark discoveries in the 1980's linking *ERBB2* amplification to oncogenic transformation by HER2 (Di Renzo et al., 1992; Kuniyasu et al., 1992; Slamon et al., 1987; Wullich et al., 1993). *MET*-amplified cell lines grown continuously in tissue culture have been established as a model of Met-dependent cancer, in which selective inhibition of Met activity induces a cytostatic or cytotoxic response *in vitro* and in xenograft tumours *in vivo* (Lai et al., 2014; Smolen et al., 2006). To address our understanding of the cellular requirements for Met-dependent oncogenic transformation, the goal of this thesis was to use *MET*-amplified cancer cell lines, known to be dependent on Met activity for proliferation and survival, as a model system to identify molecular determinants of Met-dependent transformation as selected in cancer cells. This information could then be used to improve patient stratification by identifying patients more likely to respond to Met inhibitors in the clinic, and to provide insight into the molecular mechanisms underlying oncogenic Met signaling to improve drug efficacy or identify appropriate combination therapies.

Using a combination of selective kinase inhibitors and structure-function approaches, we show that signaling between Met and the EGFR family, known to be a feature of *MET*-amplified cell biology, preferentially induces HER3 phosphorylation. This occurs independently of EGFR and HER2. Heterotypic signaling between Met and HER3 contributes to proliferation and the early outgrowth of *MET*-amplified cells *in vivo*. We subsequently demonstrated that this depends on the transcription of MPZL3, a predicted cell adhesion protein. We show that HER3 forms a complex with MPZL3, and that MPZL3 expression is elevated in *MET*-, *EGFR*- and *ERBB2*-amplified cancers, indicating that it may play a more general role in oncogenic transformation downstream of RTKs that signal through HER3.

Using a phosphoproteomic approach to identify phosphoproteins in *MET*-amplified cancer cells dependent on Met activity, we identified MPZL1, a homologue of MPZL3, among a large number of previously undescribed atypical adhesion and junctional proteins phosphorylated downstream of Met in *MET*-amplified KatoII cells. We also demonstrate an HGF-dependent acceleration of early tumour outgrowth that does not correlate with increased Met phosphorylation or canonical Met-dependent proliferative signaling, indicating that additional HGF-dependent pathways can contribute to early tumour outgrowth in *MET*-amplified cells. We have developed Met kinase domain mutants designed to accept bulky ATP analogues allowing for direct substrate-labeling of Met substrates. These mutants can also be used to generate cell lines in which Met can be inhibited by highly selective TKI analogues (discussed below). Thus, this thesis contributes to the understanding of Met-dependent crosstalk with the EGFR family through HER3. We add genes to the repertoire of HER3-dependent signaling partners with a role in promoting cancer and candidate targets of Met-dependent signaling associated with a proliferative signal output. Furthermore, we show HGF-dependent roles for increasing tumour aggressiveness in this context, and finally, we validate biochemical tools for further elaboration of Met substrates in a variety of signaling contexts.

3.2 HER3 as a central node for crosstalk between Met and the EGFR family RTKs

Signaling between the Met and EGFR family of RTKs has been reported in a variety of cellular contexts. Most of these studies have focused on mechanisms of EGFR-dependent phosphorylation of Met, or combinatorial inhibition of Met and EGFR in an attempt to improve patient response to therapy and prevent the outgrowth of *MET*-amplified and therapy resistant tumours in xenograft models (Dulak et al., 2011; Engelman et al., 2007; Xu et al., 2012; Zhang et

al., 2013). Some of these experiments have shown benefit for combinatorial inhibition of Met and EGFR in preclinical model systems, but clinical trials have consistently failed to show improvement in therapeutic outcomes when Met-directed therapies are applied to patients with EGFR-activating mutations in lung cancer. In some cases clinical trials have been closed early due to signs of increased mortality in the combination therapy arm compared to controls. It is from the few patients with clear objective responses in these clinical trials that *MET* gene amplification was identified as predictive of response to Met-directed therapies in a clinical setting (Gainor et al., 2016).

Our experiments show that in *MET*-amplified cell lines studied, heterotypic signaling between the Met and EGFR family primarily involves HER3 (Figures 2.2, 2.4 and 2.5). Intriguingly, in HCC827 cells with acquired resistance to EGFR inhibition through *MET* amplification, Met was shown to co-immunoprecipitate with HER3, and HER3 phosphorylation become dependent on Met (Engelman et al., 2007). Experimental approaches to improving EGFR-directed therapy by combinatorial targeting of EGFR, HER2 and HER3 also resulted in acquired resistance through elevated Met activity, and the induction of a Met-HER3 interaction was shown in this context by co-immunoprecipitation (Mancini et al., 2015). Conversely, inhibition of Met signaling in cells with *de novo MET* amplification increased expression of both HER3 and its ligand NRG1, suggesting that HER3 may play a role in adaptation to Met inhibition as well (Lai et al., 2014). Moreover, co-amplification or copy number gain of *ERBB3* along with amplification of *EGFR* has been observed in patients with Met exon 14 mutations who have acquired resistance to Met-directed TKIs (Recondo et al., 2020a). Our work provides additional context to these studies and suggests that the Met-HER3 axis is under strong positive selection in the context of Met-

dependent transformation. The recurrent induction of Met-HER3 complexes in cells escaping EGFR inhibition further indicates that HER3 is a critical component of oncogenic Met signaling and is required to replace the EGFR-induced signal.

3.3 HER3 promotes cancer cell proliferation through novel non-canonical signaling

A key question that we address in this thesis is the contribution of HER3 to Met-dependent signaling in the context of oncogenic transformation. Met and HER3 both activate the Akt pathway and Met generally activates signaling pathways that overlap with EGFR, such as the Ras-MAPK pathway, through the Grb2, SHC1 and Crk adaptor proteins and the phosphatase SHP2 (Furcht et al., 2015; Johnson et al., 2013). Nonetheless, the integration of HER3 into Met-dependent signaling could increase activation of shared pathways or contribute to the activation of unknown downstream effectors. Our hypothesis in investigating heterotypic signaling between Met and the EGFR family of RTKs was to identify how crosstalk expands signal output and cancer cell proliferation over invasive growth, a predominant Met-dependent phenotype.

The PI3K-Akt, Ras-MAPK and STAT3 signaling pathways are known to contribute to oncogenic transformation downstream of Met in *MET*-amplified gastric cancer cells (Lai et al., 2014). Ras-MAPK and PI3K-Akt signaling are also required for Met-dependent transformation in fibroblasts, and for 3D invasion in cell culture resulting in tubulogenesis (Schaeper et al., 2000). The phosphorylation of the Akt, Erk and STAT3 molecules are not altered by HER3 depletion, supporting the conclusion that potentially novel, non-canonical mechanisms of HER3-dependent signaling are required for oncogenic proliferative signals downstream of a Met/HER3 signaling axis (Figure 2.9). Our RNA sequencing data identified genes that had not previously been reported

to be regulated in a HER3-dependent manner and whose RNA level was decreased following HER3 knockdown. One of these, MPZL3, which decreased in expression following HER3 knockdown in multiple *MET*-amplified cell lines, partially rescued the consequent loss of HER3-dependent proliferation upon its overexpression (Figure 2.11 and 2.12). We demonstrated that MPZL3 was a HER3 binding protein and that Met activity regulates the MPZL3-HER3 interaction in *MET*-amplified cells (Figure 2.14). While MPZL3 was not known to associate with oncogenic signaling downstream of HER3 or the other EGFR-family RTKs, the MPZL3 homologue MPZL1 has been reported to contribute to HER2-dependent proliferation in breast cancer and modulate the adaptation to the HER2-targeted antibody trastuzumab (Lapin et al., 2014). MPZL1 can associate with Grb2 and the tyrosine phosphatase SHP2 in HER2-amplified breast cancer cells (Beigbeder et al., 2017). While the role of MPZL1 in cancer has primarily been associated with migration and invasion, it has been shown to promote proliferation in ovarian cancer as well through Src activation (Chen et al., 2019a). Thus, accumulating evidence implicates MPZL1 in cancer, and we show that its paralogue MPZL3 is implicated in oncogenic signaling downstream of Met and HER3 (Figure 3.1A). Moreover, the association between HER3 and MPZL3 may contribute to proliferation in other contexts that *MET*-amplified cancer (Figure 2.15).

