Mechanisms of MET-dependent tumour initiation and progression

by

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ABSTRACT

MET is a receptor tyrosine kinase that, when dysregulated, contributes to oncogenic progression in a wide range of human cancers. When bound by its ligand hepatocyte growth factor (HGF), MET coordinates a program of invasive growth and migration that largely overlaps with the process of epithelial-mesenchymal transition (EMT). The induction of an EMT program can generate cells with increased tumourigenicity and properties associated with stem cells. Cells enriched with tumour-initiating capacity are commonly termed tumour-initiating cells (TICs) and have been found to be resistant to conventional therapies and thus can contribute to recurrence. Indeed, TICs possess a gene signature that converges with cells that have undergone an EMT. MET is implicated in TIC regulation in a number of cancers, however the mechanisms by which it promotes the propagation of TICs has yet to be fully elucidated.

Breast cancer is a heterogeneous disease with multiple distinct subtypes that differ both in gene expression and prognosis. Triple negative breast cancer (TNBC) is an aggressive subtype that is negative for expression of estrogen receptor, progesterone receptor, and HER2 amplification, and therefore lacks targeted therapies and has poor prognosis. Elevated levels of MET are observed in 15-20% of all breast cancers and is associated with TNBC as well as poor survival.

Using a MMTV-*Met*^{mt};*Trp53fl/+*;*Cre* murine model of the claudin-low subtype of TNBC, which features highly mesenchymal tumours with amplified and constitutively active Met protein, we show that a Met-dependent EMT program is required for an enhanced tumour-initiating capacity. We uncover a mechanism by which signaling through Met and fibroblast growth factor receptor 1 (FGFR1) can both independently regulate tumourigenic potential, and combinatorial targeting of both receptors is needed to abrogate TICs in both *in vitro* and *in vivo* studies. We find co-expression of MET and FGFR1 in human TNBC cell lines and in patient-derived xenografts of

TNBC, and that dual inhibition of both receptors likewise depletes TIC populations. Importantly, human TNBCs with highly mesenchymal characteristics are significantly enriched for expression of HGF and FGFR1 expression, and co-expression predicts poor prognosis among these patients. These findings provide a role for MET in TNBC tumour initiation and maintenance, where it sustains a mesenchymal program that promotes a state of cellular plasticity conducive to tumourigenesis.

Co-regulation of TICs by MET and FGFR1 is facilitated by pathways mediated through FGFR substrate 2 (FRS2), a scaffold protein first identified in FGFR signaling. While MET is not conventionally known to signal through FRS2, we find that FRS2 is in fact required for MET-dependent cellular processes such as invasion and survival in a number of MET-amplified cancer cell lines. We further show in breast cancer cell lines that HGF-stimulated activation of non-amplified and wildtype MET promotes FRS2 phosphorylation and maintains ERK1/2 phosphorylation in the absence of FGFR signaling. These findings describe a previously uncharacterized role for FRS2 in MET-driven biology.

Taken together this work provides new understanding on how MET can, in both amplified and non-amplified settings, contribute to TIC regulation, and provides a rationale for targeting MET and its signaling partners as TNBC therapies. Importantly, the characterization of FRS2 as a MET signaling substrate highlights a novel mechanism by which this RTK can converge with other pathways to regulate its downstream cellular processes.

RÉSUMÉ

MET est un récepteur tyrosine kinase dont la dérégulation contribue à la progression tumorale de multiples types de cancers. Lorsque son ligand, le facteur de croissance « hepatocyte growth factor » (HGF), s'y lie, MET coordonne un programme d'invasion et de migration qui suit en grande partie le processus de transition épithélio-mésenchymateuse (TEM). L'induction d'un programme TEM peut générer des cellules présentant une tumorigénicité accrue et des propriétés de cellules souches. Les cellules possédant des propriétés d'initiation tumorale sont communément appelées cellules initiatrices de tumeur (CIT) et peuvent se révéler résistantes à la thérapie. En effet, les CIT ont une signature d'expression génique similaire aux cellules ayant subies une TEM. Le récepteur MET est impliqué dans la régulation des CIT dans plusieurs types de cancer. Cependant, les mécanismes par lesquels il favorise la propagation des CIT n'ont pas encore été élucidés.

Le cancer du sein est une maladie hétérogène comportant de multiples sous-types tumoraux distincts qui diffèrent en matière d'expression génique et de pronostic. Le cancer du sein triple négatif (CSTN) est un sous-type agressif, négatif pour l'expression des récepteurs aux œstrogènes, des récepteurs de la progestérone et l'amplification de l'oncogène HER2. Les CSTN ne possèdent donc pas de traitements ciblés et montrent un mauvais pronostic clinique. Entre 15 et 20% des cancers du sein possèdent une expression élevée de MET. Cette expression élevée de MET est associée au CSTN et à un mauvais pronostic clinique.

Les souris MMTV-*Met*^{mt};*Trp53fl*/+;*Cre* modélisent le sous-type « claudin-low » des CSTN. Ces souris présentent des tumeurs très mésenchymateuses avec une amplification et une activation constitutive de la protéine Met. L'utilisation de ce modèle murin nous a permis de montrer qu'un programme de TEM dépendant de Met est nécessaire à l'augmentation de la capacité

d'initiation tumorale. Nous avons également pu mettre en évidence la nature d'un mécanisme par lequel la signalisation via Met et le récepteur 1 du facteur de croissance des fibroblastes (FGFR1) peuvent réguler indépendamment le potentiel tumorigène cellulaire. De plus, le ciblage des deux récepteurs est nécessaire pour abroger les CIT dans des études *in vitro* et *in vivo*. MET et FGFR1 sont co-exprimés dans des lignées de cellules humaines de CSTN et dans des xénogreffes sur souris dérivées de tumeurs de patientes. La double inhibition de ces deux récepteurs induit la déplétion de la population des CIT. Il est important de noter que, chez l'humain, les CSTN présentant des caractéristiques très mésenchymateuses sont considérablement enrichis pour l'expression de HGF et de FGFR1. Cette co-expression prédit un mauvais pronostic chez ces patients. Ces découvertes confèrent un rôle clé à MET dans l'initiation et la progression tumorale des CSTN par l'initiation d'un programme mésenchymateux qui favorise un état de plasticité cellulaire propice à la tumorigène.

La co-régulation des CIT par MET et FGFR est facilitée par des voies de signalisation impliquant le substrat 2 du FGFR (FRS2), protéine d'échafaudage originellement identifiée par son rôle dans la voie de signalisation FGFR. FRS2 est la principale protéine d'échafaudage pour la signalisation de FGFR. Bien que MET ne soit pas connu pour signaler via FRS2, nous avons pu mettre en évidence que FRS2 est nécessaire aux processus cellulaires dépendant de MET tels que l'invasion et la survie dans plusieurs lignées de cellules cancéreuses amplifiées pour MET. Nous avons également pu montrer que l'activation de MET non amplifié, non muté et stimulée par du HGF peut également entrainer la phosphorylation de FRS2 dans les lignées cellulaires de cancer du sein et maintenir la phosphorylation de ERK1 / 2 en l'absence de signalisation FGFR. Ces résultats décrivent un rôle jusqu'ici non caractérisé pour FRS2 dans la biologie sous jacente aux processus biologiques et voies de signalisation en aval de MET.

En conclusion, ces travaux permettent de mieux comprendre la façon dont MET, amplifié ou non, peut contribuer à la régulation des CIT. Ces travaux identifient MET et ses partenaires de signalisation pour un ciblage thérapeutique dans le traitement des CSTN. Il est important de noter que la caractérisation de FRS2 en tant que substrat de signalisation de MET met en évidence un nouveau mécanisme par lequel ce RTK peut converger avec d'autres voies de signalisation pour réguler différents processus cellulaires.

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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. General Discussion
Chapter 4. Experimental Procedures
Chapter 5. Bibliography

Publications arising from this thesis

Chapters 2 and 3 contain material presented in the following articles:

Vanessa Y.C. Sung, Jennifer F. Knight, Radia Johnson, Yaakov Stern, Sadiq Saleh, Anie Monast, Paul Savage, Dongmei Zuo and Morag Park. MET regulates tumour-initiating cells in highly mesenchymal triple-negative breast cancers *Manuscript in preparation.*

Vanessa Y.C. Sung, Paula P. Coelho, Anie Monast and Morag Park. The scaffold protein FRS2 is required for invasion and migration in MET-amplified cancer cells. *Manuscript in preparation.*

Contributions of authors

JFK generated the *MMTV-Met^{mt};Trp53fl/+;Cre* mouse model. RJ performed bioinformatic analyses on RNA sequencing data (Figure 2.6, Table 2.2 and 2.3) and clinical data (Figure 2.14, 2.15, 2.16). SS performed bioinformatic analyses on cell lines in Figure 2.9. AM performed cell injection surgeries and measured tumour growth for Figures 2.7 and 2.8, and also performed the tumour transplantation surgeries in Figure 2.13B. PS generated the patient-derived xenografts used in Figure 2.12 and 2.13. DZ performed the staining and imaging in Figure 2.12B and provided technical support for other immunohistochemical and immunofluorescence studies. PPC performed and analyzed the collagen invasion assay in Figure 3.4B. All other data were generated, analyzed, and assembled by VYCS.

Additional publications

Knight JF, **Sung VYC**, Kuzmin E, Couzens A, De Verteuil DA, Johnson RM, Gruosso T, Saleh SM, Zuo D, Guiot MC, Davis RR, Zhao H, Gregg JP, Gingras AC, Park M. (2018) Kibra (WWC1) is a metastasis suppressor gene affected by chromosome 5q loss in human triple negative breast cancers. *Cell Reports* 22(12): 3191-3205.

Thompson, C., Lai, A.Z., Chenard, V., Gravel, S.-P., Golenar, T., Monast, A., **Sung, VYC.**, Bridon, G., Johnson, R., Zuo, D., Zahr, S., Gigoux, M., Avizonis, D., St-Pierre, J., Park, M. Inhibition of GLUT1-mediated glycolysis promotes lactate oxidation and OXPHOS dependency in drug resistant MET-amplified gastric cancer. *Manuscript in preparation.*

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LIST OF ABBREVIATIONS

ABC – ATP-binding cassette
AKT – Ak strain transforming
ALDH – aldehyde dehydrogenase
ALK – anaplastic lymphoma kinase
AML – acute myeloid leukemia
BL1 – basal-like 1
BL2 – basal-like 2
CAR-T cell – chimeric antigen receptor T cell
CBL – casitas B-lineage lymphoma
CK – cytokeratin
CRK – c-10 regulator of kinase
CSC – cancer stem cell
CTLA-4 – cytotoxic T lymphocyte associated protein 4
DCIS – ductal carcinoma *in situ*
DOK – downstream of kinase
ECM – extracellular matrix
EGFR – epidermal growth factor receptor
EMT – epithelial-mesenchymal transition
ER – estrogen receptor
ERK – extracellular signal-regulated kinase
FACS – fluorescence-activated cell sorting
FGF – fibroblast growth factor
FGFR – fibroblast growth factor receptor
FRS2 – fibroblast growth factor receptor substrate 2
GAB1 – GRB2-associated protein 1
GRB2 – growth factor receptor-bound protein 2
HER2 – human epidermal growth factor receptor 2
HGF – hepatocyte growth factor
HGFA – hepatocyte growth factor activator
HGFR – hepatocyte growth factor receptor

HOS – human osteosarcoma
HR – hormone receptor
IDC – invasive ductal carcinoma
IL – interleukin
InClust – integrative clusters
IM – immunomodulatory
IRS – insulin receptor substrate
LAR – luminal androgen receptor
M – mesenchymal
MAPK – mitogen-activated protein kinase
MBD – MET-binding domain
MMP – matrix metalloproteinase
MMTV-LTR – mouse mammary tumour virus long terminal repeat
MNNG – *N*-methyl-*N*'-nitro-*N*-nitroso-guanidine
MSL – mesenchymal stem-like
NSCLC – non-small cell lung carcinoma
PAK4 – p21-activated kinase 4
PAM50 – prediction analysis of microarrays 50
PD-1 – programmed death-1
PD-L1 – programmed death ligand-1
PDX – patient-derived xenografts
PI3K – phosphoinositide 3-kinase
PKC – protein kinase C
PLCγ – phospholipase C gamma
PR – progesterone receptor
PTB – phosphotyrosine binding
RET – rearranged during transfection
RFS – relaspse-free survival
RON – recepteur d'origine nantais
ROS – reactive oxidative species
RTK – receptor tyrosine kinase
SF – scatter factor
SHP2 – SRC homology 2 domain-containing phosphatase 2
SOS – son of sevenless
TGFβ – transforming growth factor β
TIC – tumour-initiating cell
TNBC – triple negative breast cancer
tPA – tissue-type plasminogen activator
TS – trophoblast stem
uPA – urokinase-type plasminogen activator

1 LITERATURE REVIEW

1.1 Tumour-initiating cells

1.1.1 Tumour-initiating cell model overview

The tumour-initiating cell (TIC) model posits that cancers possess unique subpopulations of cells that are enriched in tumour-initiating capacity. The model proposes a hierarchical organization of cancer cells, with self-renewing TICs at the apex where they sustain tumour growth and give rise to phenotypically and functionally diverse cells with limited tumourigenic capacity (Magee et al. 2012) (Figure 1.1A). The understanding that some cancers contain tumourigenic cells that differentiate into non-tumourigenic progeny is an old one. As early as the 1960's, studies on germ lineage cancers (Kleinsmith & Pierce 1964), neuroblastomas (Shimada et al. 1984), and myeloid leukemias (Fearon et al. 1986) documented that these cancers featured undifferentiated cells that were primarily responsible for tumour progression. Poorly differentiated neuroblastomas had extremely poor outcome (K. K. Matthay et al. 1999), while highly differentiated neuroblastic tumours could often be cured by surgery (Nitschke et al. 1988). In some infant cases of neuroblastoma, tumour cells spontaneously underwent differentiation, which significantly improved survival (Baker et al. 2010). While these cases of overt differentiation provided evidence that a structure analogous to a stem cell differentiation hierarchy existed in cancer, it was unclear how applicable this model was to adult cancers.

TICs were then described in acute myeloid leukemia in a series of studies where the authors identified a rare subset (0.01-1%) of acute myeloid leukemia (AML) cells, displaying cell surface phenotypes similar to that of normal hematopoietic stem cells, that could sustainably regenerate leukemias through multiple transplantations in immunodeficient mice (Bonnet & Dick 1997; Lapidot et al. 1994). The existence of TICs has since been established in many solid tumours,

including in the breast (Al-Hajj et al. 2003; Ginestier et al. 2007), brain (Singh et al. 2003), prostate (Collins et al. 2005), ovary (Bapat et al. 2005), colon (O'Brien et al. 2006), liver (Z. F. Yang et al. 2008), lung (Eramo et al. 2007), and pancreas (Hermann et al. 2007), though their frequency and characteristics vary among the different cancer types. Certain cancers, such as melanoma, do not appear to follow the TIC model. Operationally, TICs are defined by their ability to self-renew and simultaneously generate non-TICs to populate the tumour bulk. Other common, though not required, characteristics of TICs include rarity, stem cell markers, and differentiation capacity.

TICs are distinct from the cell of origin, which refers to the specific cell type that underwent the initial oncogenic transformation. A common alternative name for this subset of cells is "cancer stem cells" (CSCs). While the term TICs refers to the competency of these cells to give rise to a tumour, the term CSCs reflects the partial overlap with normal stem cell properties. CSCs can also incorrectly illicit an association with a stem cell as the cell of origin, which, while possible, is not a requirement of the TIC model. For the purposes of clarity and consistency, the term TICs will be used exclusively in this work.

1.1.2 Tumour-initiating cells vs clonal evolution

The clonal evolution model, in which stochastic genetic (Nowell 1976) and epigenetic (Baylin & Jones 2011) alterations confer heritable properties that provide survival and proliferative advantages upon cells, differs from the TIC model in that it is not hierarchical. Rather, any cell has the potential to accumulate mutations that lead to its selection and expansion into a dominant population that drives tumour growth (Figure 1.1B). Microenvironmental factors can also confer changes upon cancer cells in different locations (Polyak et al. 2009). Evidence supports the existence of both paradigms in cancer and that they are not mutually exclusive. Indeed, TICs

themselves can undergo clonal evolution, resulting in the emergence of a more dominant TIC population (Shipitsin et al. 2007; Barabé et al. 2007) (Figure 1.1C).

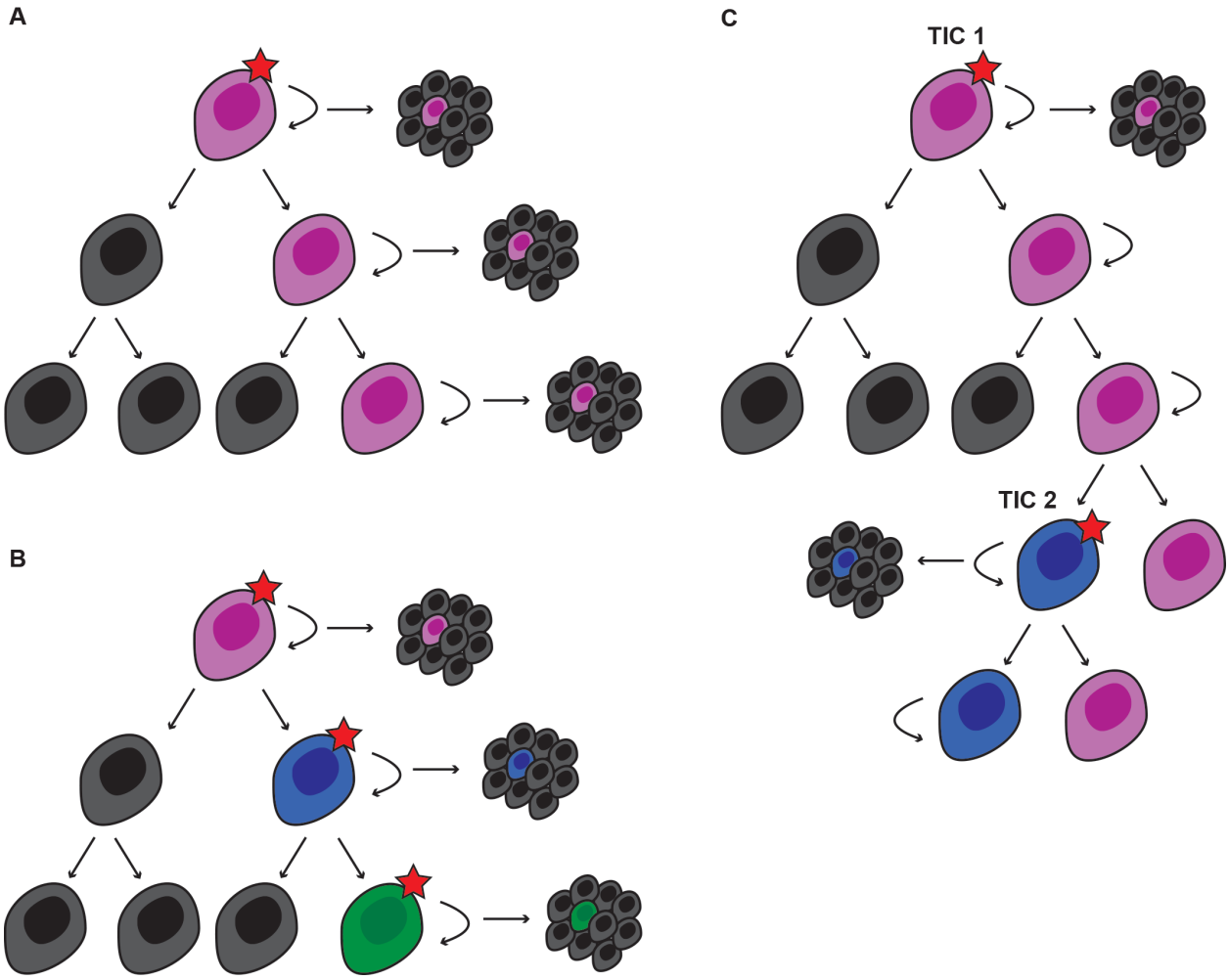


Figure 1.1 Models of tumour initiation and heterogeneity. (A) In the TIC model, only TICs can generate a tumour, based on their self-renewal and proliferative potential. (B) In the clonal evolution model, all cells have similar tumourigenic potential through acquisition of mutations that confer survival and proliferative advantage. (C) The two models are not mutually exclusive and can combine to drive tumourigenesis. Initially, tumourigenesis is driven by a specific TIC (TIC1). Over time as the tumour progresses, clonal evolution may produce another more dominant TIC (TIC2) which will drive tumour growth.

1.1.3 Experimental assays for tumour-initiating cells

A handful of experimental techniques have been developed over the years to allow investigation of the functional and phenotypic properties of TICs (Figure 1.2). The gold standard experiment for TICs is single cell injections *in vivo*, either at limiting dilutions to estimate the proportion of cells capable of generating a tumour or serial transplantations to evaluate self-renewal capacities (Bonnet & Dick 1997; Al-Hajj et al. 2003). Lineage tracing techniques have also allowed clear establishment of a hierarchical tumour initiation model in a number of syngeneic models of cancer (Schepers et al. 2012; Driessens et al. 2012; J. Chen et al. 2012). The *in vitro* tumoursphere assay is used to study putative TICs (Ponti et al. 2005; Hemmati et al. 2003). This technique was originally developed to enrich for neural stem cells based on their ability to survive and propagate in serum-free and anoikis conditions by growing as spheroid structures (neurospheres) in suspension (Reynolds & Rietze 2005). The number of tumourspheres formed, and their ability to proliferate and self-renew through multiple rounds of serial passaging, are used to predict TIC number and robustness, respectively. Finally, fluorescence-activated cell sorting (FACS) can be employed to identify and isolate cells based on their expression of stem or TIC-related cell surface markers or intracellular aldehyde dehydrogenase (ALDH) activity (examples and references given in Table 1.1). While reports suggest that tumoursphere assays and cell surface marker do not on their own consistently correlate with tumourigenicity *in vivo* (Calvet et al. 2014; C. Lehmann et al. 2012; Fillmore & Kuperwasser 2008), they remain commonly used techniques for interrogating TIC biology. This highlights the need for using multiple experimental approaches when studying TICs, and for results to be interpreted carefully.

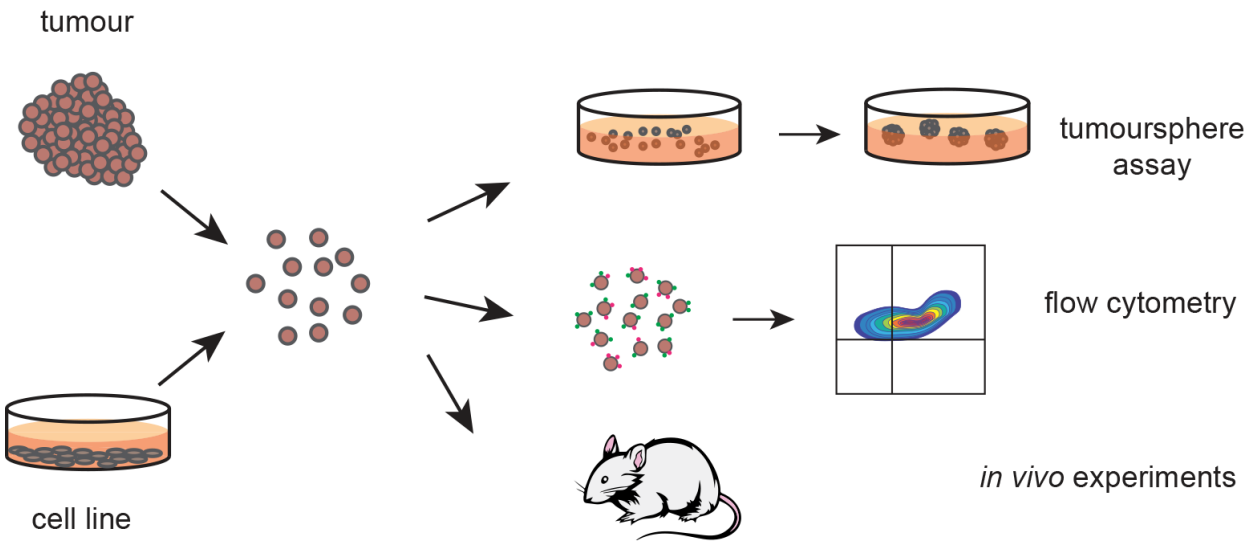


Figure 1.2 Experimental assays for tumour-initiating cells. Single cells are derived either from tumour fragments or from established cells lines for TIC assays, including tumoursphere cultures, flow cytometry, or in vivo tumour initiation studies. See text for details.

Marker	Normal function	Tumour type	References
CD34	Cell adhesion	Blood	(Bonnet & Dick 1997)
CD24	Cell adhesion, B cell proliferation and maturation	Breast	(Al-Hajj et al. 2003)
		Gastric	(C. Zhang et al. 2011)
		Pancreas	(C. Li et al. 2007)
CD44	Cell adhesion, migration, cell-cell interactions, cell signaling	Breast	(Al-Hajj et al. 2003)
		Colon	(Dalerba et al. 2007)
		Gastric	(Takaishi et al. 2009)
		Head and neck	(Prince et al. 2007)
		Liver	(Z. F. Yang et al. 2008)
		Pancreas	(C. Li et al. 2007)
		Prostate	(Collins et al. 2005)
CD90	Cell adhesion, signal transduction	Brain	(He et al. 2012)
		Liver	(Z. F. Yang et al. 2008)
		Lung	(Donnenberg et al. 2007)
CD133	Poorly understood	Brain	(Singh et al. 2003)
		Colon	(O'Brien et al. 2006)
		Endometrium	(Rutella et al. 2009)
		Liver	(Ma et al. 2007)
		Lung	(Bertolini et al. 2009)
		Ovary	(FERRANDINA et al. 2008)
		Pancreas	(Hermann et al. 2007)
		Prostate	(Collins et al. 2005)
		Skin	(Monzani et al. 2007)
ALDH	Catalyzes oxidation of aldehydes to carboxylic acids, functions as antioxidant	Breast	(Ginestier et al. 2007)
		Colon	(E. H. Huang et al. 2009)
		Head and neck	(Y.-C. Chen et al. 2009)
		Liver	(Ma et al. 2008)
		Pancreas	(M. P. Kim et al. 2011)
		Skin	(Boonyaratanakornkit et al. 2010)
Hoechst 33342 dye exclusion (side population)	N/A	Brain	(Bleau et al. 2009)
		Gastric	(Haraguchi et al. 2006)
		Lung	(Ho et al. 2007)
		Thyroid	(Mitsutake et al. 2007)

Table 1.1 Commonly used TIC markers and their normal functions.

1.1.4 Tumour-initiating cells and cellular plasticity

1.1.4.1 Tumour-initiating cells and "mesenchymal-ness"

Epithelial-mesenchymal transition (EMT) is a cellular program whereby epithelial cells decrease cell-cell junctions, lose apical-basal polarity, increases cell matrix adhesions, and display increased migratory and invasive capacities associated with mesenchymal cells (Kalluri & Weinberg 2009). EMT, and its reverse process mesenchymal-epithelial transition, were originally studied during organogenesis where this process underlies the ability of primitive cells to interconvert between cell types and travel long distances (Shook & Keller 2003; Hay 2005). The EMT program is also initiated during various pathological processes, including wound healing and tissue fibrosis (Kalluri & Weinberg 2009). Accumulating evidence supports that EMT is a key process through which both normal and neoplastic cells acquire stem-like properties (Mani et al. 2008; Morel et al. 2008), though the relationship appears to be complex. The EMT program is executed by a set of transcription factors, mainly of the SNAIL, TWIST, and ZEB families. Interestingly, cancer cells that have engaged an EMT program are typically in a "partial EMT" state where some epithelial characteristics are retained and mesenchymal characteristics are gained (Bierie et al. 2017; Yu et al. 2013). Cells that have progressed through a full epithelial to mesenchymal transdifferentiation exhibit decreased tumour-initiating capacity (Celià-Terrassa et al. 2012), suggesting that cellular plasticity is an important trait of TICs. Several lines of evidence support that cellular plasticity is a key regulator of TIC capacity. By definition, TICs have the capacity to both self-renew and differentiate into non-TICs. Transcriptional profiling has consistently revealed TICs to express gene signatures associated with cells in a less differentiated state compared to non-TICs that make up the tumour bulk (Creighton et al. 2009; Savage et al. 2017; Y. Li et al. 2011). TICs also display phenotypic and functional properties of stem and mesenchymal cells (Shibue & Weinberg 2017).

Furthermore, numerous studies have demonstrated that epithelial cells can spontaneously transition between cellular states (S. Liu et al. 2014; Chaffer et al. 2011; Gupta et al. 2011; Shaffer et al. 2017). These observations likely reflect a state of cellular plasticity that is permissive to survival under unfavourable conditions, including oncogenic transformation and tumourigenesis (Figure 1.3).

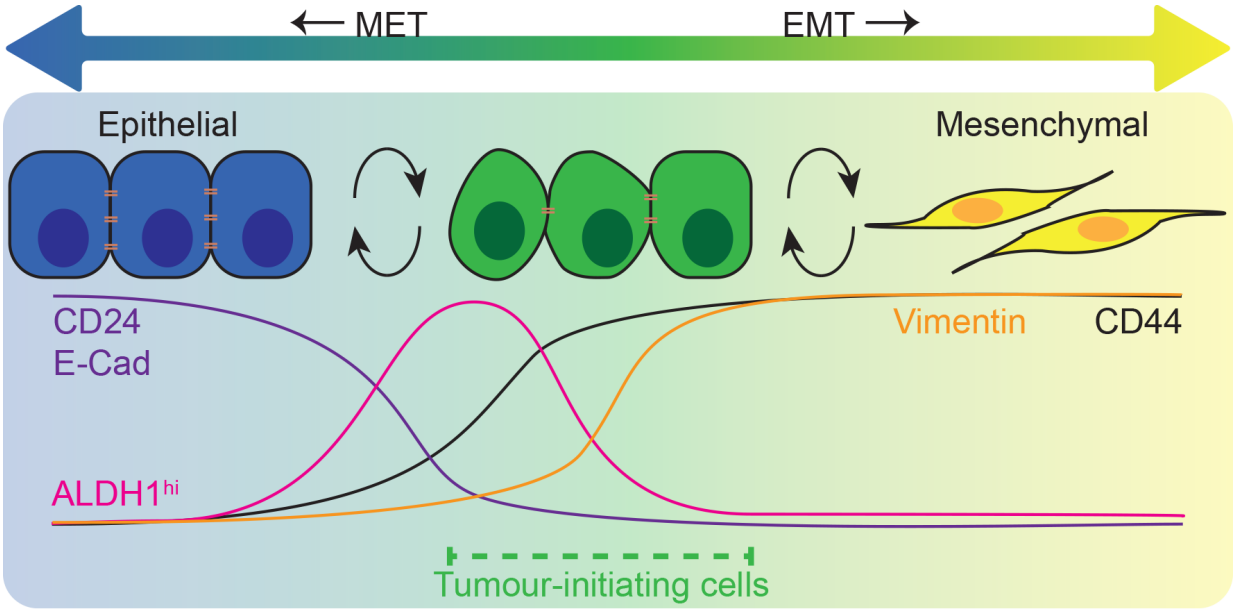


Figure 1.3 Tumour-initiating cells exist in a state of cellular plasticity. The ability to transition between more epithelial and more mesenchymal states is a key feature of TICs. Cellular plasticity underlies the ability of TICs to self-renew, differentiate, and survive conventional anti-cancer therapies. See text for details.

1.1.4.2 Tumour-initiating cells and "stemness"

Stem cell markers are frequently used to identify and physically isolate TICs. For example, the antigen PROMININ-1 (CD133) is used as a marker for stem and progenitor cells in the brain (Uchida et al. 2000), hematopoietic system (Bühring et al. 1999), kidney (Bussolati et al. 2005), and prostate (Richardson 2004), as well as for TICs in the brain (Singh et al. 2003), colon (O'Brien

et al. 2006), liver (Ma et al. 2007), ovary (FERRANDINA et al. 2008), and prostate (Collins et al. 2005). Another stem cell property that is adapted to identify TICs is the expression of ATP-binding cassette (ABC) transporters (Goodell 1996; Dontu 2003; M. Kim & Morshead 2003). These transporters efflux Hoechst 33342 dye and create a TIC-enriched "side population" that can be isolated via FACS (Bleau et al. 2009; Haraguchi et al. 2006; Ho et al. 2007). TICs also share the defining functional characteristics of stem cells, namely self-renewal and the ability to differentiate into more specialized cells, in this case non-TICs that make up the bulk of the tumour (Magee et al. 2012). This overlap in phenotypic and functional properties does not necessarily mean that TICs are invariably transformed stem cells. Normal stem cells are rare cell subsets that are typically senescent or slow-cycling, and therefore less susceptible to genetic mutations (Reya et al. 2001). Given the plasticity demonstrated by these cells, it is possible, and indeed likely, that most TICs are progenitors or further differentiated cells that have acquired stem-like properties in the altered, neoplastic state.

1.1.5 Tumour-initiating cells and the tumour microenvironment

Activation of an EMT program relies upon heterotypic signaling between neoplastic cells and the non-neoplastic cells in the stroma immediately surrounding the tumour, collectively termed the tumour microenvironment. Stromal cells can be resident or recruited, and can be categorized into three major classes – angiogenic vascular cells, infiltrating immune cells, and cancer-associated fibroblasts (Hanahan & Coussens 2012). Stromal cells can secrete a wide range of EMT-promoting factors, including growth factors (eg. TGFβ, HGF, FGFs), enzymes (eg. MMPs), and cytokines (eg. IL-6, IL-8) (Hanahan & Coussens 2012). Given that cell plasticity conferred by partial EMT appears to be important for TIC function, it is likely that stromal cells are key to creating a

supportive niche for TICs. For example, interactions between cancer and stromal cells have been shown to be important for induction and maintenance TICs in colon (H.-J. Li et al. 2012; Vermeulen et al. 2010) and breast cancers (Jinushi et al. 2011; J. Yang et al. 2013).

1.1.6 Resistance to conventional therapies and recurrence

Enhanced resistance to radiation and chemotherapy has been observed in the TICs of multiple solid cancers (Dallas et al. 2009; S. Bao et al. 2006; Creighton et al. 2009). The known causes of the intrinsic resistance of TICs to conventional therapeutic modalities include low proliferative rate (Pece et al. 2010; Roesch et al. 2010), high expression of antiapoptotic proteins (Peters et al. 1998) and ABC transporters capable of drug efflux (S. Zhou et al. 2001), and low levels of reactive oxidative species (ROS) (Diehn et al. 2009). Thus, treatment with radiation and chemotherapy may leave behind a pool of resistant, tumourigenic cells from which recurrence can develop (Figure 1.4A). Treatment regimens that exclusively target highly proliferative tumour cells are likely to fail in the long-term unless combined with TIC-targeting therapies (Figure 1.4B).

1.1.7 Therapeutics targeting tumour-initiating cells

Multiple strategies to therapeutically target TICs are currently under investigation. Signaling pathways on which TIC functions have been found to be dependent, such as TGFβ (Bhola et al. 2013), WNT (Vermeulen et al. 2010), Notch (Bouras et al. 2008), BMI-1 (Kreso et al. 2013), and Hedgehog (S. Liu et al. 2006) are attractive targets. Other approaches seek to interfere with tumour-stromal interactions by either treating with compounds that inhibit stromal secretion of EMT-promoting factors (Lin et al. 2009) or preventing the recruitment of stromal cells (MacDonald et al. 2010). As much of the functional characteristics of TICs is due to their relatively

dedifferentiated state, differentiation therapy presents another potential avenue for making TICs more susceptible to conventional therapies. These approaches typically involve epigenetic modulators that allow the expression or re-expression of genes that promote more epithelial phenotypes (Bernt et al. 2011; Munster et al. 2001; Cao et al. 2008). DNA-methylating agents have also been shown to target TICs by inducing double-stranded DNAs that lead to an anti-viral response (viral mimicry) (Roulois et al. 2015). Other studies have sought to exploit the unique metabolic requirements of TICs, where it has been reported that blockade of glycolysis prevents EMT-induced generation of breast TICs (Dong et al. 2013). Finally, large-scale drug screens have been conducted to identify compounds that could be effective at targeting TICs, with one study in breast cancer finding that pre-treatment with the antimicrobial agent salinomycin decreased the proportion of EMT-induced TICs by approximately 100-fold compared to paclitaxel (Gupta et al. 2009).