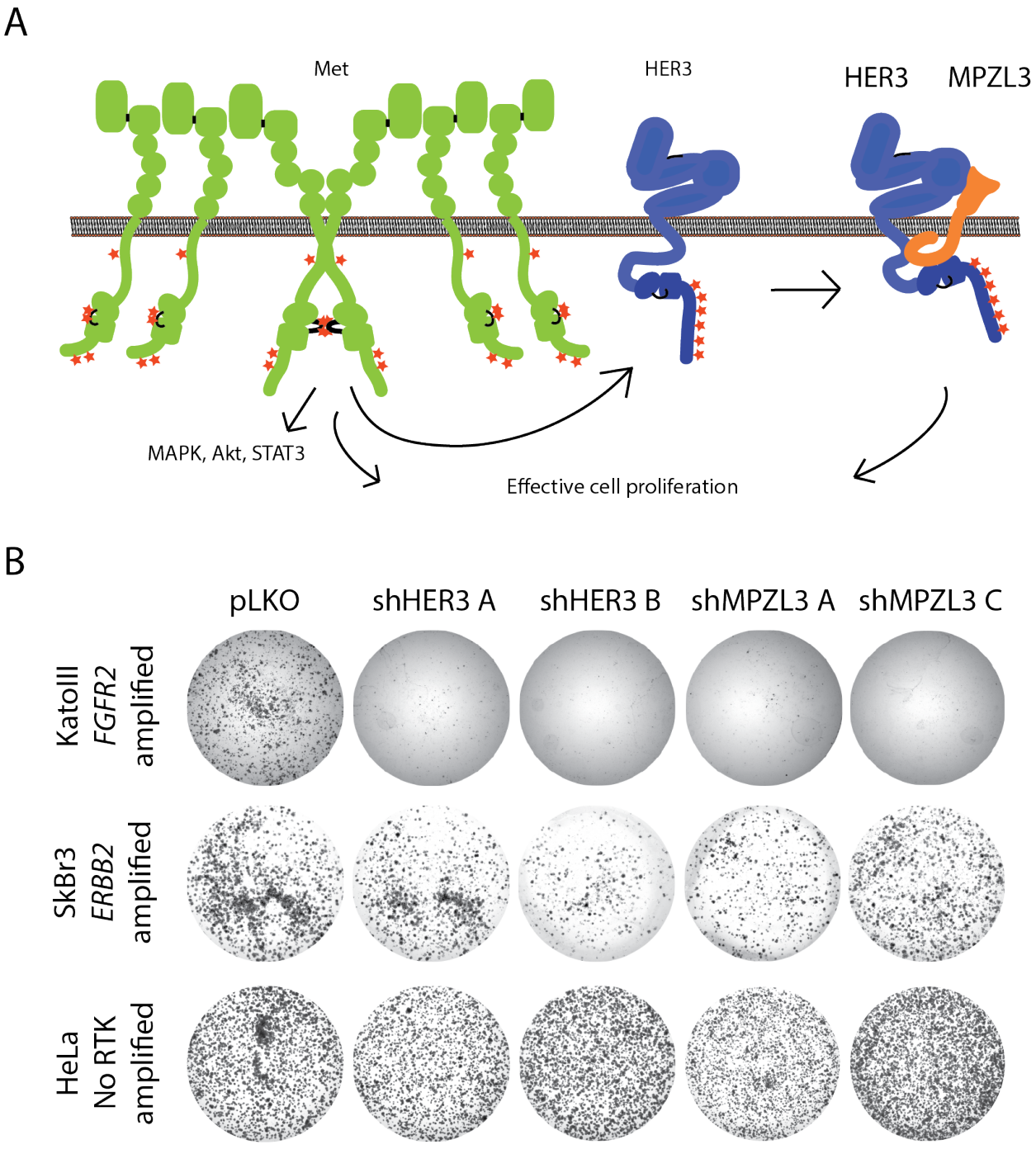


Figure 3.1: Non-canonical HER3-dependent signaling through MPZL3 promotes proliferation. *(A) Canonical proliferative signaling pathways downstream of Met are not impacted by HER3 depletion, but proliferative function is impaired due to the loss of MPZL3 levels and the HER3-MPZL3 complex. (B) HER3 and MPZL3 are required for colony formation in* FGFR2- *and* ERBB2-*amplified cell lines (n=3).* FGFR2-*amplified KatoIII cells and* ERBB2-*amplified SkBr3 cells form fewer colonies upon HER3 or MPZL3 depletion by shRNA than control cells (pLKO). By contrast, HeLa cells, in which no RTK amplification is observed, colony formation is unaffected by HER3 or MPZL3 depletion.*

3.4 A role for MPZL3 in promoting cancer cell proliferation

MPZL3 depletion phenocopies the loss of colony formation upon HER3 depletion in KatoII *MET*-amplified cells, and overexpression of MPZL3 attenuates the loss of proliferation upon HER3 depletion in EBC1 cells (Figure 2.12). To our knowledge, this is the first report of a role for MPZL3 in cancer cell proliferation or other cancer-associated phenotypes. MPZL3 is primarily understood through its role in differentiation in the epidermis (Barsam et al., 2015; Cao et al., 2007; Leiva et al., 2014; Palanza et al., 2017; Wikramanayake et al., 2017). MPZL3 knockout mice exhibit epidermal hyperplasia of immature progenitor cells within sebaceous cysts. MPZL3 is expressed during the process of epidermal differentiation and has been suggested to play a role as a tumour suppressor in cutaneous squamous carcinoma in connection to this function (Bhaduri et al., 2015).

Our observation of high MPZL3 expression in *EGFR*- and *ERBB2*-amplified cell lines in the CCLE suggests that these RTKs may share a requirement for HER3 in order to sustain MPZL3 levels (Figure 2.15). Therefore, the identification of Met-dependent mechanisms of resistance to EGFR inhibition may in fact reflect a central dependence on the ability of Met to maintain MPZL3 levels through HER3. Based on our observations of higher MPZL3 expression in the *ERBB2*-amplified SkBr3 breast cancer cell line as well as the *FGFR2*-amplified KatoIII gastric cancer cell line in the CCLE dataset, we tested the ability of these cells to form colonies upon depletion of either HER3 or MPZL3 (Figure 3.1B). We observed decreased colony formation in both cell lines for both HER3- and MPZL3-depleted cells, while the non-RTK-amplified HeLa cell line showed no difference in colony formation under these conditions. Thus, the contribution of HER3 to HER2- or FGFR2-dependent oncogenic signaling may also reflect its interaction with MPZL3 or its maintenance of

MPZL3 mRNA levels, further indicating that MPZL3 may play a central role in explaining the importance of HER3 downstream of multiple RTKs.

3.5 The biology of the HER3-MPZL3 axis is consistent with an intracellular mechanism of action

Met and HER3 are reported to co-localize within intracellular compartments of the secretory pathway in MCC97-H cells with acquired resistance to EGFR inhibition (Frazier et al., 2019). This also occurs in Cos7 cells in which Met and HER3 are overexpressed, although co-overexpression of HER2 and HER3 results in phosphorylated HER3 accumulating at the plasma membrane instead of the Golgi. Met and HER3 co-immunoprecipitate under these conditions. Mutations in the HER3 N- and C-lobes are dispensable for Met-dependent HER3 phosphorylation in these cells, and phospho-HER3 accumulates in the Golgi instead of trafficking to the plasma membrane (Frazier et al., 2019). This reflects our observations in HEK293T cells with co-overexpression of Met and HER3, where N- and C-lobe HER3 mutants exhibit Met-dependent tyrosine phosphorylation (Figure 2.4). Our MPZL3-V5 overexpression construct does not traffic to the plasma membrane but instead accumulates in intracellular compartments (Figure 2.13). Intriguingly, curation efforts by the Human Protein Atlas Project have shown that MPZL3 can localize to the endoplasmic reticulum and Golgi, while biotin-labeling of MPZL3 interactors using an MPZL3-BirA fusion protein revealed enrichment for mitochondrial proteins, which traffic through the endoplasmic reticulum as well (Bhaduri et al., 2015). Thus, the Met-dependent phosphorylation of HER3 may similarly occur in the endoplasmic reticulum or Golgi, promoting HER3 retention within the secretory pathway and thereby its association with MPZL3. The induction of Met-HER3 complexes and Met-dependent HER3 phosphorylation has been reported

in cells treated with antibodies directed against EGFR, HER2 and HER3 as an adaptation mechanism (Mancini et al., 2015). Our results and the findings of a Met-HER3 interaction within the secretory pathway suggest that this may be a mechanism of protecting HER3 from antibody-induced degradation by preventing its trafficking to the cell surface while reactivating HER3-dependent oncogenic signaling from intracellular compartments where it can bind MPZL3.

3.6 HGF-dependent internalization of Met promotes early tumour outgrowth and actin reorganization in MET*-amplified cells*

Comparison of the transforming and proliferative activity of human Met in response to human versus mouse HGF in fibroblasts or hepatocytes has demonstrated that human HGF is required to fully activate the human Met receptor (Rong et al., 1992). We were able to replicate previous reports of accelerated tumour growth of *MET*-amplified cancer cells in mice humanized for HGF (Pennacchietti et al., 2014; Zhang et al., 2005). Surprisingly, this did not correlate with an increase in Met tyrosine phosphorylation or the phosphorylation of the major oncogenic signaling effectors downstream of Met in cell culture. Thus, the activity of HGF in promoting rapid tumour outgrowth may proceed through mechanisms that do not involve increased kinase activity, or at least not as detectable by levels of tyrosine phosphorylation. This is consistent with a model in which the *MET* gene amplification maximally activates the kinase and suggests that any effect HGF has on Met may be a function of redistributing the active receptor within the plasma membrane or the endosomal pathway. Previous work in our group has shown that subcellular relocalization of activated Met complexes to dorsal ruffles regulates Met trafficking and degradation (Abella et al., 2010a). The induction of phosphatidyl-inositol-4, 5-bisphosphate recruits the Met-phosphorylated Gab1-cortactin complex to initiate invadopodia at specific

locations in the plasma membrane in another example of HGF-dependent relocalization of Met-dependent signaling (Rajadurai et al., 2012). Sustained signaling through the Ras-MAPK pathways in response to HGF requires Met signaling from endosomal complexes (Maroun et al., 2000; Parachoniak et al., 2011). Dynamic regulation of the small GTPase Arf6 across different subcellular compartments is also critical to cell migration in response to HGF (Zaoui et al., 2019a; Zaoui et al., 2019b). Thus, we hypothesized that HGF may regulate signaling downstream of Met by altering Met trafficking in *MET*-amplified cells.

In order to analyze Met trafficking upon HGF stimulation, we treated *MET*-amplified H1993 cells with fluorescently tagged HGF. Cells were fixed after stimulation and Met localization was determined by indirect immunofluorescence (Figure 3.2A). A subset of Met was internalized upon HGF stimulation, while the majority of the receptor remained at the plasma membrane. Met and HGF accumulate at punctate intracellular compartments resembling endosomes within 15 minutes and continue to accumulate in these structures up to 4 hours after stimulation. Met and HGF remain colocalized in endosomal compartments, indicating that the receptor and ligand do not dissociate as is observed in GGA3-dependent recycling of a non-amplified Met to the plasma membrane (Parachoniak et al., 2011). Thus, despite no detectable increase in Met phosphorylation, stimulation with HGF does induce Met internalization in *MET*-amplified cells.