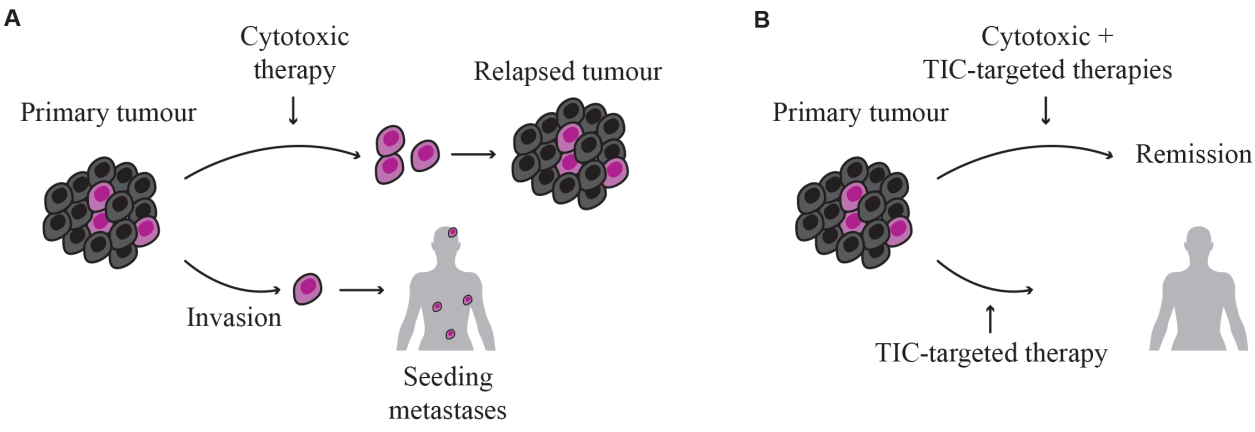


Figure 1.4 Tumour-initiating cells can escape conventional therapies and seed relapse and metastases. (A) Treating primary tumours with cytotoxic therapies alone can result in the selection of TICs that exhibit enhanced resistance to these therapies. (B) Treating primary tumours with a combination of cytotoxic and TIC-targeted therapies are more likely to achieve lasting remission. See text for details.

1.2 Receptor tyrosine kinase signaling

1.2.1 Receptor tyrosine kinase overview

Receptor tyrosine kinases (RTKs) are single-pass transmembrane proteins that convert stimulation by extracellular ligands into intracellular signaling cascades. A RTK is typically composed of an extracellular, transmembrane, juxtamembrane, and kinase domain with a flexible C-terminal tail (Hubbard 1999). The human genome encodes 58 known RTKs and these can be categorized into 20 subfamilies (Lemmon & Schlessinger 2010). While modes of activation vary among different RTKs, a general model for receptor activation initiates following ligand engagement with the extracellular domain, inducing receptor dimerization or oligomerization. This results in conformational changes that promote access of ATP to the catalytic cleft and subsequent transphosphorylation of tyrosine residues in the activation loop of the kinase domain. These charged residues retain the activation loop in an open conformation allowing continued access of ATP to the catalytic cleft, followed by phosphorylation of tyrosine residues in the juxtamembrane domain and C terminus outside of the kinase domain (Lemmon & Schlessinger 2010). These phosphotyrosines create binding sites for scaffold and adaptor proteins (discussed further below) critical for downstream signal transduction (Hubbard & Miller 2007). Negative regulation and signal termination of ligand-activated RTKs typically involves internalization and lysosomal degradation of the activated receptor, as well as dephosphorylation of the RTKs by protein phosphatases (Lemmon & Schlessinger 2010).

The signaling pathways initiated by RTKs at the cell membrane are essential for regulating diverse cellular processes including cell proliferation, differentiation, survival, and metabolism during development and normal homeostasis. Dysregulation of these pathways is implicated in

many human cancers (Blume-Jensen & Hunter 2001), motivating the development of a large number of RTK-targeted drugs that have been deployed as anti-cancer therapies to varying levels of success (Krause & Van Etten 2005). One of the major challenges that RTK-directed therapies face is drug resistance, either innate through the selection of existing resistant clones or acquired over the course of treatment (JAnne et al. 2009). RTKs converge on a limited set of downstream signaling substrates to activate their target pathways. Thus, compensatory signaling induced by the activation of other RTKs is a common mechanism of resistance (Wilson et al. 2012).

1.2.2 Protein-protein interactions in signaling cascades

1.2.2.1 Protein-protein binding domains

Upon activation, many RTKs nucleate the formation of signaling complexes by directly recruiting signaling proteins such as phospholipase c gamma (PLCγ) and non-receptor tyrosine kinases such as SRC. However, signal diversity is frequently initiated through recruitment of scaffold and adaptor proteins that themselves have no enzymatic activity but contain evolutionarily conserved binding domains (Hubbard & Miller 2007). Many of these key protein-protein interactions occur via the binding of phosphorylated tyrosine residues. Src-homology 2 (SH2) domains are small modules of approximately 100 amino acids that are evolutionary conserved, that engages in a sequence-specific manner phosphorylated tyrosine residues on other proteins (Songyang et al. 1993). There are 115 SH2 domains encoded by the human genome, constituting the largest class of phosphorylated tyrosine recognition domains (Pawson & Nash 2003). Phosphotyrosine binding (PTB) domains also facilitate binding to phosphorylated tyrosine residues, and consist of ~150 amino acids (M. M. Zhou et al. 1995). They are more diverse in sequence than SH2 domains, and are capable of binding both phosphorylated and nonphosphorylated tyrosines with high affinity

(Uhlik et al. 2005). Src-homology 3 (SH3) domains, containing around 60 amino acids, bind short proline-rich motifs with the minimum consensus Pro-X-X-Pro in target proteins (Ren et al. 1993). SH3 domains are ubiquitous, found in over 300 human proteins (Zarrinpar et al. 2003). SH2, SH3, and PTB domains are found in proteins in a variety of combinations, enabling the formation of signaling networks that both amplify and diversify the signaling initiated following RTK activation.

1.2.2.1 Scaffold and adaptor proteins

Scaffold proteins are multi-domain proteins that lack intrinsic catalytic activity, but upon activation by a RTK provide critical docking sites for other proteins that play key roles in downstream signal transduction (Lemmon & Schlessinger 2010). They typically contain SH2 and PTB domains, as well as polyproline motifs, that facilitate assembly of multi-protein complexes via phosphorylated tyrosine residues. Some scaffold proteins, such as FRS2 and GAB1, which are critical for FGFR and MET signaling respectively (discussed further below), also contain lipid-binding motifs that localize them to the plasma membrane. Adaptor proteins are smaller proteins that connect two molecules during signal transduction and typically only contain SH2 and SH3 domains (Pawson 2007). These include the GRB2 and the CRK family of proteins. Scaffold and adaptor proteins function to both consolidate and integrate signals from diverse upstream sources and amplify them into downstream specific cellular processes.

1.2.3 HGF/MET signaling

1.2.3.1 MET and the hepatocyte growth factor

The MET RTK was discovered as part of a transforming fusion protein, translocated promoter region (TPR)-MET, from a mutagenesis screen in human osteosarcoma (HOS) cells (Cooper et al. 1984; M. Park et al. 1987). The name MET is not an acronym, but comes from the methyl group of the mutagen used in this screen, *N*-methyl-*N*'-nitro-*N*-nitroso-guanidine (MNNG). MET is a member of a subfamily of RTKs that includes Recepteur d'Origine Nantais (RON) (Huff et al. 1993). Structurally, MET is produced as a single chain precursor that requires proteolytic cleavage to produce a short (50 kDa) alpha chain and a transmembrane (150 kDa) beta chain (Komada et al. 1993). The two chains constitute a disulphide-liked heterodimer and fold into a sema domain to form the extracellular ligand-binding region of MET. Following the sema domain, the beta chain also contains a cysteine-rich region, four immunoglobulin-like domains, a single-pass transmembrane domain, a juxtamembrane domain, a kinase domain, and a C-terminal tail containing multiple tyrosine residues that serve as docking sites for downstream signaling proteins (Gherardi et al. 2003) (Figure 1.5).

The hepatocyte growth factor (HGF) was identified as a serum-derived liver mitogen (Nakamura et al. 1989). A separate group independently identified the scatter factor (SF), a fibroblast-derived factor that caused colonies of epithelial cells to scatter (Stoker et al. 1987). HGF and SF were later determined to be the same protein (Naldini et al. 1991) and the ligand for MET, or hepatocyte growth factor receptor (HGFR) (Bottaro et al. 1991). HGF is secreted as a single-chain precursor that is cleaved by a number of extracellular cell surface and soluble proteases, including urokinase-type plasminogen activator (uPA) (Naldini et al. 1992), tissue-type plasminogen activator (tPA) (Mars et al. 1993), HGF activator (HGFA) (Miyazawa 2010), hepsin

(Owen et al. 2010), and matriptase (S.-L. Lee et al. 2000) to produce an active alpha-beta disulphide-linked heterodimer (Naka et al. 1992). Modulation of HGF processing provides an additional layer of regulation for HGF/MET signaling, allowing tissues to respond quickly to stimuli like injury. MET is typically expressed by epithelial cells, while HGF is secreted by mesenchymal cells and activates the receptor in a paracrine manner. MET and HGF bind in a 2:2 ratio (Gherardi et al. 2006) and together they activate an EMT program of invasive growth and migration that is essential during both development and in adulthood (Birchmeier et al. 2003).

Genetically modified mouse models provided insight into the biological function of MET signaling. *Hgf*- embryos die *in utero* due to defective placental development that compromises the exchange of oxygen and nutrients between maternal and fetal blood (Uehara et al. 1995). MET signaling is also crucial for the long range migration of muscle progenitor cells. HGF is expressed in front of the path of MET-expressing myogenic precursor cells which delaminate from the dermomyotome and travel to form limb, tongue, and diaphragm muscles (X. M. Yang et al. 1996). Ablation of *Hgf* or *Met* in mice results in the absence of these muscles (Bladt et al. 1995; Dietrich et al. 1999). In adults, MET signaling is mainly involved in organ regeneration and wound healing. HGF levels increase sharply in response to injury, and the ensuing activation of MET signaling is important in organs such as the liver (Miyazawa et al. 1989), kidney (Kawaida et al. 1994), lung (Ohmichi et al. 1996), heart (Nakamura et al. 2000), and skin (Cowin et al. 2001). Accordingly, mice with conditional deletion of *Met* in the liver exhibit defective organ regeneration upon toxic insult and hepatectomy (Borowiak et al. 2004; Huh et al. 2004). MET also promotes the proliferation and migration of keratinocytes to repopulate and close wound sites (Yoshida et al. 2003).

1.2.3.2 MET signaling cascade

Upon activation via HGF binding and receptor dimerization, MET becomes phosphorylated on tyrosine residues Y1234, and Y1235 in the activation loop in the kinase domain, stabilizing an open conformation allowing for ATP-binding (Gherardi et al. 2012), leading to the phosphorylation of tyrosine residues Y1349 and Y1356 in the C-terminal tail which serve as substrate-binding sites (Fournier et al. 1996; Zhu et al. 1994; Ponzetto et al. 1994). Serine985 and Y1003 in the juxtamembrane domain are also phosphorylated to facilitate binding of protein kinase C (PKC) (Gandino et al. 1994) and casitas B-lineage lymphoma (CBL) E3 ubiquitin ligase (Abella et al. 2005; Peschard et al. 2001), respectively, both of which negatively regulate MET signaling.

Signaling proteins that are directly recruited to MET include growth factor receptor-bound protein 2 (GRB2), p85 subunit of phosphoinositide 3-kinase (PI3K), the GRB2-associated protein 1 (GAB1), as well as the GRB2-associated ubiquitin ligase CBL. GAB1 is the major scaffold protein recruited to MET and contains a unique MET-binding domain (MBD) that allows it to bind directly to the receptor (Lock et al. 2000; Weidner et al. 1996). This robust and prolonged interaction is essential for many of the cellular processes downstream of MET. Indeed, *Gab-1-/-* mice exhibit similar phenotypes to *Met-/-* and *Hgf-/-* mice, including embryonic lethality (Sachs et al. 2000). GAB1 recruits additional signaling substrates like the p85 subunit of phosphoinositide 3-kinase (PI3K) (Maroun et al. 1999) and SRC homology 2 domain-containing phosphatase 2 (SHP2) (Maroun et al. 2000), PLCγ (Gual et al. 2000), c-10 regulator of kinase (CRK) adaptor protein (Garcia-Guzman et al. 1999), and p21-activated kinase 4 (PAK4) (Paliouras et al. 2009) to the MET signaling complex. GAB1 is also indirectly recruited by the adaptor protein GRB2, which binds to MET via its SH2 domain (Schaeper et al. 2000) (Figure 1.5).

Recruitment of the p85 subunit of PI3K to GAB1 permits phosphorylation of Ak strain transforming (AKT) and activation of its downstream pathways that are required for HGF-dependent cell survival and migration (Royal & M. Park 1995; Maulik et al. 2002). Recruitment of SHP2 to GAB1 is important for the sustained activation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, crucial for promoting proliferation and cell invasion (Maroun et al. 2000). GRB2 also facilitates activation of the MAPK pathway by recruiting son of sevenless (SOS) guanine exchange factor to the plasma membrane to activate the RAS GTPase, which initiates the cascade of phosphorylation events resulting in the phosphorylation of ERK (Dhillon et al. 2007). PLCγ, CRK, and PAK4 are all involved in the facilitating the actin cytoskeleton remodeling that is required for driving cell motility (Frigault et al. 2008).

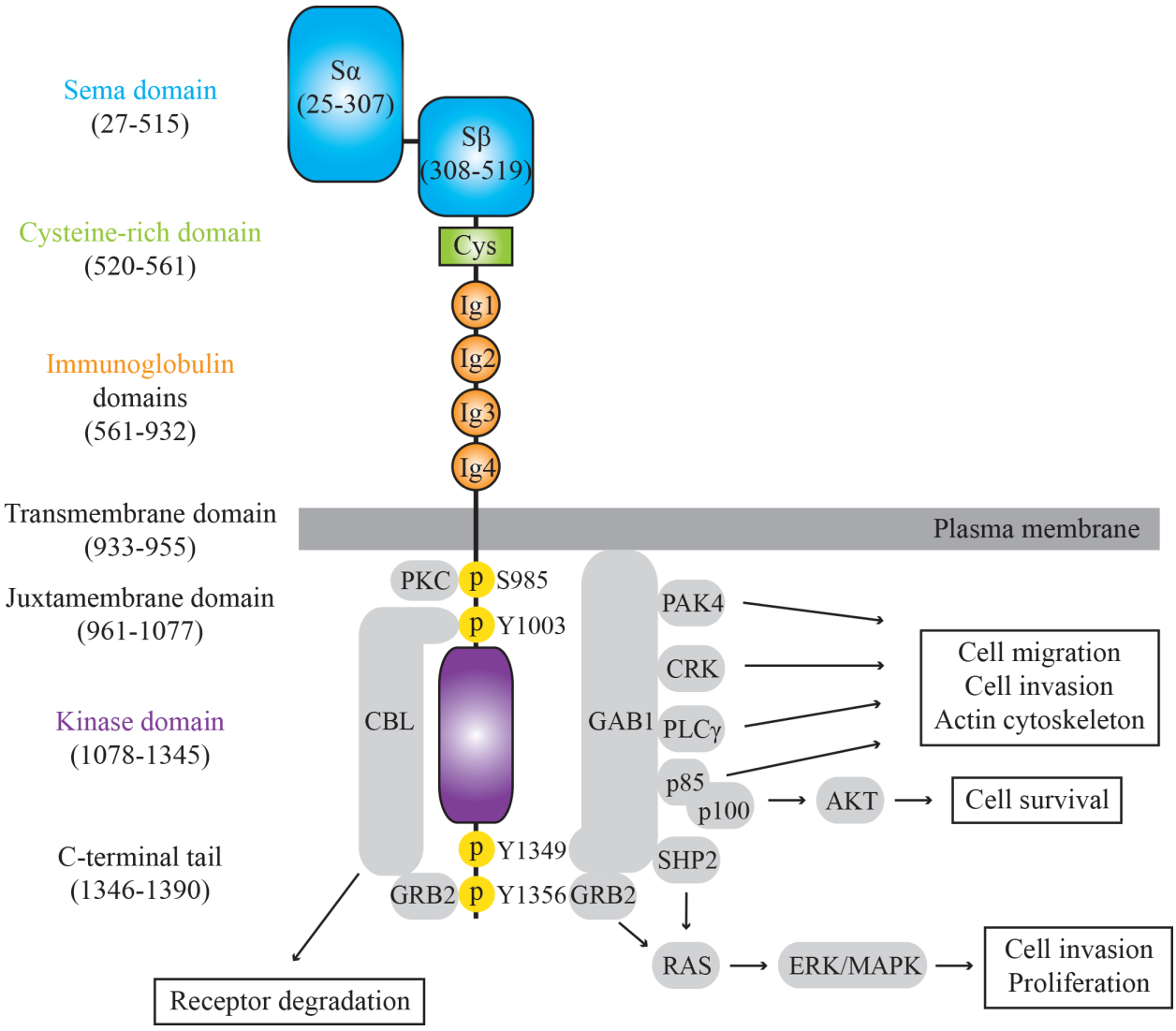


Figure 1.5 MET receptor tyrosine kinase structure and major signaling pathways. The extracellular domains of MET include a Sema domain, a cysteine-rich domain, and four immunoglobulin domains. The intracellular domains include a juxtamembrane domain, a kinase domain, and a C-terminal tail. Amino acids of each domain are indicated in brackets. The intracellular domains of MET contain phosphorylated residues (indicated in yellow) that create substrate-binding sites. Recruitment of downstream signaling substrates are shown, along with their resulting biological processes. See text for details.

1.2.3.2 MET signaling in cancer

The biological functions that MET promotes can contribute to tumour growth and metastasis when dysregulated. Aberrant MET signaling is found in a variety of human cancers and is typically associated with poor prognosis (www.vai.org/met). The mechanisms that promote elevated MET signaling are many (Figure 1.6). Overexpression of the wildtype MET receptor is frequently observed, and can be attained through transcriptional upregulation stimulated by other tumour-associated events such as hypoxia (Pennacchietti et al. 2003), pro-angiogenic factors and mitogens including HGF itself (Boccaccio et al. 1994), and loss of the p53 tumour suppressor (Hwang et al. 2011; Rong et al. 1995). Amplification of *MET* has been observed in cancer types such as gastric cancer (Kuniyasu et al. 1992), melanomas (Hayward et al. 2017), non-small cell lung carcinoma (NSCLC) (Lutterbach et al. 2007), glioblastomas (Snuderl et al. 2011), and colorectal cancer (Di Renzo et al. 1995). Mutations in MET are also detected, though less frequently than overexpression or amplification. Activating missense mutations have been identified in both sporadic and hereditary renal cell carcinoma (Schmidt et al. 1997), hepatocellular carcinomas (W. S. Park et al. 1999), and head and neck squamous cell carcinoma (Di Renzo et al. 2000). Mutations and deletions within the juxtamembrane domain (exon 14), observed in gastric (J. Lee et al. 2015) and lung cancers (Paik et al. 2015; Kong-Beltran et al. 2006), disrupt negative regulation of MET and result in delayed degradation and sustained signaling. Genomic rearrangements of *MET* also occur in cancer; indeed, as previously described, MET was discovered as a fusion protein with the TPR promoter, mostly occurring in gastric cancer (Soman et al. 1991). More recently, several new MET fusion proteins have been discovered in different types of brain tumours, occurring at remarkably high frequencies (10-15%) (Z.-S. Bao et al. 2014; International Cancer Genome Consortium PedBrain Tumor Project 2016). While all of the above mechanisms lead to ligand-independent

MET activation, increased HGF-activated MET signaling is also observed in cancer, occurring in both autocrine (Q. Xie et al. 2012; Kentsis et al. 2012; Toiyama et al. 2011) or paracrine manners (Boccaccio et al. 1994). More recently, MET was shown to be required for the recruitment and cytotoxic functions of infiltrating neutrophils in response to HGF, highlighting a novel anti-tumour role for HGF-stimulated MET signaling (Finisguerra et al. 2015).

Another role that MET alterations play in cancer is an acquired mechanism of resistance against inhibitors of other oncogenic RTKs, such as the epidermal growth factor receptor (EGFR). In NSCLC that harbor *EGFR* mutations, treatment with EGFR inhibitors (erlotinib, gefitinib) results in the selection of *MET*-amplified clones that confer therapy resistance by activating downstream survival pathways (Turke et al. 2010). *MET*-amplified clones can either be pre-existing due to tumour heterogeneity (Turke et al. 2010) or induced *de novo* by treatment due to genomic instability (Engelman et al. 2007). Accordingly, treatment of these resistant tumours with a combination of MET and EGFR inhibitors led to a therapeutic response (Pietrantonio et al. 2016; Bardelli et al. 2013). In cancer cell lines, HGF-stimulated MET-signaling can also provide at least partial compensatory signaling for a number of other oncogenic kinases when they are targeted for inhibition (Wilson et al. 2012).

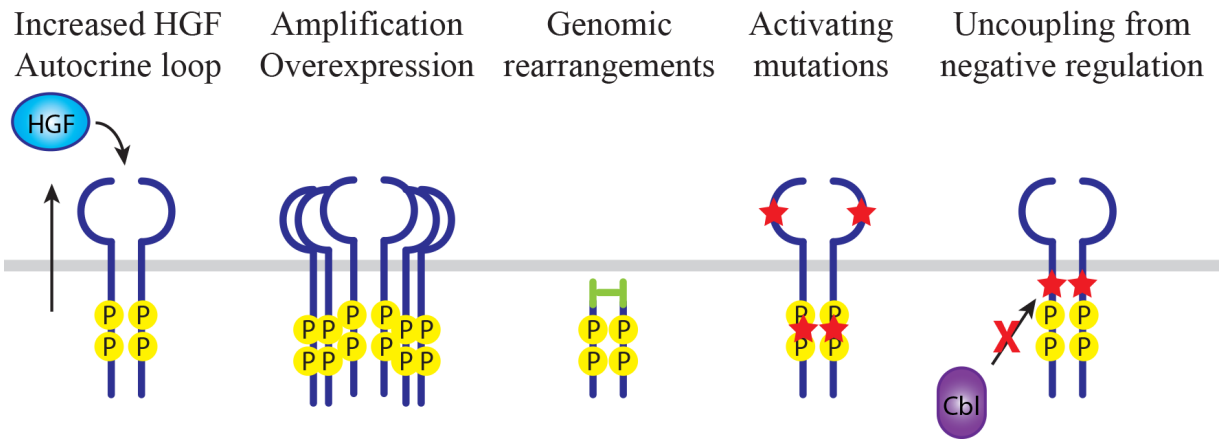


Figure 1.6 Mechanisms of aberrant MET signaling in cancer. Diverse mechanisms promote elevated MET signaling, including paracrine and autocrine HGF stimulation, overexpression of the receptor due to transcriptional upregulation or genomic amplification, genomic rearrangement, and mutations. These aberrations are found in a wide range of human cancers. See text for details.

1.2.3.3 Therapeutic targeting of MET signaling

Given that MET signaling is implicated in a wide range of human cancers, numerous therapeutic agent inhibiting MET activity have been devised and are in clinical trials (Comoglio et al. 2018). These mainly include antibodies against HGF/MET and small molecule MET inhibitors.

Anti-HGF antibodies act by binding and sequestering HGF, and preventing it from binding MET, while anti-MET antibodies compete with HGF for MET binding. The clinical trial results for both of these type of agents have been mixed. One trial involving AMG102 (Rilotumumab), an antibody against HGF, had to be terminated early due to an increased number of deaths in the antibody arm versus the placebo (Catenacci et al. 2017). Antibodies against MET were initially problematic because their bivalent structure caused them to have agonistic instead of antagonistic

properties, inducing the biological effects of HGF stimulation (M. Prat et al. 1998; Greenall et al. 2012). The problem has since been circumvented by the development of monovalent antibodies and bivalent antibodies that induce MET internalization and degradation without activation, most notably MetMab (Onartuzumab) (Martens et al. 2006) and LY2875358 (Emibetuzumab) (L. Liu et al. 2014), respectively.

Small molecule kinase inhibitors against MET have attracted by far the largest effort from the pharmaceutical industry (Comoglio et al. 2018). These molecules typically compete with ATP for binding to the active kinase site of MET, preventing signal transduction (J. Zhang et al. 2009). Both multi-target tyrosine kinase inhibitors and inhibitors specific for MET have shown promising results in clinical trials when patients were pre-selected for *MET* alterations (Paik et al. 2015; T. M. Bauer et al. 2016). The broad spectrum inhibitor PF02341066 (Crizotinib), which is approved for clinical use in NSCLC with genomic rearrangements involving ALK and ROS1 receptors (Ou 2011; Joshua et al. 2018), has shown substantial anti-tumour effects in esophageal (Lennerz et al. 2011), lung (Melody A Mendenhall MSN & MD 2015), and glioblastoma (Chi et al. 2012) tumours that harbour MET amplifications and/or exon 14 deletions.

1.2.4 MET signaling in tumour-initiating cells

The role of MET signaling in tumourigenesis has been investigated in several cancers (Luraghi et al. 2014; C. Li et al. 2011; Kentsis et al. 2012), most extensively in glioblastomas where it has been found to be a functional marker of TICs, conferring metastatic potential and radiation resistance (Y. Li et al. 2011; De Bacco et al. 2016; Joo et al. 2012; De Bacco et al. 2012). High MET expression is associated with the mesenchymal subtype of glioblastoma, and MET-positive neurospheres (tumorspheres generated from glioblastoma cells) are also enriched for the

gene signature of mesenchymal glioblastoma (De Bacco et al. 2012). This is consistent with MET signaling initiating a cellular program associated with the mesenchymal phenotype and promoting plasticity. Given that in normal tissues MET tends to be expressed by stem or progenitor cells (Boccaccio & Comoglio 2006), the association with TICs likely indicates either an expansion of transformed stem-like cells or a reversion of transformed cells into a less differentiated state. Interestingly, various isoforms of CD44, a cell adhesion protein that is commonly used as a cell surface marker for TICs, are known binding partners for MET. CD44 variant 3 (CD44v3) binds heparin sulfates which bind HGF, functioning as a co-receptor for MET and enhancing its signaling activity (Wielenga et al. 2010; van der Voort et al. 1999). Co-expression of MET and CD44v3 correlates with poor prognosis in colon cancer (Wielenga et al. 2010). CD44v6 is required in some cell lines for full HGF-dependent MET phosphorylation and efficient activation of the MAPK pathway (Orian-Rousseau et al. 2002). Targeting TPR-MET to the plasma membrane induces an autocrine signaling network involving hyaluronic acid and CD44, enhancing cellular transformation (Kamikura et al. 2000). This functional partnership further implicates MET signaling as a key player in sustaining TIC populations.

1.2.5 FGFR signaling in tumour-initiating cells

Fibroblast growth factor receptors (FGFRs) are a family of four highly conserved single pass transmembrane RTKs (FGFR1-4) that bind 18 known FGF ligands (Turner & Grose 2010). A fifth related receptor, FGFR5 (also known as FGFRL1), can also bind FGFs but contains no tyrosine domain (Wiedemann & Trueb 2000). The binding specificity of FGFs to FGFRs is established through the differing ligand-binding capacities of each receptor (X. Zhang et al. 2006), alternative splicing of FGFRs, and tissue-specific expression of ligands, receptors, and other cell-surface

proteins (Turner & Grose 2010). Binding of a FGF to FGFR1-4 induces receptor dimerization, enabling an active conformation of the receptor which allows ATP-binding and subsequent phosphorylation of tyrosine residues in the juxtamembrane, kinase, and C-terminal domains (Lemmon & Schlessinger 2010). These phosphotyrosine residues function as docking sites for signaling substrates and allow the activated FGFR to assemble a signaling complex that includes PLCγ, as well as GRB2 and SHP2 via recruitment by fibroblast growth factor receptor substrate 2 (FRS2) (discussed further below). The biological outputs of FGFR signaling are context-dependent, and include proliferation, migration, and regulation of differentiation (Turner & Grose 2010).

FGFR signaling regulates fundamental pathways during embryonic development, and is important in adulthood for processes such as angiogenesis (Javerzat et al. 2002) and wound repair (Meyer et al. 2012). Dysregulation of FGFR signaling due to genetic alterations is implicated in many types of human cancers (Babina & Turner 2017). Importantly, FGF2 (or bFGF)-activated FGFR signaling is one of the most significant regulators of self-renewal capacity and maintenance of the undifferentiated state in stem cells (Dvorak et al. 2005; Amit et al. 2000; Xu et al. 2001). Consistent with this function in normal physiology, FGF2 (as well as other FGFs) is capable of promoting the tumourigenic potential of cancer cells by inducing a mesenchymal program (Fillmore et al. 2010; Marek et al. 2008; Shirakihara et al. 2011). Triple negative breast cancer cell lines with a mesenchymal phenotype exhibit autocrine secretion of FGF2 (Sharpe et al. 2011). Finally, FGFR signaling has been directly shown to expand TIC populations in a range of human cancers (S. Kim et al. 2013; Maehara et al. 2017; McDermott et al. 2018; Song et al. 2017)

1.3 FRS2 scaffold protein

1.3.1 FRS2 protein family

The FRS2 family of proteins has two members, FRS2α/FRS2 and FRS2β/FRS3. Both are similar in structure, containing a myristoylation site at the N-terminus which binds them to the plasma membrane constitutively and a PTB domain for binding specific tyrosine residues on RTKs (Schlessinger 2000). FRS2 has six tyrosine phosphorylation sites, four of which bind GRB2 and two of which bind SHP2 (Kouhara et al. 1997; Hadari et al. 1998). In contrast, FRS3 has one fewer GRB2 binding site but has a direct binding domain for ERK (L. Huang et al. 2006). FRS2 and FRS3 appear to have some differences in RTK affinities, but activation of either protein results in robust activation of predominantly the RAS/ERK pathway (Gotoh 2008). The following sections focus exclusively on signaling through FRS2α, referred to as FRS2.

1.3.2 Regulation of FRS2 signaling

FRS2 is phosphorylated by a limited species of RTKs, most prominently FGFRs, neurotrophin receptors (Ong et al. 2000; Gotoh, Laks, et al. 2004), but also RET (Kurokawa et al. 2001; Melillo et al. 2001), and ALK (Degoutin et al. 2007). FRS2 binding to FGFRs is unique in that it can occur stably with or without activation of the receptors (Ong et al. 2000). In contrast, binding between the PTB domain of FRS2 and other RTKs requires activation-dependent tyrosine phosphorylation in the juxtamembrane domain.

Upon activation, GRB2 and SHP2 are recruited to FRS2 via their respective binding sites (Figure 1.7). GRB2 forms a complex with FRS2 along with SOS (Kouhara et al. 1997), GAB1 (Ong et al. 2001), CBL (Wong et al. 2002), which enables activation of the RAS/ERK pathway,

PI3K pathway, and ubiquitination and degradation of FRS2, respectively. The FRS2-SHP2 complex induces strong activation of the ERK pathway, stronger than that by GRB2 (Hadari et al. 1998). In turn, activated ERK negatively regulates FRS2 by phosphorylating it at several threonine residues (Lax et al. 2002). ERK-mediated negative regulation can occur in the absence of FRS2 activation, providing a feedback mechanism for regulating FRS2-dependent pathways (Lax et al. 2002).

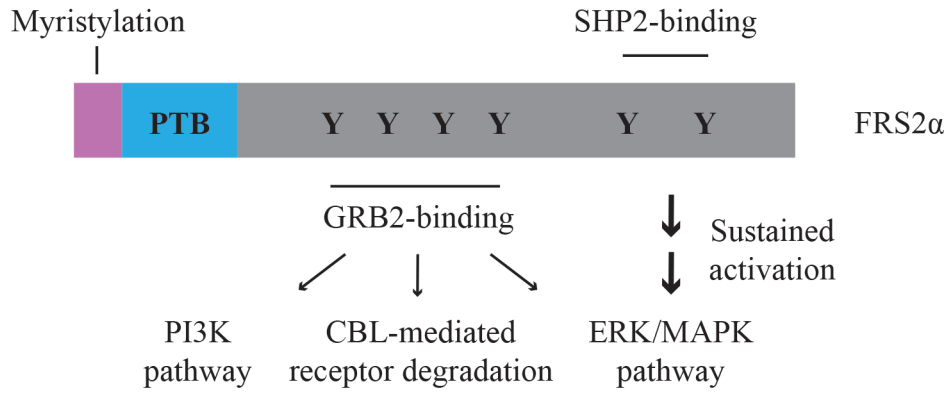


Figure 1.7 FRS2 structure and downstream signaling pathways. FRS2α/FRS2 contains a myristylation site that anchors it to the plasma membrane, a PTB domain to bind to RTK, four GRB2-binding sites, and two SHP2-binding sites. SHP2 induces a strong, sustained ERK signal, whereas GRB2 recruits additional proteins allowing it to also activate the PI3K pathway and receptor degradation. See text for details.

1.3.3 FRS2 signaling in development

FGF signaling plays a fundamental role in many aspects in development (Turner & Grose 2010). FRS2 is required to facilitate many of these FGF-stimulated developmental activities (Gotoh 2009), as well as those regulated by its other associated RTKs (Easton et al. 2006; Ong et al. 2000). Studies in mouse embryos specifically investigating Frs2 revealed that it is strongly expressed in

the extraembryonic ectoderm where trophoblast stem (TS) cells reside and ubiquitously expressed throughout development (Gotoh, Laks, et al. 2004; Gotoh et al. 2005). *Frs2*-null mice show early embryonic lethality due to a failure to maintain self-renewing TS cells that are dependent on FGF4-stimulated signaling (Gotoh et al. 2005). Interestingly, mutant mice expressing Frs2 lacking the four Grb2-binding sites ($Frs2\alpha^{4F/4F}$) exhibited only mild development defects, while Frs2 lacking the two Shp2-binding sites ($Frs2\alpha^{2F/2F}$) resulted in severe defects and perinatal death (Gotoh, Ito, et al. 2004). Subsequent studies found that the SHP2-binding sites on FRS2 play a critical role in maintaining the self-renewal and proliferative capacity of neural stem/progenitor cells by acting as the primary regulator of ERK activity (Yamamoto et al. 2005; Sato et al. 2010).

1.3.4 FRS2 signaling in cancer

FGFs and their receptors have well-established pro-tumourigenic functions in a variety of human cancers (Babina & Turner 2017). As the main scaffold protein downstream of FGFRs, FRS2 has predictably been found to contribute to FGF-dependent oncogenic signaling (Manuvakhova et al. 2006; Valencia et al. 2011; J. Liu et al. 2016). More recently, FRS2 was identified as a recurrently amplified gene in high-grade serous ovarian cancer (HGSOC) in two independent studies (Luo et al. 2015). *FRS2*-amplified ovarian cancer cell lines are dependent on FRS2 for survival, and FRS2 overexpression in immortalized human embryonic kidney and ovarian epithelial cell lines confer anchorage-independent growth *in vitro* and tumourigenesis *in vivo* (Luo et al. 2015). The 12q15 chromosomal region containing *FRS2* is also amplified in high grade liposarcomas (Wang et al. 2011), gliomas (Fischer et al. 2008), and breast adenocarcinomas (Luo et al. 2015). Interestingly, consistent with observations of other scaffold proteins and their associated RTKs (Dunn et al. 2014; Cheung et al. 2011), amplification of *FRS2* and *FGFR*s are typically mutually exclusive

(Luo et al. 2015), further supporting that FRS2 can function as an oncogene independently of an RTK. A recent study identified the N-terminal myristoylation site of FRS2 as a potential therapeutic target, where treatment with a myristoyl-CoA analog inhibited FGF-mediated tumour progression in a variety of cancer cell types (Q. Li et al. 2018).

1.4 Human breast cancer tumourigenesis

1.4.1 Breast cancer overview and statistics

Breast cancer is the most common cancer (excluding non-melanoma skin cancers) and the second-leading cause of death from cancer in Canadian women (Canadian Cancer Society 2018). It is also possible, but rare, for men to develop breast cancer. 1 in 8 women are expected to develop the disease in her lifetime and 1 in 31 will die from it. In 2017, breast cancer accounted for 25% of all new cancer cases and 13% of all cancer-related deaths in women in Canada (Canadian Cancer Society 2018). While breast cancer incidence has held relatively steady since the 1980's, mortality rates have decreased by 44% due to a combination of increased screening and therapeutic advancements (Canadian Cancer Society 2018). Currently, the 5-year survival rate for women diagnosed with breast cancer in Canada is 87%. However, breast cancer is a highly heterogeneous disease comprised of multiple subtypes exhibiting distinct histological and molecular characteristics, leading to different therapeutic options and prognoses (discussed further below).

Upon diagnosis, breast tumours are assessed using several established clinical parameters to predict patient prognosis and inform treatment decisions (Haybittle et al. 1982). Tumour stage is determined based on the size of the disease and the extent to which it has spread locally or to the rest of the body. Higher stage cancers are associated with worse patient outcome (Elkin et al.

2005; Woodward et al. 2003). Tumour grade refers to the level of differentiation exhibited by the cancer cells, as defined by how morphologically similar they are to normal, non-cancerous breast epithelial cells. High grade, poorly differentiated tumours are indicative of aggressive disease and are also associated with worse prognosis (BLOOM & RICHARDSON 1957).