We measured actin polymerization using fluorescent phalloidin, a dye that incorporates into filamentous actin. HGF stimulation induced actin polymerization along the outer edge of each cell colony (Figure 3.2A). We also observed the induction of circular actin-enriched structures consistent with the formation of dorsal ruffles as observed in response to HGF in other cell lines

(Abella et al., 2010a). Extensive remodeling of the cortical actin cytoskeleton is also seen in the induction of invadopodia downstream of Met (Rajadurai et al., 2012). Actin polymerization peaked at 1-hour post-treatment and decreased after this point, indicating that HGF stimulation in cell culture induces a transient signal. In mice expressing humanized HGF, the induction of HGF-dependent actin reorganization would presumably occur on a continuous basis. We performed HGF stimulation in HER3-depleted H1993 and Mkn45 *MET*-amplified cells but did not observe a difference in Met internalization or actin polymerization by fluorescence microscopy under these conditons (data not shown). Thus, the rapid outgrowth of *MET*-amplified cells grown in the presence of humanized HGF may be dependent on actin remodeling that accompanies Met internalization but is not regulated by HER3-dependent signaling.

We further assessed HGF-dependent Met internalization biochemically (Figure 3.2B). Cell surface proteins were biotinylated using thiocleavable biotin before stimulation with HGF. Cell surface biotin was then stripped on ice before lysis over a time course following HGF treatment. Biotinylated proteins, protected from stripping by internalization during chase, were isolated by precipitation on a streptavidin resin. Untreated cells and cells treated for the full 1-hour time course were also left unstripped as labeling controls (first lane and last two lanes). Met internalized basally in unstimulated cells, but HGF treatment notably increased internalized Met up to 15 minutes after stimulation. Thus, both biochemical and cytochemical approaches confirm that HGF promotes Met internalization.

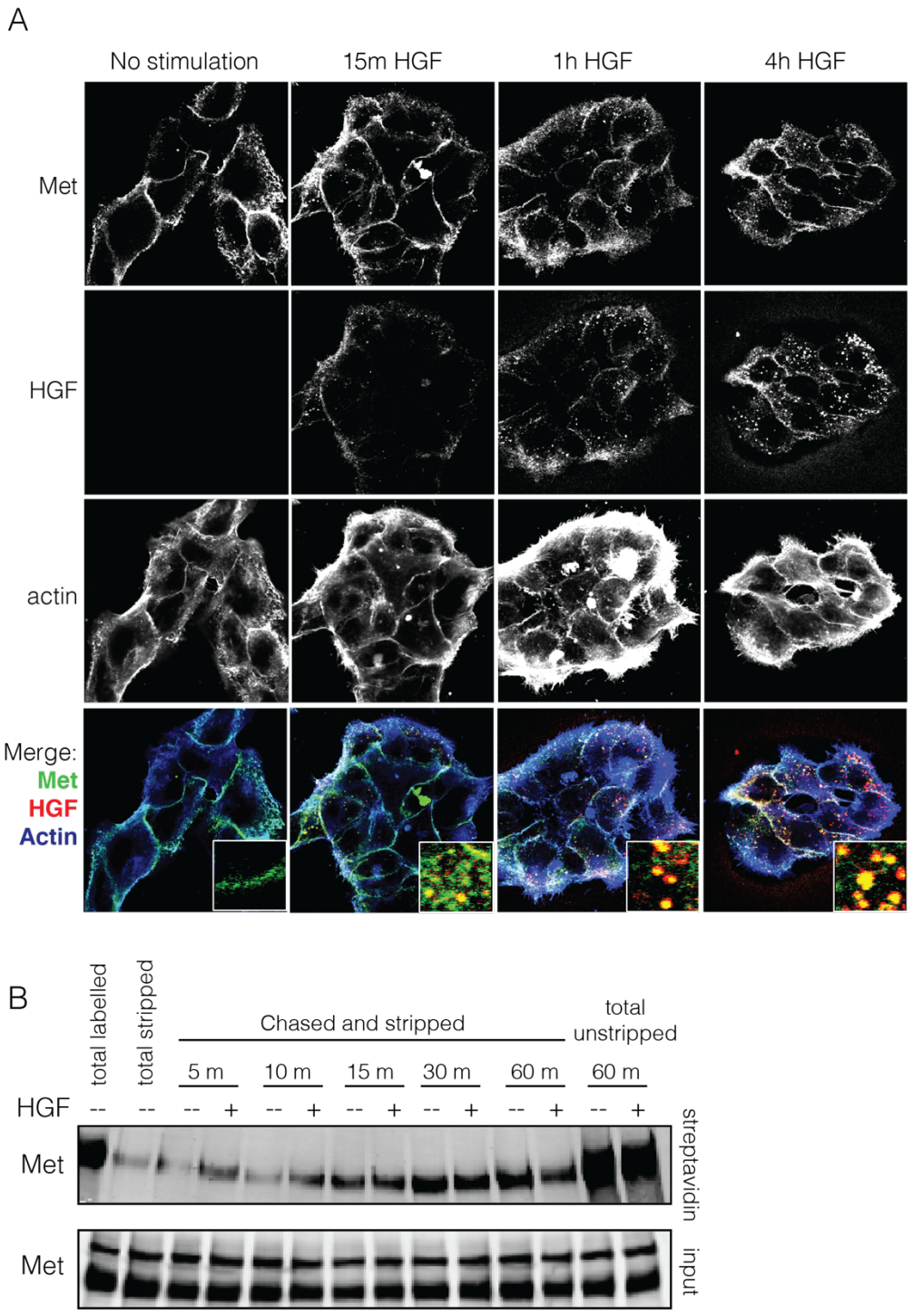


Figure 3.2: HGF increases Met internalization in* MET*-amplified cells. *(A)* MET*-amplified H1993 cells were stimulated with fluorescent HGF followed for the indicated times at 37°C (n=2). Met localization and actin polymerization were determined in after fixation by indirect immunofluorescence and fluorescent phalloidin, respectively. (B) H1993 cells were surface-labeled with cleavable biotin, stimulated with HGF, and stripped of surface biotin after chase (n=2). Internalized proteins was enriched by streptavidin resin and probed for total Met. Cells lysed before stripping (without HGF treatment, first lane; with 60m treatment or control, last two lanes) show total labeled Met, while cells stripped immediately (second lane) show stripping efficiency.*

The increased actin polymerization we observe upon HGF stimulation suggests that the acceleration of tumour outgrowth in HGF-expressing mice may be due to signaling impinging on the cytoskeleton instead of canonical proliferative signaling pathways. This further supports the possibility that the induction of Met-dependent signaling through adhesion-related pathways contributes to the proliferative signal output observed in *MET*-amplified cells.

3.7 Met Leu1157Ala selectively undergoes autophosphorylation **in situ** *using N6-modified ATPγS*

In order to model signaling downstream of the amplified receptor in a simplified setting, we developed mutants of the Met receptor capable of selectively utilizing N6-modified ATP analogues with a gamma-thiophosphate group in order to conduct substrate-labeling experiments (Figures 2.20 and 2.21). Our goal in this was to validate whether HER3 is in fact a direct substrate of Met phosphorylation in the constitutively activated setting. We overexpressed wild-type Met or the Met Leu1157Ala mutant in HEK293T cells to constitutively activate their kinase activity as in Figure 2.20, in which both constructs are autophosphorylated and promote the tyrosine phosphorylation of other proteins (Figure 2.20C).

ATPγS is a charged molecule and does not cross the plasma membrane on its own. We permeabilized cells with the detergent digitonin in order to facilitate the use of ATPγS or N6-modified analogues of ATPγS *in situ* in cells. We optimized kinase buffer conditions to ensure that signal detected by the anti-thiophosphoester detection antibody, 51-8, could specifically recognize thiophosphorylated proteins in cell lysates (Figure 3.3A).