1.4.2 Breast tumour initiation and progression

1.4.2.1 Normal mammary gland development

Most of our understanding of mammary development comes from studies conducted in murine mammary glands, which bear remarkable similarities to the human breast. The mammary gland is organized into lobes filled with hollow ducts that channel milk from milk-secreting alveolar sacs out through the nipple during lactation (Inman et al. 2015). The mammary ducts and alveoli are made up of a bilayer inner luminal epithelial cells and outer myoepithelial cells. While the basic ductal structure is already in place at birth, the ductal epithelium undergoes extensive branching morphogenesis into the mammary adipose tissue at puberty to generate the ductal tree that will fill up the mammary gland (Topper & Freeman 1980). Throughout adulthood, the mammary gland is continuously remodeled in response to hormonal signals during menstrual cycles and pregnancies. This ability to repeatedly remodel through proliferation, differentiation, and apoptosis supports the existence of self-renewing mammary stem cells, the main evidence for which is that a single cell injected into a cleared mammary fat pad can reconstitute an entire functional mammary gland containing both luminal and myoepithelial cells (Kordon & Smith 1998; Stingl et al. 2006; Shackleton et al. 2006). While the earliest lineage tracing study suggested that distinct unipotent stem cells contribute to luminal and myoepithelial cells in the mammary gland (Van Keymeulen et al. 2011), a more recent study combining lineage tracing with 3D *in situ* imaging provided

evidence for the now broadly accepted model that the differentiation hierarchy likely begins with a mammary stem cell at the apex, which gives rise to a bipotent progenitor that differentiates into lineage-committed luminal and myoepithelial progenitor cells (Rios et al. 2014) (Figure 1.8). The cellular signals that maintain undifferentiated progenitors and dictate lineage determination are poorly understood.

1.4.2.2 Breast cancer initiation

The most common form of breast cancer originates in the ductal epithelium. The disease is considered a premalignant ductal carcinoma *in situ* (DCIS) while the abnormally proliferating cells are contained within the lumen of the mammary duct (Schnitt 2003). The breast cancer has progressed to invasive ductal carcinoma (IDC) once malignant cells infiltrate the basement membrane that surround the ducts and invade into the nearby breast tissue (Bombonati & Sgroi 2011). Longitudinal studies have found that 20-50% of DCIS patients later developed IDC at the same site in the breast, suggesting that DCIS is a precursor for IDC (Page et al. 1995; Sanders et al. 2005). Furthermore, DCIS is frequently found adjacent to invasive disease at diagnosis (Evans et al. 1997), and numerous studies have identified genetic similarities between DCIS and IDC (Buerger et al. 1999; Burkhardt et al. 2009; O'Connell et al. 1998). The inability to reliably distinguish between DCIS that will progress to an invasive state and those that will not has meant that nearly all DCIS patients undergo intensive treatment with potentially harmful side effects, whether or not they need it (Vatovec et al. 2014). Recent studies have begun to identify molecular features associated with disease progression that are unique to the different intrinsic subtypes of breast cancer (discussed further below), and established subtype-specific gene signatures that could predict progression to IDC in a subset of patients (Lesurf et al. 2016).

1.4.2.3 Breast cancer metastasis

Progression to metastatic disease is the primary cause of cancer mortality. Metastasis is a multi-step process, requiring cancer cells to break down cell-cell junctions, invade and migrate through the extracellular matrix (ECM), intravasate into the blood stream, survive in circulation in anchorage-independent conditions, and ultimately extravasate into and colonize the parenchymal tissue of a distal organ (Chambers et al. 2002). Breast cancers metastasize most frequently to the lungs, brain, bones, and liver, and exhibit subtype-specific patterns of metastatic spread (Kennecke et al. 2010).

The steps required for successful metastasis by epithelial cancer cells involve specialized cellular functions typically associated with mesenchymal cells, including the dissolution of junctional proteins, production of ECM-degrading enzymes, increased capacity for invasion and migration, and anoikis resistance. Therefore, while overt EMT is rarely observed in human cancers, metastatic cells employ a partial EMT program that typically involves upregulation of one or a combination of the transcription factors SNAIL1/2, TWIST1/2, and ZEB1/2 (Korpal et al. 2008; Jolly et al. 2015; J. Yang et al. 2004; Barrallo-Gimeno 2005). Loss of apical-basal cell polarity has also been reported to promote metastasis in breast cancer (McCaffrey et al. 2012; Xue et al. 2013). While the loss of cell polarity proteins alone is not sufficient to induce an EMT, it compromises cell-cell adhesions, which disrupts epithelial tissue organization and response to regulatory signals (Chatterjee & McCaffrey 2014).

At the same time, the mesenchymal phenotype that facilitates the successful dissemination to a secondary site is postulated to create a major bottleneck in metastatic progression, as it is not conducive to proliferation. A reversion to a more epithelial phenotype, via mesenchymal-to-epithelial transition, may be required for efficient outgrowth at the metastatic site (Bockhorn et al.

2014; Ocaña et al. 2012). Given the complexity of this process, it is hypothesized that only a select population of cells from a primary tumour possess the capacity to successfully metastasize. In breast cancer, TICs with enhanced ALDH activity have been demonstrated to be enriched in metastatic potential (Marcato et al. 2011; Croker et al. 2008).

1.4.3 Breast cancer subtypes

1.4.3.1 Histopathological subtypes

Breast cancers are categorized into subtypes based on immunohistochemical detection of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). ER+/PR+/HER2- tumours, representing the majority of all breast cancers diagnosed (70%), tend to be low grade, with low metastatic burden and rate of recurrence (Onitilo et al. 2009). These hormone receptor-positive (HR+) tumours are considered hormone-dependent and have the best clinical outcome. HER2+ tumours constitute around 20% of all breast cancers and is characterized by HER2 protein overexpression and amplification (Slamon et al. 1987). This subtype is associated with poor prognosis, but has benefited from therapies directed against HER2 (Slamon et al. 1987; Piccart-Gebhart et al. 2005; Romond et al. 2005). Around 12-17% of all breast tumours are ER-/PR-/HER2-, and these diseases are collectively termed triple negative breast cancers (Foulkes et al. 2010). The TNBC subtype has the worst overall outcome and frequently occur in young women (K. R. Bauer et al. 2007; Foulkes et al. 2010). Since TNBC is a classification based on exclusion from the other 2 subtypes, it is a highly heterogeneous group of diseases, a feature that has been further highlighted by the advent of gene expression profiling of breast cancers (B. D. Lehmann et al. 2011; B. D. Lehmann et al. 2016).

1.4.3.2 Molecular "intrinsic" subtypes

Global gene expression analysis has provided further insights into breast cancer heterogeneity. Using a 496-gene "intrinsic" gene set, breast cancers were divided based on their gene expression profiles into luminal-like, HER2-enriched, basal-like, and normal-like subtypes (Perou et al. 2000). Subsequent studies further divided the luminal-like subtype into luminal A and luminal B (Sørlie et al. 2001), and identified an additional subtype called claudin-low (Herschkowitz et al. 2012). These molecular subtypes show distinct patterns of clinical outcome, with luminal A breast cancers having the best prognosis and basal-like breast cancers having the worst (Sørlie et al. 2001; Sorlie et al. 2003). The original 496 genes have since been refined to 50 genes, known as the prediction analysis of microarrays (PAM) 50 (Parker et al. 2009), and are routinely used in the clinical research to classify breast cancers and predict response to treatment and patient outcome.

The molecular subtypes show remarkable, albeit incomplete, concordance with histopathological subtypes of breast cancer (Malhotra et al. 2010). Most ER+ tumours fall into the luminal A and luminal B subtypes, which also feature relatively high expression of many gene expressed by normal breast luminal cells, such as cytokeratin (CK)8/18 (Perou et al. 2000). The main difference between the luminal subtypes is that luminal B breast cancers exhibit a stronger proliferative profile and have poorer prognosis compared to luminal A (A. Prat & Perou 2011). The HER2-enriched subtype is, as expected, driven by a gene signature associated with the amplification or overexpression of HER2, as well as high expression of genes located in the *HER2* amplicon on 17q21 (Perou et al. 2000). The basal-like and claudin-low subtypes make up approximately 50% and 30% of triple negative breast cancers, respectively (A. Prat & Perou 2011). Basal-like breast cancers are characterized by markers of breast basal/myoepithelial cells, such as CK5/6, 14, and 17, EGFR, and vimentin (Rakha et al. 2008). These tumours are highly

proliferative, genomically unstable, and associated with poor prognosis. The claudin-low subtype features tumours with low expression of tight junction and cell adhesion proteins, such as Claudin 3, 4, 7 and E-Cadherin, and are enriched in expression of stem and mesenchymal gene signatures (A. Prat et al. 2010; Sabatier et al. 2014). Claudin-low breast cancers are also associated with tumours with rare histopathologies like metaplastic and medullary (Sabatier et al. 2014).

Beyond the classic "intrinsic" subtypes of breast cancer, a more recent study divided TNBC into 6 subtypes based on gene expression, including basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) (B. D. Lehmann et al. 2011). After elimination of stromal and immune contamination, these were subsequently refined down to BL1, BL2, M, and LAR, with each subtype showing distinct patterns of response to treatment and clinical outcome (B. D. Lehmann et al. 2016). Furthermore, efforts to identify molecular drivers of breast cancer by integrating genomic and transcriptomic analysis resulted in the classification of 10 novel subgroups, called integrative clusters (InClust), with distinct clinical outcomes (Curtis et al. 2012). These additional subtyping schemes resolve the considerable heterogeneity observed in both the histopathological and "intrinsic" subtypes and will hopefully help to better inform patient stratification and treatment decisions.

1.4.4 MET in breast cancer

Aberrant MET signaling in breast cancer most commonly takes the form of MET protein overexpression. Elevated levels of MET are associated with poor prognosis invasive breast cancers across subtypes (Camp et al. 1999; Ghoussoub et al. 1998; Ho-Yen et al. 2013; Kang et al. 2003; Y. J. Kim et al. 2014; Lengyel et al. 2004; Tolgay Ocal et al. 2003; Yan et al. 2015; Zagouri et al.

2013; Raghav et al. 2012). Increase in both autocrine and paracrine HGF-stimulated MET signaling is also observed in breast cancer, often strongest at the infiltrating margins of tumours (Tuck et al. 1996; Edakuni et al. 2001; Jin et al. 1997). Increased HGF levels in the serum of breast cancer patients is correlated with shorter disease-free survival post-surgery (Yamashita et al. 1994), and with metastasis (Taniguchi et al. 1995). Genetic mutations and genomic amplifications of *MET* are rare in breast cancer. One study found increased copy numbers of *MET* in around 8% of the cases studied, and these were correlated with lower recurrence-free survival and the TNBC subtype (Gonzalez-Angulo et al. 2013). *MET* and *HGF* amplifications have also been linked to HER2+ breast cancer patients who progress on anti-HER2 therapy (Minuti et al. 2012).

High MET levels are associated with basal-like and triple negative subtypes of breast cancer (B. D. Lehmann et al. 2011; Ho-Yen et al. 2013; Y. J. Kim et al. 2014; Charafe-Jauffret et al. 2006). This association is supported by genetically engineered mouse models targeting expression of oncogenic variants of *Met* specifically to the mammary epithelia, which resulted in the development of basal-like mammary tumours (discussed further below) (Ponzo et al. 2009; Knight et al. 2013; Graveel et al. 2009). There is also evidence that constitutive activation of Met in luminal progenitor cells of the mammary epithelium leads to their population expansion and redirects their lineage commitment towards a basal phenotype, potentially contributing to the tumourigenesis of basal-like tumours (Gastaldi et al. 2013). Collectively, these lines of evidence strongly suggest that MET plays a role in the tumourigenesis of basal-like and triple negative breast cancers, however the mechanisms by which this may occur remain poorly understood.

1.4.5 Identification of TICs in breast cancer

Putative populations of breast TICs can be isolated by FACS using cell surface markers including CD24, CD44 (Al-Hajj et al. 2003) and α6 integrin (CD49f) (Gómez-Miragaya et al. 2017), as well as ALDH activity (Ginestier et al. 2007). The most commonly used combination of cell surface markers to delineate breast TICs is $CD44^{+}CD24^{-/low}$, but to date no markers have been found to be universal. There appears to be little overlap between $CD44^{+}CD24^{-/low}$ and $ALDH^{+}$ populations in individual tumours, suggesting that intratumoural heterogeneity in breast cancer may include distinct TIC populations with different proliferative and metastatic capacities (Charafe-Jauffret et al. 2009; S. Liu et al. 2014). There is also significant variability among breast cancer cell lines in both the percentage of cells with ALDH activity and cells expressing the $CD44^{+}CD24^{-/low}$ phenotype (Charafe-Jauffret et al. 2009; Ricardo et al. 2011). These observations highlight the importance of employing multiple experimental techniques when studying breast TICs, including *in vitro* tumoursphere assays, and *in vivo* tumour initiation and limiting dilution assays.

In mouse models of breast cancer, a different set of cell surface markers are used to identify TICs. Many of these are integrins, mainly β1 integrin (CD29) and β3 integrin (CD61), used in combinations that mirror the identification of stem and progenitor cells during normal mammary gland development. CD29, a marker of mammary stem cells, is used in combination with epithelial markers to enrich for TICs in models of basal breast cancer, correlating with the less differentiated pathologies of these tumours (M. Zhang et al. 2008). CD61, a marker of mammary luminal progenitor cells, is used in combination with epithelial markers to enrich for TICs in models of luminal breast cancer, correlating with the more differentiated pathologies of these tumours (Vaillant et al. 2008).

Subtype-dependent patterns of gene expression have provided insight into the potential cellular origins of breast TICs. A comparison of the transcriptional profiles of cells at different stages along the normal mammary development hierarchy with the transcriptional profiles of the intrinsic subtypes of breast cancer found the mammary stem cell signature to be most closely associated with the normal-like and claudin-low subtypes, the luminal progenitor cell signature with the basal-like subtype, and the mature luminal cell signature with the HER2 and luminal subtypes (Lim et al. 2009) (Figure 1.8). These associations are also found to be conserved in murine models of the breast cancer subtypes (Lim et al. 2010). While these findings suggest that claudin-low and basal-like TICs could arise from transformed stem or progenitor cells respectively, it is also possible a more differentiated cell could aberrantly acquire stem-like properties. Likewise, luminal and HER2 tumours could also arise from mammary stem or progenitor cells that continue to differentiate. Regardless of the cellular origins of breast TICs, it is clear that there are distinct tumour progression pathways for each breast cancer subtype.

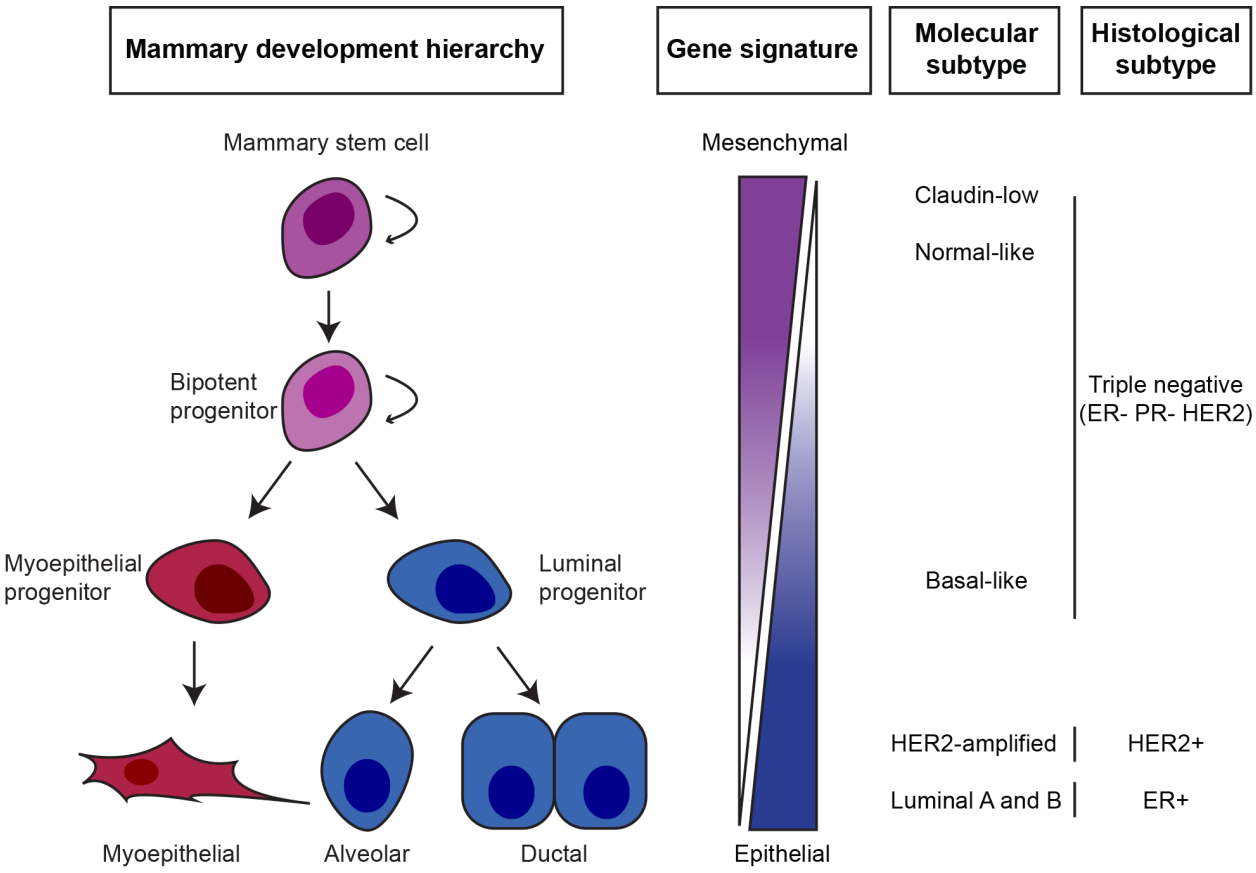


Figure 1.8 The mammary gland development hierarchy and relation to breast cancer subtypes. Normal mammary gland development features a hierarchy of cells that starts with a highly mesenchymal mammary stem cell and ends with terminally differentiated ductal, alveolar, and myoepithelial cells. Transcriptional profiling has found that different subtypes of breast cancer exhibit gene expression signatures that are associated with specific stages along the mammary development hierarchy. See text for details.

1.4.6 Current and emerging treatment options

1.4.6.1 Conventional therapies

Conventional therapies for patients with breast cancer include surgery, radiation, chemotherapy. The actual course of treatment depends primarily on the staging of the breast cancer. Surgical excision of the disease can involve resection of only the tumour (lumpectomy) or removal the

entire breast (mastectomy) (Fisher et al. 2002). Because cancer cells can metastasize through the lymphatic system, sentinel or axillary lymph nodes are also commonly removed and biopsied (Nelson 2004). Radiation therapy is frequently administered in an adjuvant (after surgery) setting to lower the risk of recurrence or in cases of locally advanced breast cancer where the tumour is unresectable (Cuzick 2005). Chemotherapy refers to a range of cytotoxic drugs, including alkylating and anti-microtubule agents, anti-metabolites, and topoisomerase inhibitors. These can be administered adjuvantly, neo-adjuvantly (before surgery) to shrink the tumour, or to treat metastatic breast cancer (Saloustros et al. 2008). Radiation and chemotherapy are both accompanied by considerable side effects, including nausea, hair loss, fatigue, nervous system and organ damage, fertility problems, and in rare cases increased risk of secondary cancers (Partridge et al. 2001). Increased understanding of the cellular and molecular biology of breast cancer has enabled the development of new therapies that are more specifically targeted toward cancer cells and are therefore associated with less severe side effects.

1.4.6.2 Targeted therapies

HR+ breast cancers are treated with endocrine therapies, commonly with compounds that compete with estrogen for ER binding (eg. tamoxifen, fulvestrant) (Jordan 2012) or aromatase inhibitors, which prevent the synthesis of estrogen (eg. letrozole, anastrozole) (Hiscox et al. 2009). HER2+ breast cancers are treated with monoclonal antibodies (eg. trastuzumab) (Slamon et al. 2001) or tyrosine kinase inhibitors (eg. lapatinib) against HER2. Importantly, it is now clear that trastuzumab also functions as an immunotherapy (discussed further below) by activating the immune system via a mechanism called antibody dependent cellular toxicity (Clynes et al. 2000; S. Park et al. 2010).

Currently, treatment for TNBC typically involves combinations of anthracycline and taxane-based chemotherapeutic agents along with surgery, with no clear consensus on therapeutic regimens (Foulkes et al. 2010). These breast cancers lack hormone receptors and HER2 amplification, and therefore do not have well-defined therapeutic targets. The heterogeneity within this subtype has hindered the development of targeted therapies, however several promising therapeutic approaches for TNBC are being investigated (Bianchini et al. 2016). Sequencing of TNBC tumours has revealed their inherent genomic instability, prompting numerous clinical trials attempting to exploit this feature using platinum salts and PARP inhibitors to cause DNA double-strand breaks (Abramson et al. 2015; Tutt et al. 2010). This strategy has seen some success in tumours with defects in DNA repair, including in the nearly 20% of TNBC that harbor *BRCA1/2* mutations. Despite the genomic instability, TNBCs do not carry mutations in established oncogenes at high frequencies; *TP53* mutations or loss occur most frequently (60-70%), followed by *PIK3CA* (~10%) (Koboldt et al. 2012). Inhibitors targeting PI3K, as well as its downstream targets Akt and mTOR, are under investigation to be used in combination with chemotherapies (Yunokawa et al. 2012; S.-B. Kim et al. 2015). The LAR subtype of TNBC shows sensitivity to blockade of the androgen receptor (AR) pathway, with some patients showing partial or complete responses to an AR inhibitor (Gucalp et al. 2013; Traina et al. 2015). The overexpression or activation of several RTKs have also been demonstrated to promote tumour progression in TNBC and are potential therapeutic targets, including EGFR, FGFR, and MET (Jerusalem et al. 2016; Ponzo et al. 2009). Effective treatment of TNBC will require better understanding of the molecular drivers of these diseases in order to inform rational patient stratification and therapeutic decisions.

1.4.6.3 Emerging therapies

TICs are attractive therapeutic targets given the evidence supporting their resistance to conventional and targeted therapies, and their ability to give rise to metastatic lesions (Wei & Lewis 2015). Multiple approaches targeting pathways that regulate breast TICs are under ongoing investigation. These include pathways with well-established functions in normal stem cells, such as Hedgehog (S. Liu et al. 2006), Notch (Harrison et al. 2013), and Wnt (Kakarala et al. 2010). Signaling pathways, like TGFβ (Bhola et al. 2013) and PKCα (Tam et al. 2013), that regulate EMT are also often found to regulate breast TICs. Activation of cell surface receptors like EGFR (Savage et al. 2017), HER2 (Ithimakin et al. 2013), and CXCR1 (Ginestier et al. 2010) also promote breast TICs. Therapies that inhibit these pathways can potentially eradicate TICs but their durability may be hindered by cellular plasticity. There is accumulating evidence to suggest that breast TICs display plasticity that enable interconversion between TIC and non-TIC states. The interconversion can be spontaneous (Chaffer et al. 2011) or induced (Morel et al. 2008), and are facilitated by the EMT program. Thus, targeting the EMT mechanism is a promising therapeutic strategy, as blockade of EMT could both reduce breast TIC populations and inhibit plasticity. This added complexity of TIC plasticity underscores the need to target both TICs and non-TICs in breast cancer treatment. An important consideration for employing therapies that block EMT or induce a mesenchymal-to-epithelial transition is the potential risk of improving conditions for metastatic outgrowth by promoting a more epithelial phenotype.

Approaches that aim to activate or re-activate the immune system to enable enhanced elimination of tumour cells have shown promising results in breast cancer (Solinas et al. 2017). The immune system plays a dual role in breast cancer progression. During early stages of tumourigenesis, acute inflammation at the tumour site activates an innate immune response and

results in tumour cell killing. Cells that escape this immune response eventually cause a shift from acute to chronic inflammation, creating a tumour microenvironment inhabited by suppressive immune cells that are permissive to tumour progression (Gil Del Alcazar et al. 2017; DeNardo & Coussens 2007). Importantly, multiple cell types in the tumour microenvironment can suppress the anti-tumour immune response through immune checkpoint pathways, the most widely studied being the programmed death-1 (PD-1)/programmed death ligand-1 (PD-L1) axis. Tumour and immune cells express PD-L1, which bind PD-1 on the surface of activated T cells and induce an inhibitory signal (Pardoll 2012). Many clinical trials are in progress for antibodies that block the PD-1/PD-L1 pathway, used either as single agents or in combination with chemotherapy (Solinas et al. 2017). Importantly, higher levels of immune infiltration in the tumour at diagnosis predicts better response to chemotherapies and survival (Dushyanthen 2015), particularly in aggressive subtypes like HER2 and TNBC, suggesting that immune activation plays a role in the anti-tumour effects induced by conventional therapies (Teschendorff et al. 2007; Ignatiadis et al. 2012; Desmedt et al. 2008). Indeed, clinical trials using chemotherapy as a trigger to activate the immune system and sensitize patients to immune checkpoint blockade therapy are ongoing and showing promising preliminary results in TNBC (Kok et al. 2018). Other forms of immunotherapy under investigation for breast cancer include blockade of another immune checkpoint protein cytotoxic T lymphocyte associated protein 4 (CTLA-4) (Vonderheide et al. 2010; McArthur et al. 2016), engineered T cells (chimeric antigen receptor T (CAR-T) cell therapy) (Bajgain et al. 2018; Adusumilli et al. 2014), and cancer vaccines against tumour-specific antigens (Clifton et al. 2017; Heery et al. 2015).

1.5 Experimental models of breast cancer

1.5.1 Genetically engineered mouse models of breast cancer

Mouse models have proved extremely useful for studying how aberrations in specific signaling or tumour suppressor pathways altered in breast cancer contribute to tumour initiation and progression, and affect response to treatment (Kersten et al. 2017). While mouse models do not perfectly recapitulate human cancers, they facilitate the study of important aspects of cancer biology that are not possible in other model systems. First, mouse models provide a way to elucidate the step-wise progression from a benign lesion to a malignant tumour. Second, because most mouse models are generated in immune-competent mice, they allow for the study of the complex co-evolution and interactions between tumour cells and their immune environment.

The use of mouse models to study genetic alterations in mammary tumourigenesis has typically followed two strategies – transgenic or knockout mice. Transgenic mice are used to study the effects of gain of function alterations in a gene, while knockout mice are used to study loss of function. A combination of both of these strategies can also be used to generate mice with multiple altered pathways. More recently, the CRISPR/CAS9 genome editing system has been adapted for cancer modeling in mice, including for breast cancer (Annunziato et al. 2016).

1.5.1.1 Tissue-specific promoters

Tissue-specific promoters confer the ability to create genetic alterations only in the desired tissue. This is important if manipulation of a gene of interest in the germline tissue is embryonic lethal or if it results in the development of abnormalities that prevents the investigation of a tissue-specific disease. One of the most commonly used tissue-specific promoters is the mouse mammary tumour

virus long terminal repeat (MMTV-LTR). MMTV is a retrovirus that when infected in mice results in spontaneous mammary tumours. Due to its specificity for the mammary epithelium, its promoter can be used to target expression of genes of interest to the mammary gland (Cardiff & Muller 1993). The MMTV promoter is expressed by luminal and myoepithelial cells, and drives expression of transgene in both virgin and post-partum mammary glands (Wagner et al. 2001). Another frequently used mammary tissue-specific promoter is the whey acid promoter (WAP) which, in contrast to MMTV, is only transcriptionally active mid-pregnancy (Lipnik et al. 2005).

1.5.1.2 Cre-recombinase/loxP system

The Cre-recombinase/*loxP* system was developed to create animals with conditional gene knockouts, since loss of certain genes can result in severe developmental defects or embryonic lethality (Sauer 1998). In this system, the target gene is flanked by palindromic sequences known as *loxP* sites, which are recognized by the Cre-recombinase enzyme (Sauer 1998). In the presence of Cre-recombinase, the *loxP* sites recombine and allow for excision of the flanked gene of interest. Thus, by expressing Cre-recombinase using the MMTV promoter, ablation of the target gene can be isolated to the mammary epithelium.

1.5.1.3 MMTV-Met^{mt} model of basal-like breast cancer

To examine the effect of aberrant Met activation specifically in the mammary epithelium, our lab previously generated transgenic mouse models where the expression of wildtype and weakly transforming mutants of *Met* (M1248T and M1248T/Y1003F) were driven by the MMTV promoter (Figure 1.9), hereafter referred to as MMTV-*Met*^{mt} (Ponzo et al. 2009). M1248T is an activating mutation in the kinase domain, originally identified in papillary renal cell carcinoma

(Schmidt et al. 1997). Y1003F is a mutation in the juxtamembrane domain, which results in the loss of the c-Cbl binding site leading to prolonged signaling due to delayed receptor degradation (Peschard et al. 2001; Abella et al. 2005). While Y1003 mutations are rare in human tumours (Cancer Genome Atlas Research Network 2014), they are comparable to genetic alterations found in non-small cell lung cancer where the exon that codes for Y1003 is partially or fully deleted (delta exon 14) (Kong-Beltran et al. 2006).

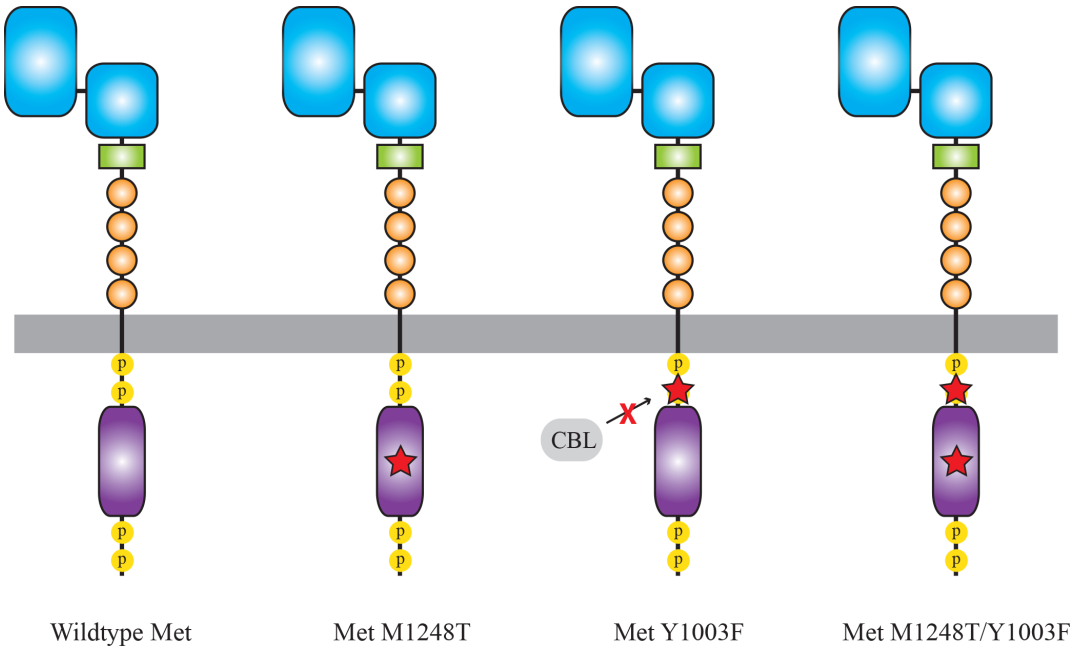


Figure 1.9 Mutant variants of the Met receptor used to generate MMTV-*Met*^{mt} mouse model. Mutant oncogenic variants of Met were overexpressed under the control of the MMTV promoter in FVB/N mice. See text for details.

All MMTV-*Met*^{mt} tumours have constitutively phosphorylated Met, resulting in elevated Akt and Erk activity that could be inhibited by a small molecular inhibitor (PHA665752) targeting Met kinase activity or siRNA against Met. ~50% of MMTV-*Met*^{mt} mice developed mammary tumours with solid nodular histopathology, similar to those commonly observed in tumours arising from mouse models of luminal breast cancers. The remaining 50% of tumours exhibited a range of pathologies, including papillary, scirrhous, adenosquamous, and spindle-cell, and expressed EMT and basal markers. A cross-species transcriptional comparison with human breast tumours confirmed that MMTV-*Met*^{mt} solid tumours clustered among human luminal tumours, whereas MMTV-*Met*^{mt} mixed-pathology tumours most closely resembled human basal-like breast tumours. Tumours with solid histopathology are most commonly generated in mouse models where oncogenes that activate the Ras pathway are driven by the MMTV promoter. The fact that the MMTV-*Met*^{mt} mouse model generated tumours with mixed pathologies suggested that the Met signal specifically promoted a less differentiated, more mesenchymal tumour phenotype. The same study also found that a MET transcriptional signature, enriched for EMT signatures, correlates both with basal-like breast cancer and poor prognosis. At the protein level, MET in combination with the EMT transcription factor SNAIL is a strong predictor of poor outcome in independent cohorts, further supporting a role for MET in basal-like breast cancer via its function as a regulator of EMT.

1.5.1.4 MMTV-Met^{mt};Trp53fl/+;Cre model of claudin-low breast cancer

MMTV-*Met*^{mt} tumours developed at long latencies and low incidence, suggesting that cooperating oncogenic events could be required for efficient tumourigenesis. As previously discussed, loss of

function mutations in *TP53* are the most frequent mutations found in TNBCs. p53-regulated microRNA miR34a is a known regulator of *MET* mRNA levels, and a mouse model of mammary tumourigenesis involving *Trp53* deletion carried spontaneous amplifications of *Met* (Smolen 2006). To investigate the effect of *Trp53* loss during Met-induced mammary tumourigenesis, our lab generated a conditional mouse model where MMTV-Met^{mt} was combined with Cre-recombinase (MMTV-*Cre*) mediated heterozygous deletion of *Trp53* alleles flanked by *loxP* sites (*Trp53fl/+*) in the mammary gland (Figure 1.10), henceforth referred to as MMTV-Met^{mt};*Trp53fl/+*;*Cre* (Knight et al. 2013). MMTV-Met^{mt};*Trp53fl/+*;*Cre* mice exhibited both a significant reduction in tumour latency and increase in tumour penetrance compared to MMTV-Met^{mt} mice. MMTV-Met^{mt};*Trp53fl/+*;*Cre* mice had increased penetrance compared to *Trp53fl/+*;*Cre* mice, however both developed tumours with a predominantly spindloid pathology (80% and 63%, respectively), with the remainder being poorly differentiated adenocarcinomas. Spindloid tumours stained strongly for the mesenchymal marker Vimentin and were negative for the epithelial marker E-cadherin, indicative of an EMT phenotype. Importantly, all MMTV-Met^{mt};*Trp53fl/+*;*Cre* and *Trp53fl/+*;*Cre* spindloid tumours underwent loss of heterozygosity for *Trp53* and selectively amplified their endogenous *Met* locus, supporting a synergy between a strong Met signal and loss of *Trp53* in driving the spindle-cell, EMT-like pathology.

Gene expression analysis revealed that spindloid tumours were characterized by signatures of strong EMT and the Hgf/Met signaling axis, and are highly correlated with the human claudin-low subtype of TNBC. The claudin-low EMT phenotype was dependent on Met activity, where inhibition with small molecule inhibitors (PHA665752 and Crizotinib) resulted in elevated expression of epithelial markers like E-cadherin and Claudin-1. Furthermore, both MMTV-Met^{mt};*Trp53fl/+*;*Cre* and *Trp53fl/+*;*Cre* spindloid tumours were dependent on Met signaling for

survival, proliferation, and metastatic potential. The fact that all *Trp53fl/+*;*Cre* tumours that correlated with human claudin-low breast cancer contained amplification of the *Met* locus suggests that loss of *Trp53* alone is insufficient to drive this phenotype. Thus, the MMTV-*Met*^{mt};*Trp53fl/+*;*Cre* mouse model represents a robust model for studying the role of Met signaling in the tumourigenesis of highly mesenchymal TNBCs.