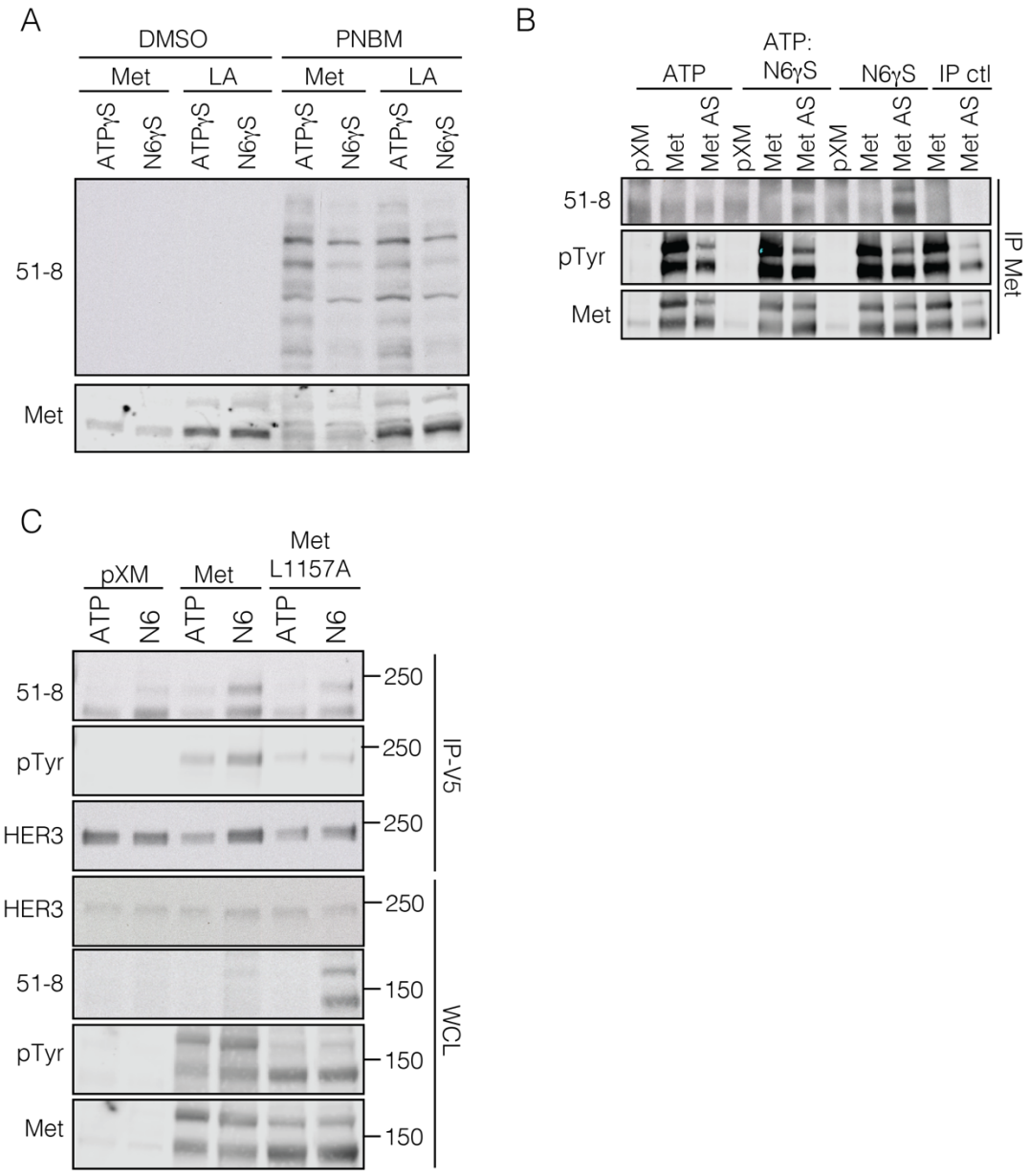


***Figure 3.3: Met Leu1157Ala selectively utilizes N6-modified ATP analogues* in situ.** *(A) Cells were permeabilized using digitonin and treated with kinase reaction buffer containing a mix of ATP and either ATPγS or N6-phenethyl-ATPγS (N6γS) (n=1). To test thioantibody selectivity we left some lysates unalkylated as in Figure 2.21C (DMSO) and alkylated others with p-nitrobenzyl mesylate (PNBM (n=1)). Lysates were then probed with anti-thiophosphoester antibody (51-8). We observed some background labeling in cells treated with N6γS. (B) We titrated N6γS against ATP in kinase buffer to optimize conditions for selective autothiophosphorylation of Met Leu1157Ala* in situ*. Immunoprecipitated Met was selectively autothiophosphorylated in samples containing N6γS, and by excluding ATP we could maximize this signal. (C) We conducted in-cell labeling in HEK293T cells expressing a HER3-V5 construct (n=3). While Met autophosphorylation was selectively observed in Met Leu1157Ala-expressing cells treated with N6γS, background thiophosphorylation of HER3, or some other background signal detected by 51-8, was observed upon immunoprecipitation using the V5 tag (IP-V5).*

We observed induction of thiophosphorylated proteins in conditions with both wild-type and Leu1157Ala Met variants whether we used ATPγS or N6-phenethyl-ATPγS (N6γS). By titrating

N6γS against ATP and other nucleotides in in-cell kinase reaction buffer we identified conditions favourable for its selective use by Met Leu1157Ala over wild-type Met (Figure 3.3B). Selective incorporation of the thiophosphate moiety into Met Leu1157Ala occurred in kinase buffer supplemented exclusively with N6γS in place of ATP. We overexpressed the Met variants in HEK293T cells expressing V5-tagged HER3 and conducted in-cell substrate labeling, but background labeling of HER3 was unexpectedly observed in the absence of Met overexpression (Figure 3.3C). Thus, we were unable to determine whether or not HER3 is a direct substrate of Met using this approach.

Chemical capture using thioreactive columns has been developed as an alternative method of identifying thiophosphorylated proteins (Hertz et al., 2010; Schaffer et al., 2015). While we were unable to demonstrate direct Met-dependent phosphorylation of HER3, the Met analogue-sensitive labeling mutants we have developed permit future analysis of candidate direct substrates of Met by this approach. Another important use of analogue-sensitive kinases involves the use of modified TKIs based on the structure of PP1, a Src inhibitor, to selectively inhibit analogue-sensitive kinase mutants while having minimal impact on the activity of non-mutated cellular kinases (Kraybill et al., 2002). This permits more specific inhibition of the mutated kinase than can be normally achieved using ATP-competitive TKIs, which have significant off-target binding (Davis et al., 2011). In order to utilize the Met Leu1157Ala mutant in this manner, we have begun optimization of TKI analogues for selective activity against Met Leu1157Ala. The ability to discriminate Met-dependent phosphosites from those affected by off-target kinases will be useful in validating candidate Met-dependent phosphoproteins such as those identified by our phosphoproteomic profiling.

3.8 Signal rewiring in MET*-amplified cancer cells*

A goal of this thesis was to establish the molecular determinants of proliferative signaling in Met-dependent cancers and explain how *MET* amplification promotes a proliferative output over cell dispersal (Figure 3.4). HGF-dependent activation of Met is primarily associated with the induction of cell migration and an increase in invasive potential in a majority of HGF-responsive cell lines (Stoker et al., 1987; Weidner et al., 1990). A proliferative response to HGF-dependent Met activation is observed in the placenta during development and in hepatocytes *in vitro* and *in vivo*, indicating that in specific contexts Met activation does in fact promote proliferation (Borowiak et al., 2004; Nakamura et al., 1986). Nonetheless, the low rate of objective response to Met-directed therapy in cancer indicates that this is not the major outcome of Met expression in most cancers (Noonan et al., 2016). In contrast to the migration observed upon HGF stimulation in *MET*-normal cells, the induction of Met activation by washing out the Met-directed TKI PHA-665752 induces cell rounding but does not activate migration in 2 out of 3 adherent *MET*-amplified cell lines tested (data not shown). As 4 of the 7 *MET*-amplified cell lines in our panel grow in suspension, these cells do not exhibit Met-dependent migration in cell culture. KatoII cells adhere to plastic and lose cell rounding in the presence of Met inhibitors such as PHA or crizotinib (data not shown).

As gene amplification has been observed to induce constitutive Met activation and Met-dependent proliferation *in vitro* and is associated with objective response to Met inhibition in the clinic, we used *MET*-amplified cells as a model system to study how proliferative signaling is

activated downstream of Met in an oncogenic context (Figure 3.4A). *MET*-amplified cells maintain high levels of protein compared to non-amplified cell lines and constitutive phosphorylation of activation loop tyrosines (Tyr1234/35), thus exhibiting high levels of kinase activity (Figure 2.1). We hypothesized that a switch to proliferative signaling could involve the inclusion of novel additional signaling partners that are not activated in HGF-dependent contexts where cell migration may predominate (Figure 3.4B). Our observation of conserved Met-dependent tyrosine phosphorylation of HER3 in *MET*-amplified cells contrasts with the lack of HER3 expression and phosphorylation in non-*MET*-amplified cells stimulated with HGF (Figure 2.2 and 2.22D). Our data support the conclusion that HER3 contributes to proliferative signaling downstream from Met, but the ability of HER3-depleted tumours and cell cultures to adapt and eventually proliferate indicates that additional mechanisms downstream of Met may be sufficient to enable cell proliferation in the absence of signaling through HER3.

Additional work in our group has identified a key role for crosstalk with the RTK FGFR1 and the large multisite docking protein FRS2, a homologue of Gab1, in promoting tumour initiation in mouse mammary tumours with *MET* amplification (Sung, Duhamel *et al.*, under review). We probed for FRS2 phosphorylation in our panel of *MET*-amplified cell lines and observed Met-dependent phosphorylation of FRS2 in KatoII, Snu5, Okajima and Mkn45 cells, indicating that the induction of Met-FRS2 signaling is a common event in the *MET*-amplified setting (Figure 3.5).

We also observed Met-dependent tyrosine phosphorylation of the adaptor proteins SHB and GAREM, and the large multisite docking protein IRS2, in *MET*-amplified KatoII cells. GAREM is a tyrosine-phosphorylated adaptor protein that interacts with Grb2 and has been shown to promote EGFR-dependent proliferation through MAPK pathway activation (Tashiro et al., 2009).

SHB is a homologue of the Met- and EGFR-interacting SHC adaptor, and is recruited to a complex with IRS1 and IRS2 to promote proliferation in pancreatic β-cells (Annerén and Welsh, 2002; Welsh et al., 2002).