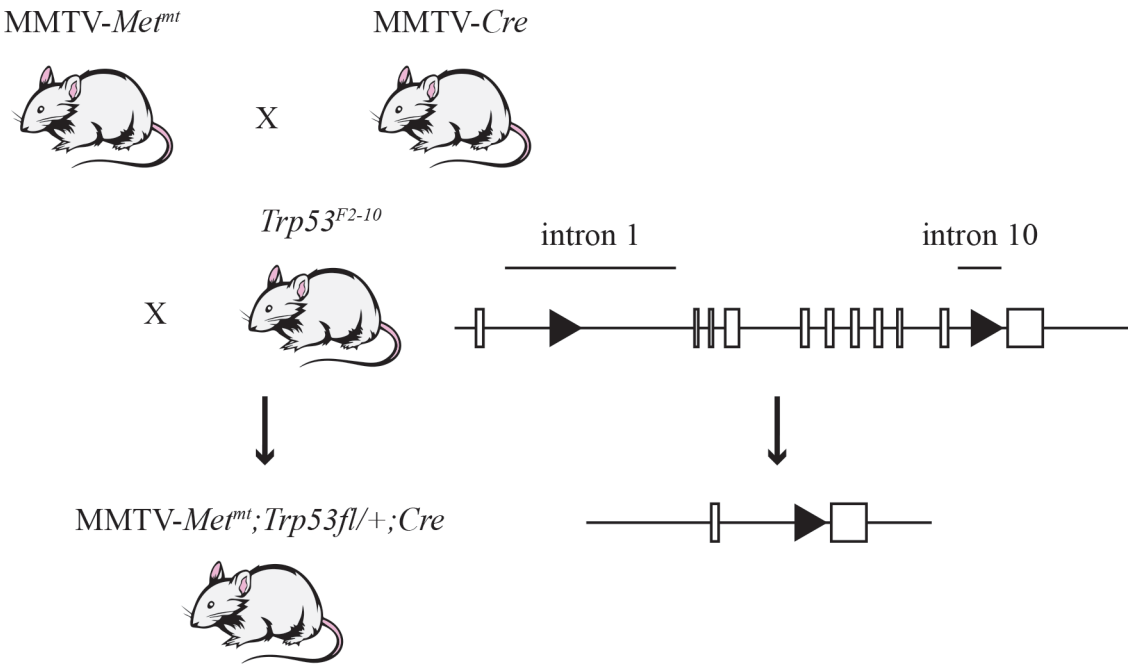


Figure 1.10 Generation of the MMTV-*Met*^{mt};*Trp53fl/+*;*Cre* mouse model of breast cancer. *LoxP* sites (indicated by ▶) were inserted in intron 1 and 10 of the *Trp53* allele, such that Cre-mediated recombination resulted in the excision of exons 2-10. As Cre expression was under the control of the MMTV promoter, the resulting MMTV-*Met*^{mt};*Trp53fl/+*;*Cre* mice exhibited loss of Trp53 only in the mammary gland.

1.5.2 Breast cancer cell lines

The first breast cancer cell line to be established was BT20 in 1958 (LASFARGUES & OZZELLO 1958). Over the past 60 years, more than 70 cell lines derived from primary tumours, pleural effusions, metastatic lesions, and normal breast tissue have been established (www.atcc.org). Molecular profiling of breast cancer cell lines has shown that they can be divided into subtypes that mirror those observed in primary breast cancer (Neve et al. 2006; A. Prat et al. 2010; Kao et al. 2009). Cell lines with basal characteristics are divided into basal A and basal B subtypes, corresponding to basal-like and claudin-low breast cancer, respectively (Neve et al. 2006). To address the heterogeneity of TNBC group, TNBC cell lines were more recently divided into additional subtypes based on their molecular signatures (B. D. Lehmann et al. 2011; B. D. Lehmann et al. 2016).

Although cell lines are used prolifically for studying molecular and cell biology, there have been ongoing debates about how representative cell lines are of human breast cancer. Due to continual passage, cell lines have undergone extensive clonal selection, lack the heterogeneity observed in primary tumours (Gillet et al. 2011), and are unreliable predictors of drug response (Johnson et al. 2001). Cell culture conditions such as culture media and dimensionality cause substantial variation in experimental outcome (Birgersdotter et al. 2005; Streuli et al. 1991; M. A. Matthay et al. 1993). Furthermore, complex *in vivo* interactions between epithelial and stromal components of the tumour are typically missing from *in vitro* cell culture. Efforts to address these issues have led to the development of 3-dimensional culture systems that better model *in vivo* tumour architecture (Debnath & Brugge 2005), and the co-culturing of tumour cells with stromal cells to study their interactions (Shekhar et al. 2000; Goswami et al. 2005; Holliday et al. 2009). Xenograft models using cell lines are also not without limitations. Cells are typically injected into

immunocompromised mice, which preclude evaluation of the important roles that immune cells play in tumour development and response to treatment. In addition, there are profound differences between human and mouse stroma (Hovey et al. 1999). In response, there have been attempts to co-inject human fibroblasts with cancer cell lines (Krtolica et al. 2001), as well as develop mice with "humanized" immune systems (Walsh et al. 2017); however, these approaches tend to be resource-intensive and have not yet been consistently adopted. Thus, while cell lines have been instrumental in advancing breast cancer research, it is important to consider their experimental limitations when interpreting data derived from them.

1.5.3 Patient-derived xenografts

Patient-derived xenografts (PDXs) are primary patient tumour fragments that are transplanted directly into immunocompromised mice and are successively propagated in mice without ever being cultured on plastic. These xenografts recapitulate the histopathology, treatment response, and metastatic potential of the original patient tumours, and are a powerful preclinical tool for studying disease. Many groups have independently established breast PDX cohorts (Savage et al. 2017; DeRose et al. 2011; X. Zhang et al. 2013; Petrillo et al. 2012; Kabos et al. 2012). While PDX models have been established for all clinical subtypes, TNBCs and high grade tumours have higher graft efficiency and are overrepresented compared to their incidence in breast cancer overall. Indeed, engraftment success itself is correlated to decreased overall and recurrence-free survival in patients (McAuliffe et al. 2015; DeRose et al. 2011).

PDXs are not without their limitations as a disease model. The need for engraftment into immunocompromised mice again precludes the study of the immune components of breast cancer. Furthermore, the human stromal component of the primary tumour are lost over successive PDX

passaging (DeRose et al. 2011), and not all murine growth factors and cytokines can stimulate their equivalent human receptors. Finally, while PDXs are found to faithfully recapitulate their matched primary tumours genomically and transcriptionally, single cell sequencing has revealed that clonal selection does occur over serial passaging (Eirew et al. 2015; Petrillo et al. 2012; Manoir et al. 2013).

1.6 Rationale

TNBC is a heterogeneous group of diseases that lacks effective patient stratification strategies and targeted therapies, resulting in poor prognosis. Evidence suggests that TNBCs are enriched with TICs, which enhance tumourigenesis and metastasis, and are resistant to conventional therapies. These functional characteristics of TICs are promoted at least in part by their mesenchymal gene signature.

MET is one of the predominant RTKs that, when activated, coordinates an EMT program. Whilst elevated levels of the MET are associated with TNBCs and predict poor clinical outcome, the functional role of MET in TNBC is poorly understood. Throughout this thesis, I employ established transgenic mouse models, cell lines, and patient-derived xenografts to address two main hypotheses. First, that **MET plays a role in TNBC tumourigenesis and progression by promoting a mesenchymal program that sustains TIC populations**. The second is that **MET can signal through a non-canonical FRS2-dependent pathway to activate pro-tumourigenic signals**. Given that there are already several inhibitors of MET approved for clinical use, establishing the MET pathway as a regulator of TICs offers new potential therapeutic targets in TNBC.

2 RESULTS

2.1 MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid tumour cells are enriched in TICs

As described in the previous chapter, MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid tumours express a strong EMT program (Knight et al. 2013), which is associated with increased tumourigenicity. To evaluate if this is reflective of a higher tumour-initiating potential, we compared the TIC populations of spindloid tumours with that of non-spindloid tumours from the same model, using a range of established assays (Figure 1.2). Using tumour-derived cell lines, we found that spindloid cells (A1005, A1129) were nearly three times more proliferative than non-spindloid cells (A1221, A1222) (Figure 2.1A). The proportion of cells bearing mouse mammary TIC markers $CD24^{+}CD29^{hi}$ or $CD24^{+}CD49f^{+}$ was evaluated in each cell line; however, no differences were observed in the proportion of cells expressing these markers (data not shown). To functionally test TIC capacity, we employed the tumoursphere assay and found that A1005 and A1129 spindloid cells formed more numerous and larger tumourspheres compared to A1221 and A1222 non-spindloid cells, supporting the idea that spindloid cell lines possess a higher proportion of TICs that form tumourspheres with greater proliferative capacity (Figure 2.1B). Limiting dilution assays performed *in vivo* remain the gold standard functional readout for TIC frequency; as such we injected A1129 and A1221 single cells at limiting dilutions (100, 50, and 10 cells) into the 4th mammary fat pads of female athymic mice. The TIC frequency of A1129 cells was four times higher than A1221 cells (1:68 and 1:283 respectively, p=0.023) (Table 2.1), further supporting that MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid, EMT tumour cells are enriched in their TIC compartment.

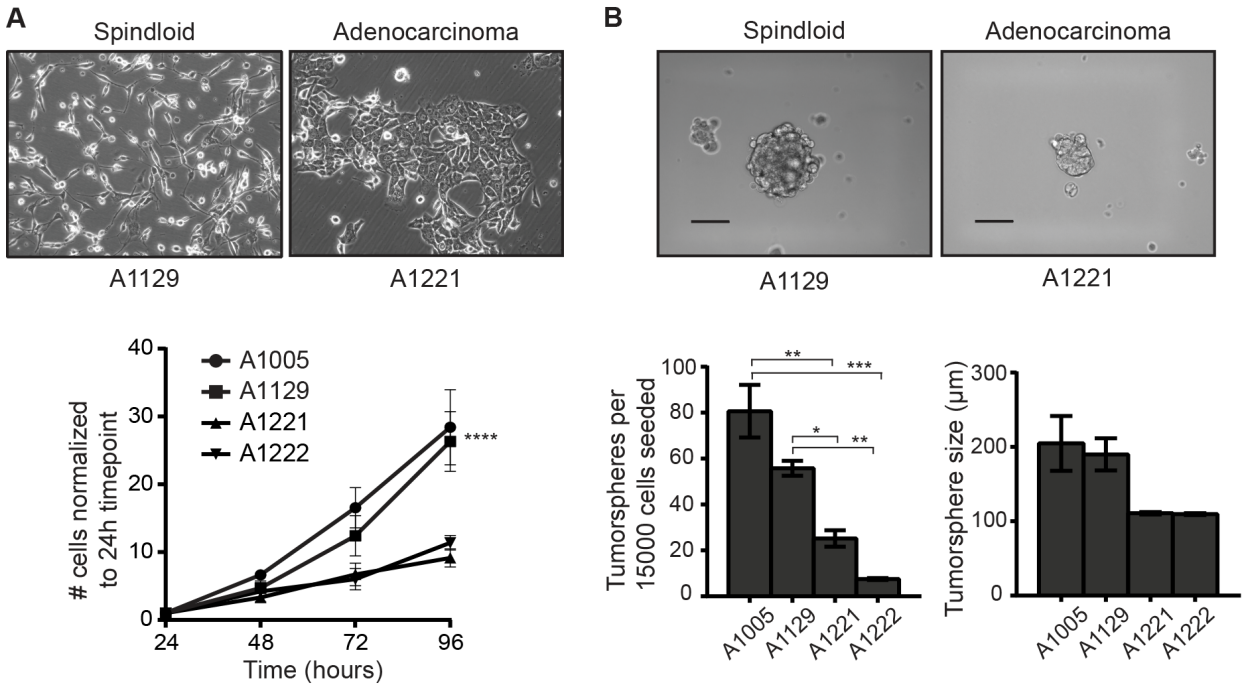


Figure 2.1 MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid tumour-derived cells are more proliferative and have higher sphere-forming efficiencies (SFE) compared to non-spindloid cells. (A) Representative images of MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid cell line A1129 and non-spindloid cell line A1221 in adherent culture are shown (top). The proliferative rates of 2 spindloid (A1005, A1129) and non-spindloid (A1221, A1222) cell lines were assessed for 96h by Trypan blue assay (bottom). (B) Representative images of tumourspheres formed from MMTV-*Met^{mt};Trp53fl/+;Cre* tumour cell lines (top). Tumourspheres were assessed on day 5 for quantity and size using AxioVision (Carl Zeiss) (bottom). Scale bar: 100µm. *p< 0.05; **p ≤ 0.01; ***p≤ 0.001; ****p≤ 0.0001. Error bars indicate SEM.

2.2 Met inhibition decreases but fails to abrogate tumoursphere formation

The EMT phenotype and proliferative capacity of MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid cells on both plastic and in soft agar are dependent on Met kinase activity (Knight et al. 2013). To determine whether active Met signaling was also required to maintain the TIC populations in these cells, A1005 and A1129 tumourspheres were cultured in the presence of the small molecule Met inhibitor Crizotinib. Unexpectedly, Met inhibition decreased but did not abrogate tumoursphere

formation (Figure 2.2A). When we examined the effect of Met inhibition on downstream signaling effectors by Western blot, we found that while Akt showed a corresponding decrease in phosphorylation, Erk activation remained unaffected (Figure 2.2B). The ability of RTKs to engage in compensatory signaling is well-documented (Wilson et al. 2012); therefore, we hypothesized that the loss of Met signaling might be compensated for by growth factors present in the sphere culture medium, namely EGF, bFGF, or insulin. To test this, tumoursphere assays were repeated with sequential removal of individual growth factors. Strikingly, in the absence of bFGF, Crizotinib fully abrogated both A1005 and A1129 tumoursphere formation (Figure 2.2C). Thus, signaling through the FGFR pathway compensates for loss of Met signaling to sustain TICs in MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid cells.

Tumor incidence in limiting dilution assay

Tumor cell lines	Number of cells injected			TIC frequency	95% CI	*P*
	10	50	100			
	Proportion of positive grafts					
A1129 spindloid	1/8	4/6	4/6	1:68	1:34-1:137	0.771
A1221 non-spindloid	0/8	1/6	2/6	1:283	1:92-1:874	0.544

Table 2.1 Tumour incidence in limiting dilution assay. Cells were injected into the mammary fat pads of female athymic mice at the indicated numbers. Tumour-initiating capacity was calculated using Extreme Limiting Dilution Analysis online software (http://bioinf.wehi.edu.au/software/elda/) 12.5 weeks post-inoculation.

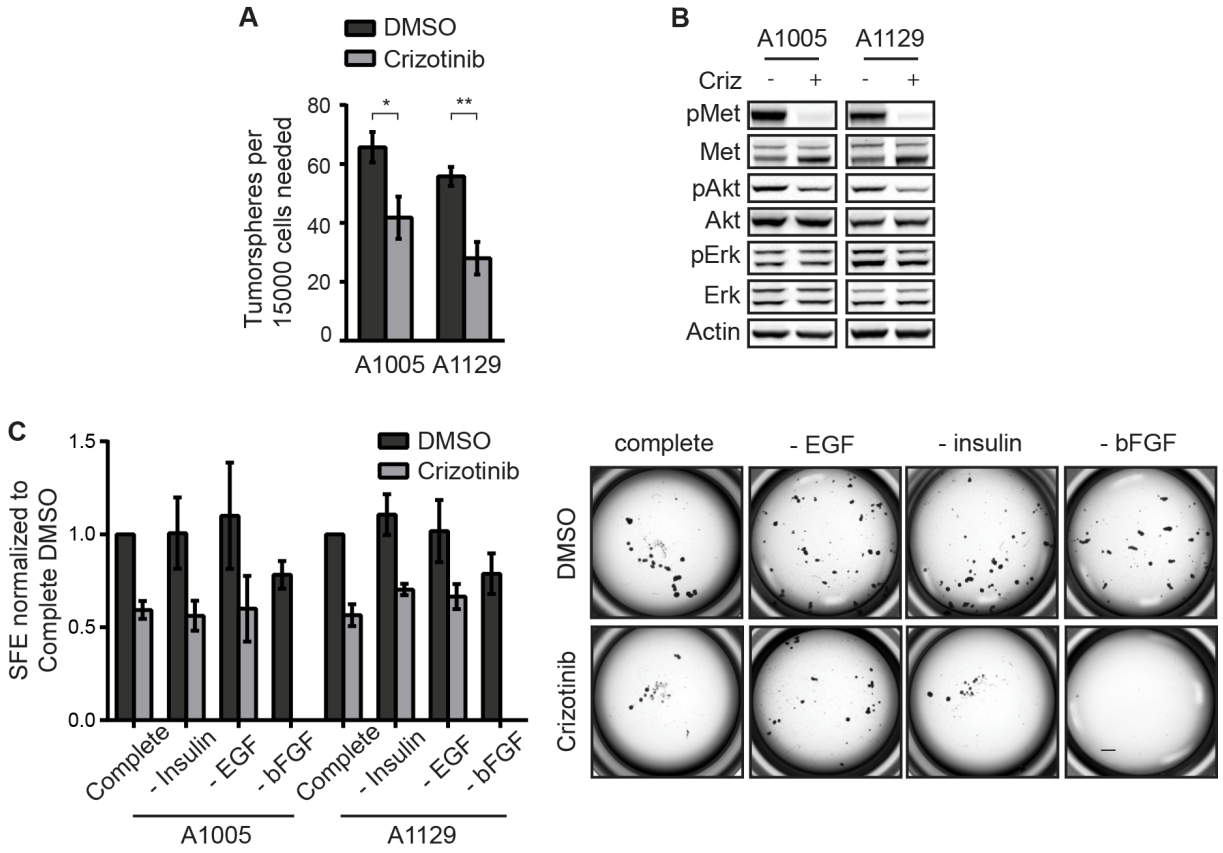


Figure 2.2 MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid tumoursphere formation is abrogated under conditions of Met inhibition but can be rescued by activated FGFR signaling. (A) A1005 and A1129 tumourspheres were cultured in the presence or absence of Crizotinib and counted after 5 days. (B) The activation of Met and its downstream signaling effectors in control and Crizotinib-treated tumourspheres were assessed by Western blot analysis. (C) A1005 and A1129 tumourspheres were cultured in media lacking EGF, insulin, or bFGF, and in the presence or absence of Crizotinib. Representative images of tumourspheres in growth factor-depleted media are shown (right). Scale bar: 500µm. *p< 0.05; **p≤ 0.01. Error bars indicate SEM.

2.3 Dual inhibition of Met and FGFR signaling targets TICs of spindloid tumours *in vitro*

To determine if the signaling interaction between Met and FGFR was specific to MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid tumours, or was ubiquitous among different models, we tested a panel of cell lines derived from MMTV-*Met^{mt};Trp53fl/+;Cre*, *Trp53fl/+;Cre*, and MMTV-*Met^{mt}*

tumours. Tumourspheres were cultured in the presence of DMSO, Crizotinib alone, small molecule FGFR inhibitor PD173074 alone, or both inhibitors in combination. Tumoursphere proliferation was measured using Cyquant proliferation assays after 7 days in culture. Interestingly, all spindloid tumour cell lines, including those derived from *Trp53fl/+;Cre* tumours (which spontaneously amplified *Met* (Knight et al. 2013)), showed a significant reduction in sphere proliferation when treated with both inhibitors, compared to either inhibitor alone (Figure 2.3A). In contrast, the combined effect of Crizotinib and PD173074 was not observed in non-spindloid tumour cell lines.