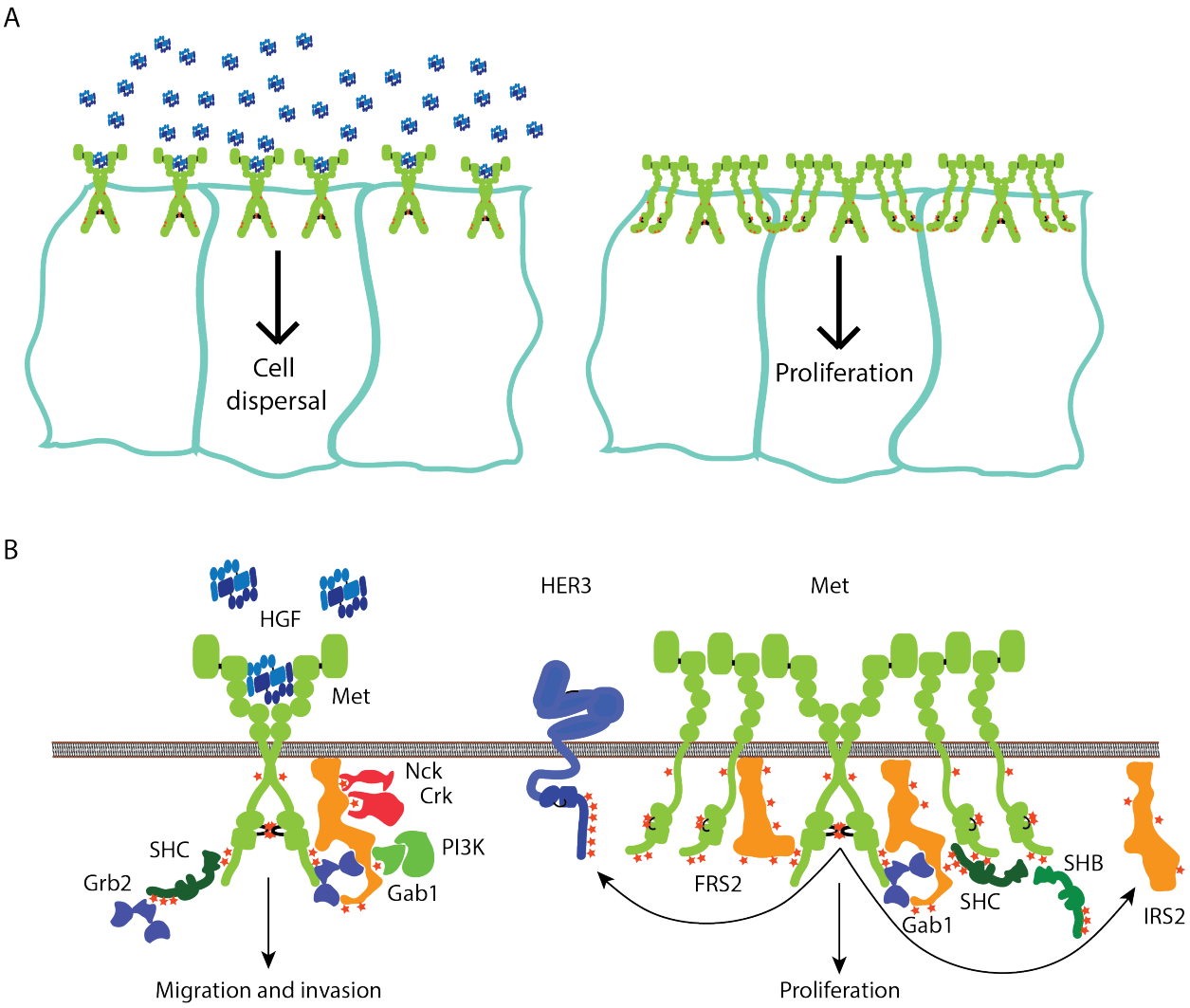


Figure 3.4: Met signal rewiring upon* MET *amplification. *(A) Unlike the cell dispersal or migration observed in most epithelial cells stimulated with HGF,* MET*-amplified cells preferentially exhibit a proliferative response to Met signaling but do not show an increase in cell migration on 2D substrates. (B) We hypothesize that the induction of cell proliferation as an outcome of Met signaling in the amplified context is due to the induction of additional phosphorylation not observed in most HGF-dependent contexts. The phosphorylation of these additional substrates would recruit additional downstream effectors required for Met-dependent proliferation. We show evidence of Met-dependent HER3 phosphorylation, and that HER3 promotes proliferation in* MET*-amplified cells. We further show Met-dependent phosphorylation of the IRS2 large multisite docking protein and the SHB*

and GAREM adaptor proteins, which are not known to be phosphorylated in HGF-dependent contexts. Recent work in our group has also shown that FRS2 is phosphorylated in MET*-amplified cells (Sung, 2019)*

IRS2 is canonically associated with signaling downstream of the insulin and insulin-like growth factor RTKs (reviewed in Copps and White, 2012; Peng and He, 2018). Phosphorylation of IRS2 has been shown to promote proliferation and cell survival downstream of a variety of kinases in the pancreas as well in several cancers, but to our knowledge has not been reported to be phosphorylated in a Met-dependent manner (Emdal et al., 2018; Gui et al., 2013; Jeong et al., 2018; Mohanty et al., 2005; Stamateris et al., 2016; Szabolcs et al., 2009). Thus, we have identified a number of novel proliferation-related proteins usually associated with RTK signaling whose phosphorylation by Met may underlie rewiring toward a proliferative signal in the amplified setting.

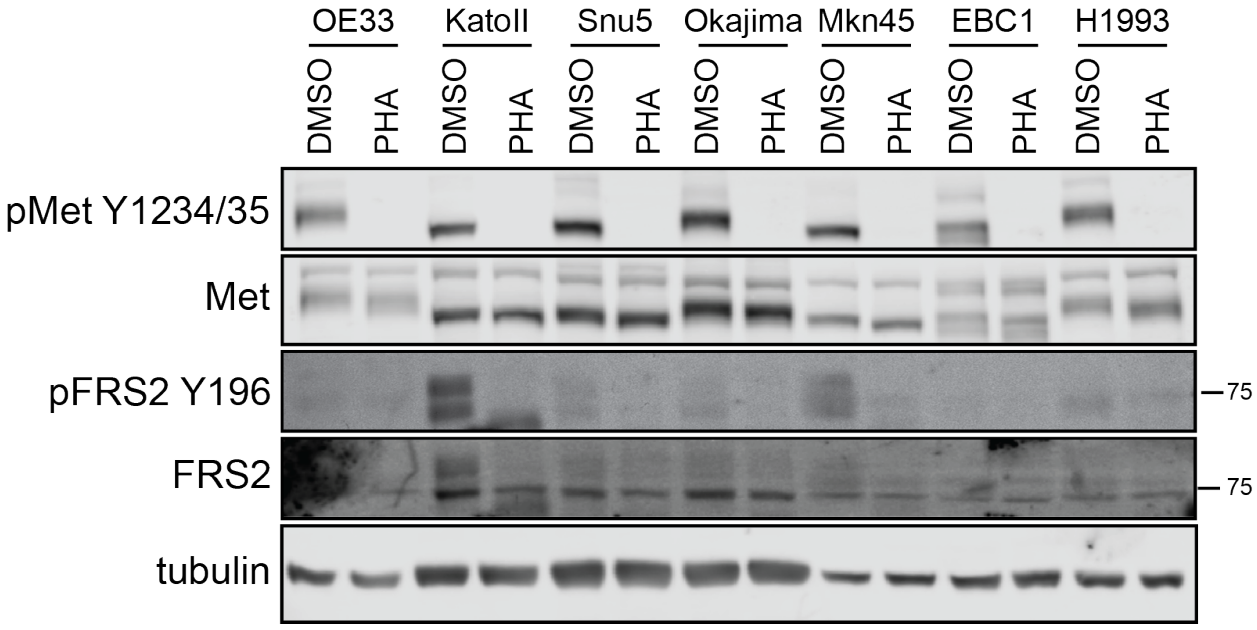


Figure 3.5: FRS2 is phosphorylated in a Met-dependent manner in* MET*-amplified cell lines*. We probed lysates from our panel of* MET*-amplified cell lines for FRS2 phosphorylation and total protein levels in the presence and absence of the Met TKI PHA-665752 (PHA) (n=2). Treatment with PHA reduced FRS2 phosphorylation at Tyr196 in KatoII, Snu5, Okajima and Mkn45 cells.*

3.9 Predicted and known adhesion receptors play central roles in MET*-amplified cells*

We observed a large number of adhesion-related genes among our list of Met-dependent phosphosites in KatoII cells and enrichment for atypical adhesion and junctional complexes (Figure 3.6). The genes driving enrichment of these pathways were desmosomal proteins including the desmocollin-2 (DSC2)-plakophilin-2 (PKP2)-plakoglobin (JUP)-desmoplakin (DSP) complex, which forms desmosomal structures involved in action potential propagation in the heart (Figure 2.18) (Lee and McGrath, 2020). To our knowledge, these proteins are not known to be tyrosinse-phosphorylated in response to Met activation. The desmosomal protein PKP3 is reported to be phosphorylated upon HGF stimulation, and the internalization of desmosomes is part of the classic cell-scattering response to HGF (Johnson et al., 2013; Royal and Park, 1995). Nonetheless, while HGF-dependent regulation of desmosomes is well characterized, phosphorylation of DSP and JUP in HGF-stimulated cells has not been observed (Li et al., 2001; Pasdar et al., 1997). Mutations in DSP and DSC2 have been shown to associate with heart arrhythmias in both human patients and genetically-engineered mouse models (Chen et al., 2019b; Gehmlich et al., 2011). Thus, the phosphorylation of a noncanonical desmosomal complex in the *MET*-amplified state implies the expansion of signaling towards adhesion complexes in comparison to the ligand-activated state.

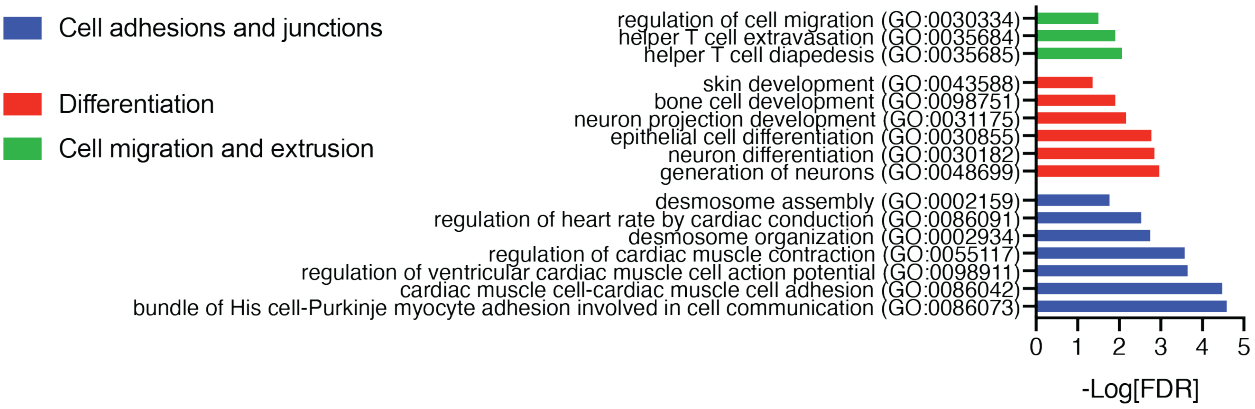


Figure 3.6: Met-dependent phosphosites enrich for atypical cell adhesion and junctional pathways. *Select highly-enriched pathways from our gene ontology analysis of genes containing Met-dependent phosphosites shows enrichment for atypical functions including T-cell extravasation by diapedesis and desmosomal structures involved in action potential propagation in the heart. Differentiation-related genes with Met-dependent phosphosites are mostly found in neuronal, bone or skin tissue-related processes.*

We also observed enrichment for pathways related to neuronal, skin and bone differentiation, and the cell migration-related pathways included several functions related to T-cell extravastion and diapedesis. Differentiation-related gene ontology was largely found within processes governing neuronal development. In both of these cases, pathway enrichment was driven by canonical Met-dependent substrates including the Crk, CrkL and Nck adaptor proteins, STAT3, and Met itself.

The activation of tissue-specific adhesion proteins downstream of amplified *MET* points to a continued and critical role for the regulation of cell junctions and adhesions while cells are in a proliferative state. Thus, our observations suggest that the induction of proliferation may not represent rewiring away from junctional signaling *per se*, but activation of additional Met-dependent signaling partners beyond its normal repertoire that enhance proliferation. The failure of a majority of *MET*-amplified cell lines to undergo Met-dependent migration and invasion may be due to an inability to dynamically regulate junctional signaling in the constitutively-activated state as opposed to the HGF-stimulated state in normal cells (Zaoui et

al., 2019a; Zaoui et al., 2019b). A key outstanding question is whether the activation of these atypical junctional and adhesion proteins impacts cell proliferation, as we have shown for MPZL3.

3.10 Conclusions

The Met RTK plays pleiotropic roles in mammalian biology in diverse tissues in normal development and physiology. In cancer, its role as a transforming oncogene is mostly associated with its ability to promote cell proliferation and thus tumour expansion. We show that this involves the induction of heterotypic signaling through HER3, but that HER3 expression promotes the stabilization of a novel binding partner, MPZL3, as opposed to maintaining the activity of proliferative signaling pathways downstream of Met. We identify several adaptors known to be involved in RTK signaling but previously unassociated with Met that are phosphorylated in a Met-dependent manner, pointing to additional rewiring of RTK signaling in the gene-amplified setting. These results explain how cells in which HER3 has been depleted are capable of eventually adapting to the loss of the HER3-MPZL3 axis. The induction of phosphorylation of several additional junctional complexes in the amplified setting shows that proliferative signaling occurs along within the context of an expansion of overall Met signaling, and not with a coordinated shift towards proliferation-related genes *per se.* Nonetheless, this may actually be important for the proliferation of *MET*-amplified cells, as exemplified by MPZL3, a predicted adhesion receptor with a proliferative role downstream of Met and HER3 in cancer. Our work suggests that MPZL3 may be involved in other RTK-dependent cancers, particularly those involving HER3 signaling.

Our work demonstrates that previously uncharacterized signaling pathways are required for efficient oncogenic proliferation downstream of Met. Thus, we can contribute potential

biomarkers for predicted Met or HER3-dependent proliferation. This work supports a central role for HER3 in *MET*-amplified cancers and provides rationale for further investigation of how HER3 contributes to oncogenesis in other RTK-dependent settings. HER3 is an important regulator of cancer-associated proliferation in cancers with EGFR- and HER2-dependent RTK activation, and we show that HER3 should be considered an important component of proliferative signaling downstream of Met as well. Increasing evidence suggests that combinatorial targeting of HER3 is critical for the potentiation of EGFR- and HER2-directed precision medicine, and our work adds to the evidence that Met-dependent resistance mechanisms may be suppressed by this as well.

CHAPTER 4: MATERIALS AND METHODS

4.1 Antibodies and reagents

Antibody 148 was raised in rabbit against a C-terminal peptide of human Met (Rodrigues et al., 1991). Antibodies against phosphorylated Met at $Tyr^{1234/1235}$, HER2 at $Tyr^{1221/1222}$, HER3 at Tyr^{1289}, Src at Tyr^{416}, Akt at Ser^{473}, Erk1/2 at Thr^{202}/Tyr^{204}, STAT3 at Tyr^{705}, and generic tyrosine peptide (pY100), as well as total EGFR, HER2, HER3, pan-Akt, Erk1/2, and STAT3 were purchased from Cell Signaling Technologies. For experiments in which HER3 deletion constructs were included, total HER3 levels were measured using the HER3-1B2E antibody (Cell Signaling), which binds an epitope in the pseudokinase domain. In all other cases, HER3-XP total antibody (Cell Signaling), raised against an epitope in the HER3 C-tail, was used. Antibody against phosphorylated EGFR at Tyr^{1173} were purchased from Santa Cruz Biotechnology. Antibodies against M2-FLAG peptide, actin and tubulin were purchased from Sigma. Antibodies against V5 peptide and the 51-8 anti-thiophosphoester antibody were purchased from Abcam, while antibodies against MPZL3 were purchased from ProteinTech. HRP-conjugated secondary antibody was purchased from Thermo Fisher, while IRDye infrared secondary antibodies were purchased from Mandel Scientific.

PHA-665752 was used at 500 nM unless otherwise indicated and was a gift from Pfizer. Gefitinib and lapatinib, used at 1 mM, and dasatinib, used at 100 nM, were gifted by Dr. W. Muller. Dimethyl sulfoxide (DMSO) was purchased from Sigma and used at a concentration of 1:1000 as a vehicle control. Crizotinib was purchased from LC Laboratories. HGF was a generous gift from Genentech. ATP, GTP, p-nitrobenzyl mesylate (PNBM), ATPγS and N6-modified ATPγS analogues were purchased from Sigma.

4.2 Cell cultures, transfections and lentiviral transduction

Malme-3M, KatoIII, HCC70 and HCC1395 cells were obtained through ATCC. Malme-3M and KatoIII cells were cultured in Iscove's Modified Dulbecco's Medium supplemented with 20% FBS, as suggested. HCC70 and HCC1395 cells were cultured in RPMI supplemented with 10% FBS, as suggested. OE33 cells were described previously (Grandal et al., 2017) and cultured in RPMI supplemented with 10% FBS and 2 mM L-glutamine (Thermo Fisher). HeLa, Snu5, KatoII, Okajima and MKN45 cells were cultured as described previously (Abella et al., 2010a; Lai et al., 2014; Lai et al., 2012). EBC1 were obtained through RIKEN BRC Cell Bank and H1993 cells were obtained from ATCC; these were cultured in MEM alpha or RPMI media, respectively, supplemented with 10% FBS (Thermo Fisher). SkBr3 cells were gifted by Dr. William Muller and were cultured in McCoy's 5A media supplemented with 10% FBS (Themo Fisher). The PDX GCRC1994 cell line was generated by Matthew Dankner, a student of Dr. Peter Siegel, by dissociation of an excised PDX grown subcutaneously and serial culture under fibroblast-depleting conditions. PDX GCRC1994 cells were cultured in DMEM supplemented with 10% FBS (Thermo Fisher). HEK293 and HEK293T cells were cultured in DMEM supplemented with 10% FBS (Thermo Fisher).

Transfections of HEK293 cells for immunoprecipitation experiments was performed using polyethylenimine (Longo et al., 2013). Transfections of HEK293T cells for immunoprecipitation, transphosphorylation assays, kinase assays or viral production were performed by the calcium phosphate method. Lentiviral cultures for shRNA or transgene overexpression were collected as described at https://portals.broadinstitute.org/gpp/public/resources/protocols. Lentiviral transduction was followed by selection in puromycin (1-2 ug/ml) or blasticidin (2-10 ug/ml) for 3-14 days, and control cells were verified for negative selection prior to the start of each

experiment. For siRNA treatments, EBC1 cells were transfected using the HiPerfect reagent (Qiagen).

4.3 Gene expression constructs and RNAi

Met and EGFR were expressed from the pXM and pXER backbones, respectively, under control of the adeno-major late promoter and in the presence of an SV40 origin of replication for episomal amplification in HEK293T cells (Countaway et al., 1989; Rodrigues et al., 1991). Met Δexon 14, tyrosine-to-phenylalanine and asparagine-to-histidine nonbinding mutants, and lysine 1110 alanine kinase-impaired mutants have been described previously (Fixman et al., 1995; Kamikura et al., 1996; Rodrigues and Park, 1994; Zhu et al., 1994). Met Leu1157Ala and Leu1157Gly substitutions were generated using the QuikChange site-directed mutagenesis kit (Agilent) (primer sequences in Table 4.1).

EBC1 cells were transfected in suspension using pooled siRNA at 50 nM and immediately seeded for proliferation and lysis, which was conducted after 72 hours. Allstars control siRNA was purchased from Qiagen. siGENOME SMARTpool pooled siRNA duplexes targeting *ERBB3* were purchased from Dharmacon (sequences in Table 4.2). Viral vectors for shRNA expression as well as empty-cassette plasmids used as vector controls were obtained from the Mission TRC library (Sigma) via the McGill Cellular Perturbation Services at McGill University (clone information in Table 4.3). Gateway entry vector for MPZL3, Gateway destination vectors pLX303, pLX304, and pLEX307 for mammalian cDNA overexpression, and pLX317 vector for GFP-V5 overexpression were obtained through the Mission TRC3 Orfeome collection via the McGill Cellular Perturbation Services. Gateway entry vector for ERBB3 was obtained through Addgene (pDONR223-ERBB3,

#23874) (Johannesen *et al.*, 2010). The Q5 site-directed mutagenesis kit (Qiagen) was used to generate Ile682Asn and Val924Arg point mutations and the short-tail and no-tail deletions in the HER3 entry vector prior to recombination (primer sequences in Table 4.1). These constructs were used to generate lentivirus for cell transduction. Split intein mediated protein ligation (SIMPL) Gateway destination vectors were generously gifted by the Stagljar laboratory at the University of Toronto. Recombined MPZL3- and HER3-SIMPL vectors were transfected for the SIMPL assay. Vector recombination was performed using the Gateway LR Clonase II enzyme kit (Thermo Fisher). Cys51Ala and Arg99Gln mutations were introduced using single-strand reactions followed by annealing and DpnI digestion (primer sequences in Table 4.1) (Edelheit et al., 2009).

4.4 Immunoprecipitation and Western blotting

Cells and were harvested for protein lysis in a Triton-glycerol-HEPES (TGH) buffer (50 mM HEPES, 150 mM sodium chloride, 15 mM magnesium chloride, 2 mM EGTA, 1% v/v Triton X-100 (Sigma), 10% v/v glycerol). Lysates were frozen, thawed and centrifuged at full speed in an Eppendorf microcentrifuge for 15 minutes and protein content was assessed by Bradford assay (Biorad). Cell lysate equivalent to 1 mg protein collected 36 hours post-transfection was diluted to 500 ml in TGH buffer for each immunoprecipitation reaction and precleared with 10 ul sepharose-protein G beads (GE Healthcare). Immunoprecipitation was performed overnight by incubation using antibody 148 against Met, anti-EGFR, HER2 or HER3 total antibody or anti-V5 antibody, followed by capture using sepharose-protein G beads. For nonspecific controls, rabbit or mouse IgG (Sigma) were used. Beads were washed three times in lysis buffer prior to analysis.

Table 4.1: Primers used for site-directed mutagenesis

Construct	Primers	
Met Leu1157Ala	forward	GCGAAGTGAAGGGTCTCCGCTGGTGGTCGCACCATACATGAAACATG GAGATCTTCG
	reverse	CGAAGATCTCCATGTTTCATGTATGGTGCGACCACCAGCGGAGACCCT TCACTTCGC
Met Leu1157Gly	forward	GCGAAGTGAAGGGTCTCCGCTGGTGGTCGGACCATACATGAAACATG GAGATCTTCG
	reverse	CGAAGATCTCCATGTTTCATGTATGGTCCGACCACCAGCGGAGACCCTT CACTTCGC
HER3 Ile682Gln	forward	CTTGGCCAGACAATTCAAAGAGACAGAGC
	reverse	ACTTTGTTAGCCTTCTCAC
HER3 Val924Arg	forward	CATGGTGATGCGCAAGTGTTGGATG
	reverse	TAGACATCAATTGTGCAGATC
HER3-short tail (Thr1141*)	forward	CCAACTTTCTTGTACAAAGTTGG
	reverse	AGTCAGCAGACTGTGGCG
HER3-no tail (Phe965*)	forward	CCAACTTTCTTGTACAAAGTTGG
	reverse	GAACTCATTGGCTAGTTCTTTAAAG
MPZL3 Cys51Ala	forward	GGTTATGTTGGAGAAAAGATCAAGTTGAAAGCCACTTTCAAGTCAACT TCAGATGTCAC
	reverse	GTGACATCTGAAGTTGACTTGAAAGTGGCTTTCAACTTGATCTTTTCTC CAACATAACC
MPZL3 Arg99Gln	forward	CCACAGCAGGCACATTTCAGGATCGGATTTCCTGGGTTGG
	reverse	CCAACCCAGGAAATCCGATCCTGAAATGTGCCTGCTGTGG

Table 4.2: Pooled HER3-targeting siRNA duplexes

Sequence (sense)
GCAGUGGGAUUCGAGAAGUG
AGAUUGUGCUCACGGGACA
GUGGAUUCGAGAAGUGACA
GCGAUGCUGAGAACCAAUA

Table 4.3: shRNA hairpins used for HER3 and MPZL3 depletion

shRNA	Clone number	Sequence
pLKO (vector)	SHC001	--
shCtl (non-targeting)	SHC002	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTTTG
shHER3 A	TRCN0000194972	CCGGCTTCGTCATGTTGAACTATAACTCGAGTTATAGTTCAACATGACGAAGTTTTTTG
shHER3 B	TRCN0000199364	CCGGCCTGTGCATGTGCTCTTATTGCTCGAGCAATAAGAGCACATGCACAGGTTTTTTG
shMPZL3 A	TRCN0000137252	CCGGGCAGCCACACAGTATCAATATCTCGAGATATTGATACTGTGTGGCTGCTTTTTTG
shMPZL3 B	TRCN0000167937	CCGGCCAGGGTGTTTATATCGTCTTCTCGAGAAGACGATATAAACACCCTGGTTTTTTG
shMPZL3 C	TRCN0000167873	CCGGGAGATCATCAGTAAAGACTTTCTCGAGAAAGTCTTTACTGATGATCTCTTTTTTG

Centrifuged whole cell lysate or proteins captured on sepharose beads were boiled for 5 minutes in Laemmli buffer. Samples were resolved using 8% or 12% SDS-PAGE (BioRad) or 4-12% gradient NuPAGE Protein Gel (Thermo Fisher). Proteins were transferred to Newblot PVDF membranes (Mandel Scientific), blocked in 3% bovine serum albumin in TBST or Odyssey Blocking Buffer (Licor) and incubated with primary antibodies diluted in Near Infra-Red Blocking Buffer (Rockland) overnight at 4° C. Membranes were washed in TBST and incubated with secondary antibodies diluted in a 1:1 TBST:Rockland blocking buffer for one hour, washed in TBST again, and scanned using a Odyssey scanning machine (Licor) or visualized using Western Lightning Plus ECL (Perkin Elmer).

4.5 Proliferation and colony-forming assays

Proliferation assays were performed in 96-well dishes with $4x10^3$ EBC1, H1993 or KatoII cells seeded per well with 18-24 replicates per condition. Proliferation was measured as a function of cell confluence by IncuCyte live-cell microscopy (Essen Biosciences). Soft agar assays were performed in 6-well dishes coated with a layer of bottom agar (6 mg/ml), with $1x10^4$ KatoII cells seeded in top agar (3.2 mg/ml). Cells were plated in duplicate per condition and fed every 4-5 days, with colonies counted after 23 days in culture. Quantification was performed from 4 biological replicates for soft agar assays. For 2D colony assays in adherent HeLa, SkBr3 and KatoIII cells, $1x10^4$ cells were plated in duplicate in 6-well dishes and monitored for 14 days. Cells were fixed in 2% paraformaldehyde and stained with 0.1% crystal violet in a 10% ethanol solution.

4.6 Subcutaneous xenograft experiments

For xenograft experiments testing HER3 depletion, $5x10^5$ (KatoII) or $1x10^6$ (H1993) cells were injected bilaterally into the flanks of immune-deficient NOD.Cg-Prkdc^{scid}Il2rg$^{tm1Wjl/SzJ}$ mice (Jackson). Five mice were injected per condition. Tumours were detected by palpation and measured every 3 days. Animals injected with KatoII cells were sacrificed for ethical considerations at 13 days post-injection.

For xenograft experiments to test KatoII and Mkn45 cell growth in mice humanized for HGF, $1x10^6$ cells were injected bilaterally into the flanks of immune-deficient NOD.Cg-Prkdc^{scid}Il2rg$^{tm1Wjl/SzJ}$ mice (control) or NOD.Cg-Hgf$^{tm1.1(HGF)Aveo}$Prkdc^{scid}Il2rg$^{tm1Wjl/}$J mice (HGF). Animals injected with KatoII cells were sacrificed for ethical considerations at 18 days post-injection. Tumours from

Mkn45 cells were resected at 18 days post-injection to monitor for distant metastases; none were observed.

Flash-frozen tumour samples removed at ethical endpoint were lysed using a TissueLyserII bead mill in TGH buffer. Haematoxylin and eosin (H&E) staining was performed on paraffin-embedded tumour sections by the Histology Innovation Platform of the Goodman Cancer Research Centre at McGill University.

4.7 Flow cytometry

EBC1, H1993 or KatoII cells selected for virally-transduced shRNA vectors (puromycin) were seeded in equal numbers and grown for 3 days before washing in PBS, followed analysis of cell surface annexin-V and cell permeability using the Annexin-V-FLUOS staining kit (Sigma). Flow cytometry was performed on live cells using a BD FACSCalibur cytometer at the McGill Life Sciences Complex Flow Cytometry Facility.

4.8 RNA sequencing

KatoII cells transduced with shHER3A, shHER3B or the pLKO empty vector were selected in puromycin and plated for experiments. Total RNA was extracted from 3 independent infections used to prepare replicates for soft agar and xenograft injection assay using the RNeasy Plus Mini Kit (Qiagen). High RNA quality in all samples was verified using the Bioanalyzer RNA Nano 600 assay (Agilent). cDNA libraries were PCR-amplified and barcoded from 500 nanograms of RNA per sample, and pooled libraries were sequenced using the HiSeq4000 next-generation sequencing platform (Illumina) by the Centre d'Expertise et de Services at Génome Québec. The pooled libraries were sequenced with an average coverage of $3.3x10^7$ reads per sample.

Fastp (version 0.19.4) was used to collect quality control metrics of the raw reads. RNA sequences were aligned and sorted by coordinates, to the NCBI human genome build 38 (GRCh38.p12) with version 94 of gene annotations, using the STAR aligner (STAR_2.6.1a_08-27) (Chen et al., 2018; Dobin et al., 2013). The removal of alignment duplicates was done with Sambamba (version 0.6.8) (Tarasov et al., 2015). Quantification of genes was performed using featureCounts (v1.6.3) (Liao et al., 2014). DESeq2 (v 1.20.0) was used to normalize feature counts and to test find the differentially expressed genes (Love et al., 2014). The HGNC symbols were extracted and added to the DESeq2 results data frame using biomaRt (version 2.36.1) using the "hsapiens_gene_ensembl" dataset and the "Ensembl Release 94 (October 2018)" mart (Durinck et al., 2005; Durinck et al., 2009). Gene expression was extrapolated from gene-normalized read counts.

4.9 Quantitative RT-PCR

RNA was isolated from three independently infected and selected lines of HER3-depleted or control EBC1 and H1993 cells or four independently-collected replicates of MPZL3-depleted or control KatoII cells using the RNeasy Plus Mini Kit (Qiagen). High RNA quality in all samples was verified using the Bioanalyzer RNA Nano 600 assay (Agilent). cDNA libraries were synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) from 400-1000 ng total RNA. qPCR reactions were performed using SYBR Green I Master on a LightCycler480 (Roche). Primers for qPCR were purchased from Integrated DNA Technologies (primer sequences listed in Table 4.4). Gene expression was normalized to the geometric mean of three housekeeping genes, *RPLP0, HPRT* and *GAPDH*, using the delta-Ct method.

4.10 Immunofluorescence

Cells were seeded on glass coverslips, washed and fixed in 2% paraformaldehyde. Cells were permeablized with 0.1% Triton X-100 (Sigma), and coverslips were blocked using 2% BSA and incubated overnight with primary antibody at 4C in the presence of 2% BSA, 0.1% Triton X-100 and 0.05% Tween-20 (Sigma). Coverslips were washed in Triton/Tween buffer, incubated for 45m at room temperature with secondary antibodies, washed further, and counterstained with DAPI. Images were acquired on an LSM800 microscope and analyzed using Zen software (Zeiss).

Table 4.4: Primers used for qRT-PCR

Gene	Primers	
ERBB3	forward	GGGGAGTCTTGCCAGGAG
	reverse	CATTGGGTGTAGAGAGACTGGAC
MPZL3	forward	ATTCCCATGACAGAGCTAACAG
	reverse	GCACAAAGACAAGGATGGAAAG
NYNRIN	forward	CTGAGCTGAGACCAGGATTAAG
	reverse	GTTGGAGAGAATGTGGGTGTAG
LGR6	forward	CTCTTCCCTTTCCTCTC
	reverse	CTGAGTTTTGGTTGTATTTG
BHLHE41	forward	AGAGGAAACGAACAGCAGTTGA
	reverse	TAGGTATCCTTGGTGTCGTCTCG
RAMP1	forward	TGGAGCCTTGGGACAGA
	reverse	GGCTTCCAGGTTAATACCAGAG
SPNS2	forward	CCATCTTCATCTGCCTGATCTT
	reverse	CAGTGATGGCCCAGTTAGAAA
PPM1K	forward	TTTCCAAACCAACAGGCAAAG
	reverse	CCTCCCAAAGTGCTAGGATTAC
RPLP0	forward	CTCAACATCTCCCCCTTCTC
	reverse	GACTCGTTTGTACCCGTTGA
GAPDH	forward	GCACCAGGTGGTCTCCTCT
	reverse	TGACAAAGTGGTCGTTGAGG
HPRT	forward	TGATAGATCCATTCCTATGACTGTAGA
	reverse	CAAGACATTCTTTCCAGTTAAAGTTG

4.11 Cell-surface biotinylation

To measure cell surface levels of HER3, Met and MPZL3-V5, surface proteins were biotinylated with EZ-link Sulfo-NHS-SS-biotin (Thermo Fisher) for 20 minutes at 37C. Cells were washed in HEPES-buffered saline prior to lysis and biotin enrichment using NeutrAvidin-agarose beads (Thermo Fisher). For measurement of internalized Met protein in the presence of HGF, cells were incubated in biotin-containing media for the times indicated at 37C, and remaining surface biotin was stripped on ice with reducing agent (100 mM sodium 2-mercaptoethanesulfonic acid in 50 mM Tris-HCl, 100 mM sodium chloride, 1 mM EDTA and 0.2% BSA) prior to lysis. Protein was eluted from NeutrAvidin-enriched samples in Laemmli buffer and was analyzed by Western blot.

4.12 SILAC labeling

SILAC labeling of KatoII cells was accomplished by serial passage in lysine- and arginine-free RPMI medium (Thermo Fisher) supplemented with 10% dialyzed FBS (Wisent), 0.87 mM proline (Sigma), 0.64 mM light (Lys), medium (Lys-4D:2HCl) or heavy (Lys-8:HCl) lysine (Silantes) and 0.38 mM light (Arg), medium (Arg-6:HCl) or heavy (Arg-10:HCl) arginine (Silantes). Cells were continuously cultured in media containing only light, medium or heavy lysine and arginine for 6 passages. Cells cultured in media containing equimolar mixtures of light, medium and heavy amino acids were grown as controls. Amino acid incorporation was measured by one-dimensional LC-MS/MS using a QExactive BioPharma mass spectrometer (Thermo Fisher) by our collaborators at the Université de Montréal.

4.13 Phosphoproteomic profiling

SILAC-labeled KatoII cells were washed in PBS after treatment, and cell pellets ($4x10^8$ cells) were flash frozen in liquid nitrogen. Protein extraction, tryptic digestion, phosphoprotein enrichment and mass spectrometry were performed by our collaborators at the Université de Montréal. Cell pellets were lysed in 8M urea in the presence of tris(2-carboxyethyl)phosphine and chloroacetamide and trypsinized overnight. Tyrosine-phosphorylated tryptic peptides were enriched using the PTMscan PhosphoTyrosine Rabbit mAb kit (Cell Signaling Technologies) or by titanium dioxide as described previously (Kanshin et al., 2013). Enriched phosphopeptide extracts were analyzed using a QExactive BioPharma mass spectrometer (Thermo Fisher). Mass spectrometry data was processed using MaxQuant and searched against the SwissProt set of *H. sapiens* uniprot database (http://uniprot.org/). Known protein interactions were identified among genes with Met-dependent phosphosites using the STRING database (version 11.0) (http://string-db.org/) (Szklarczyk et al., 2019a). Gene ontology analysis by biological process was performed using the PANTHER classification system (version 14.0) (Mi et al., 2019).

4.14 Substrate-labeling kinase assays

For *in vitro* kinase assays, HEK293T cells transfected with Met variants were lysed and full-length Met protein was immunoprecipitated from 5 mg total protein in TGH buffer. Immunoenriched proteins were washed twice in detergent-containing lysis buffer and twice in HEPES-buffered saline. Sepharose beads were resuspended in kinase buffer (20 mM HEPES, 150 mM sodium chloride, 10 mM magnesium chloride), supplemented with ATP, ATPγS, or N6-modified ATPγS analogues, and incubated at 37C for 20 minutes. Kinase assays were supplemented with DMSO

or alkylated by addition of PNBM at room temperature for one hour prior to elution in Laemmli buffer and analysis by Western blot.

For in-cell substrate labeling, HEK293T cells expressing Met or Met Leu1157Ala were washed in kinase buffer (100 mM potassium acetate, 20 mM HEPES, 5 mM sodium acetate, 1 mM magnesium acetate, 0.5 mM EDTA, 0.5 mM magnesium chloride, 5 mM β-glycerophosphate) and then incubated for 20 minutes at room temperature in kinase buffer supplemented with 5 mM GTP, 0.5 mM dithiothreitol, 30 μg/ml digitonin, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 1 mM phenylmethane sulfonate, 10 μg/ml aprotinin and leupeptin, and 0.1 mM ATPγS or N6-modified ATPγS analogue. Cells were lysed by addition of an equal volume of 2X TGH buffer (100 mM HEPES, 300 mM sodium chloride, 2% Triton X-100, 20% v/v glycerol, 4 mM EGTA, 30 mM magnesium chloride).

4.15 Statistical Analysis

Quantitative data for Western blot analysis, qRT-PCR, colony-forming assays and cell confluence are presented as the means ± SEM. Statistical significance was assessed using a two-tailed Student's *t* test, and ordinary one-way ANOVA using Prism software. Significance is as follows: $p > 0.05$, not significant (ns); $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$. In general, data distribution was assumed to be normal but this was not formally tested. For measurements of median MPZL3 expression in RTK-amplified cell lines using CCLE data, a non-parametric test was used as a normal distribution of data was not assumed. P-values and the number of experiments used for quantification and statistical analysis are indicated in the corresponding figure legends. For gene expression experiments using qRT-PCR, statistics were corrected for multiple

comparisons using the two-stage step-up method of Benjamini, Krieger and Yekutieli (Benjamini et al., 2006). In experiments with defined controls, multiple comparisons were corrected using Dunnet's method of statistical hypothesis testing (Dunnett, 1955). For MPZL3 expression in CCLE data, multiple comparisons were corrected using Tukey's range test (Tukey, 1949).

CHAPTER 5: REFERENCES

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