A key functional characteristic of TICs is the ability to self-renew, which can be assayed *in vitro* by assessing the capacity of tumourspheres to maintain sphere-forming efficiency (SFE) over multiple rounds of passaging. Since spindloid tumourspheres fail to form in the presence of Met and FGFR inhibitors in combination, A1005 and A1129 tumourspheres were allowed to establish over 2 days before inhibitors were added for a further 3 days. On day 5, we counted the number of tumourspheres in each condition, enzymatically and mechanically dissociated the tumourspheres into single cells, re-plated into fresh 6-well plates, and repeated the same treatment process. Strikingly, combination treated tumourspheres exhibited a decrease in SFE with each passage that was not observed in any other conditions (Figure 2.3B). Moreover, Met and FGFR co-inhibition resulted in the loss of Erk phosphorylation, as well as elevated levels of cleaved caspase 3 in A1129, indicative of apoptosis (Figure 2.4C). Interestingly, the compensatory signaling between Met and FGFR appeared to be dependent on the activation of Frs2, a scaffold protein typically found downstream of FGFRs. In this context, Met and FGFR can both independently activate Frs2 and phosphorylation of Frs2 is lost only under dual RTK inhibition, resulting in loss of Erk activity. These findings support a system in which Met and FGFR signaling

cooperate to sustain the proliferation, self-renewal, and survival of spindloid TICs through maintenance of an activated MAPK pathway.

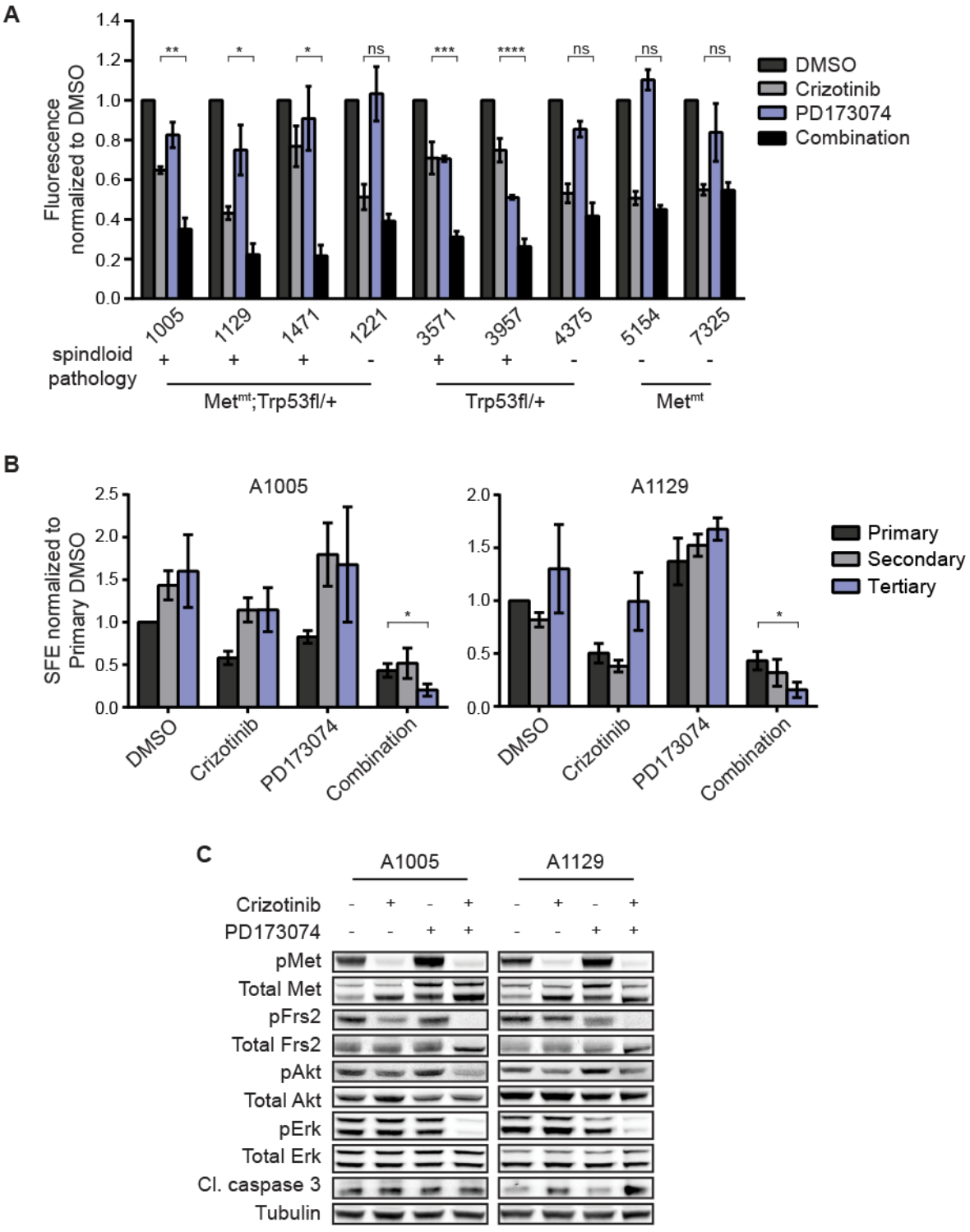


Figure 2.3 Dual inhibition of MET and FGFR signaling impairs TIC proliferation, self-renewal, and survival in spindloid tumours. (A) A panel of MMTV-*Met*^{mt}, *Trp53fl/+;Cre*, and MMTV-*Met*^{mt}*;Trp53fl/+;Cre* cells were seeded in 96-well low-attachment plates, treated with 1μM Crizotinib and/or 1μM PD173074, and tumoursphere proliferation was detected by Cyquant proliferation assay (Invitrogen). (B) A1005 and A1129 tumourspheres were allowed to form over 48h, and subsequently treated with Crizotinib and/or PD173074 for 72h. Primary tumourspheres were enzymatically and mechanically dissociated into single cells and serially passaged to assess self-renewal capacity. (C) Western blots analysis was used to detect cell death and signaling changes in tumourspheres treated with Crizotinib and/or PD173074 for 72h. *p< 0.05; **p≤ 0.01; ***p≤ 0.001; ****p≤ 0.0001. Error bars indicate SEM.

2.4 FGFR1 is preferentially expressed in spindloid tumour cells and maintains tumoursphere formation when Met is inhibited

FGFRs are a family of RTKs implicated in a variety of different cancers (Babina & Turner 2017). We found FGFR1 to be selectively upregulated in spindloid tumour cells compared to all other tumour types and normal mammary gland at both the transcript (Figure 2.4A) and protein (Figure 2.4B) levels. Consistent with our findings in tumour-derived cell lines, MMTV-*Met*^{mt}*;Trp53fl/+;Cre* and *Trp53fl/+;Cre* tumours with spindloid pathology showed stronger immunohistochemical staining for FGFR1 compared to tumours with non-spindloid pathologies (Figure 2.4C). To directly test the requirement for FGFR1 in spindloid tumoursphere formation, FGFR1 was stably silenced using shRNA in A1005 and A1129 cells (Figure 2.5A). Loss of FGFR1 did not inhibit 2D proliferation (Figure 2.5B), but restored sensitivity to Met inhibition in both cell lines, where tumoursphere formation was abrogated in the presence of Crizotinib (Figure 2.5C). Likewise, shRNA knockdown of Frs2 did not inhibit 2D proliferation but rendered both A1005 and A1129 incapable of forming tumourspheres when treated with Crizotinib (Figure 2.5D-F). These data demonstrate that FGFR1 signaling through Frs2 is required for the maintenance of MMTV-*Met*^{mt}*;Trp53fl/+;Cre* TICs in the absence of Met activation.

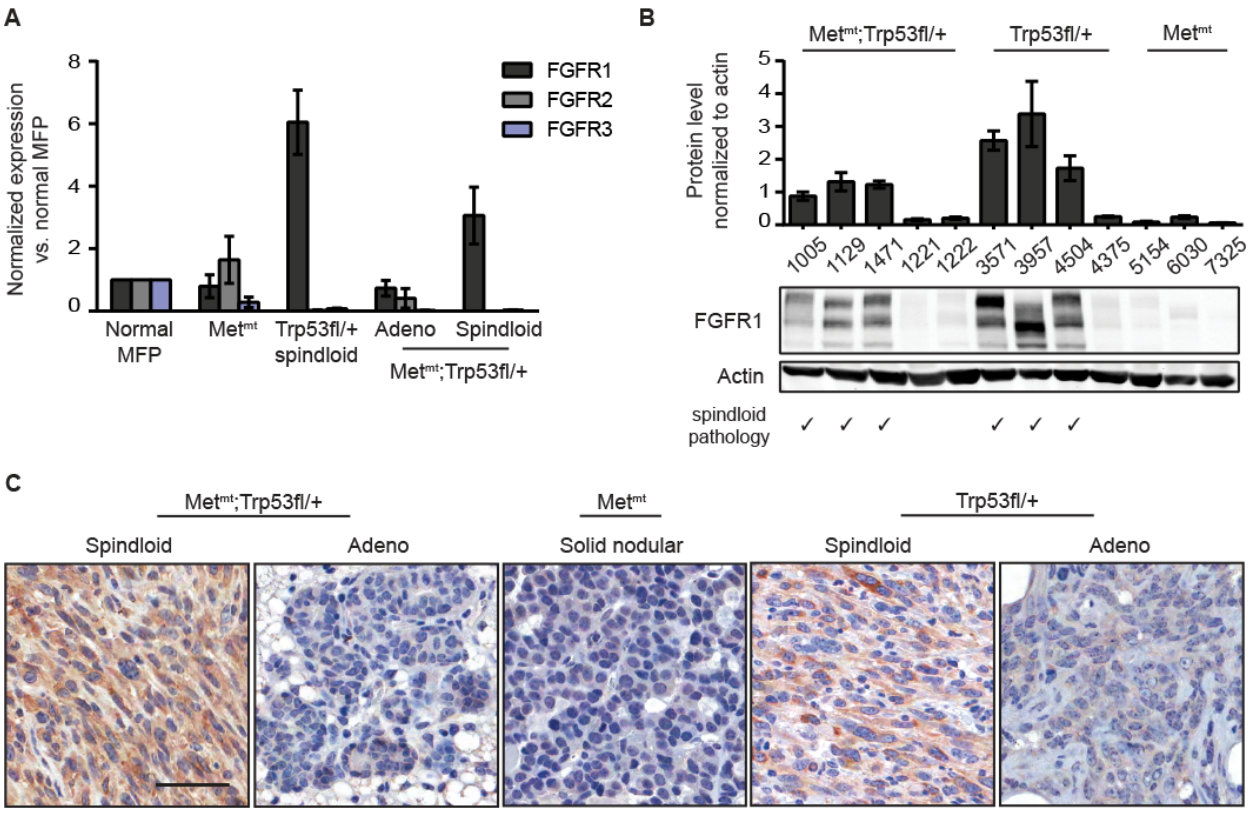


Figure 2.4 FGFR1 is preferentially expressed in spindloid tumors. (A) RNA extracted from MMTV-*Met*^{mt}, *Trp53fl/+;Cre*, and MMTV-*Met*^{mt}*;Trp53fl/+;Cre* tumour cells was used to assess relative expression levels of FGFR1, 2, and 3 using quantitative RT-PCR. FGFR4 transcripts were not detected in these tumours. (B) Western blot analysis was performed to validate FGFR1 expression at the protein level. The pathology of each tumour-derived cell line tested is indicated below the blot. (C) Paraffin-embedded sections of corresponding tumours were analyzed by immunohistochemistry for FGFR1 expression. Scale bar: 50μm. Error bars indicate SEM.

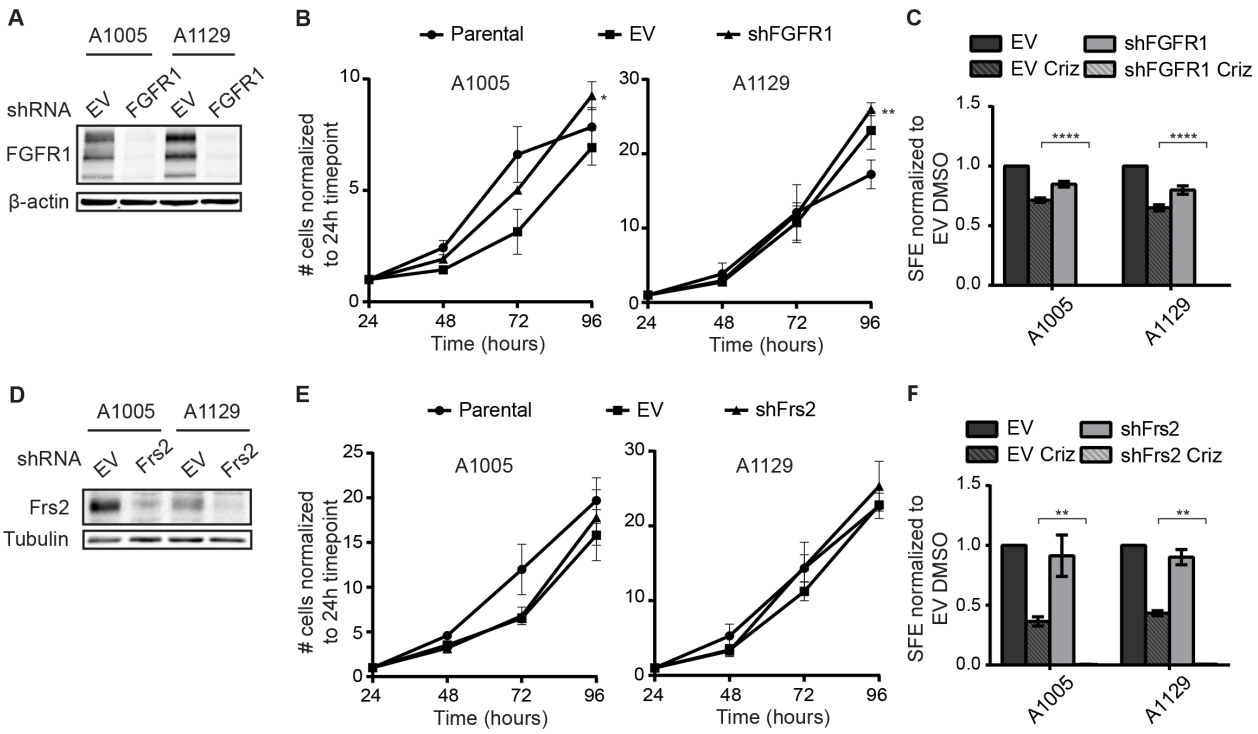


Figure 2.5 FGFR1 signaling via Frs2 compensates for loss of Met signaling to sustain tumourspheres. (A) A1005 and A1129 cells were transfected with empty vector or shRNA against FGFR1, and efficient knockdown of FGFR1 was verified by Western blot analysis. (B) The effect of FGFR1 knockdown on proliferation in adherent condition was assessed for 96h by Trypan blue assay. (C) The effect of FGFR1 knockdown on tumoursphere formation in the presence or absence of 1μM Crizotinib was assessed after 5 days of culture. (D) A1005 and A1129 cells were transfected with empty vector or shRNA against Frs2, and efficient knockdown of Frs2 was verified by Western blot analysis. (E) The effect of Frs2 knockdown on proliferation in adherent condition was assessed for 96h by Trypan blue assay. (F) The effect of Frs2 knockdown on tumoursphere formation in the presence or absence of 1μM Crizotinib was assessed after 5 days of culture. *$p < 0.05$; **$p \leq 0.01$; ****$p \leq 0.0001$. Error bars indicate SEM.

2.5 Co-inhibition of Met and FGFR1 signaling in TICs blocks proliferation and induces a program of differentiation and cell death

To better understand the events that occur in TICs upon inhibition of Met and FGFR1 signaling, we performed RNA sequencing on tumourspheres from 3 MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid cell lines (A1005, A1129, A1471) treated with Met or FGFR inhibitor alone, or in combination for 24h. All 3 cell lines treated with a MET-FGFR inhibitor combination had a gene expression signature distinct from that of cells treated with either inhibitor alone (Figure 2.6A). By contrast, and consistent with the lack of an observed phenotype, there were no statistically significant differences in gene expression between DMSO and Crizotinib alone-treated cells. Interestingly, the gene expression signature of tumourspheres treated with PD173074 alone exhibited some overlap with expression profiles observed in those treated with a combination of MET and FGFR inhibitors, suggesting that loss of FGFR signaling can be compensated for by MET activity. Gene set enrichment analysis (GSEA) showed that genes with decreased expression (FDR <0.05, $\log_2$fold change ≤-1.5) were linked to cell cycle and DNA replication pathways (Figure 2.6B, Table 2.2), with some cell cycle genes also being implicated in EMT and stemness (*Mybl2*, *Hmga2*, *Aurka*). Other genes with decreased expression were associated with metastasis (*Pea3* subfamily, *Fosl1*, *Kcnn4*) and negative regulation of MAPK pathway (*Dusp5*, *Dusp6*, *Spred2*). The observation that cell cycle and DNA replication were the pathways most significantly lost suggests that in this model Met and FGFR inhibition targeted highly proliferative cells, which may represent a population of amplifying progenitors.

Genes with increased expression in MET-FGFR dual inhibited cells compared to DMSO-treated controls (FDR <0.05, $\log_2$fold change ≥1.5) were associated with cell differentiation, cell death, and catabolism (Figure 2.6C). Elevated genes also featured GEFs (*Kalrn*, *St5*, *Rab3il1*) and,

interestingly, several genes implicated in the promotion of metastasis (*Pik3r3*, *Lamb2*) and stemness (*Foxc1*, *Dclk1*) (Table 2.3), the latter of which may reflect an enrichment of highly stem-like, slow-cycling cells that survive the initial acute loss of proliferative signal. The progressive loss of SFE through serial passaging of tumourspheres (Figure 2.3B), however, suggests that co-inhibition of Met and FGFR1 signaling is prohibitive to the self-renewal capacity of these stem-like TICs over time. Overall, the observed changes in gene expression are consistent with an inhibition of proliferation and EMT program in *Met^{mt};Trp53fl/+;Cre* spindloid cells upon loss of Met and FGFR1 signaling, resulting in reduced capacity to survive and propagate in TIC conditions.

2.6 Dual inhibition of Met and FGFR signaling reduces A1129 tumour-initiation *in vivo*

To investigate whether co-targeting Met and FGFR1 *in vivo* could suppress tumour-initiation, female athymic mice were injected with A1129 tumour cells in the 4th mammary fat pad. Mice were then randomized into four groups, receiving either vehicle control, Crizotinib, an orally available FGFR inhibitor BGJ398, or both agents in combination by daily gavage (n=7 for combination; n=10 for all other groups) (Figure 2.7A). Mice receiving combination therapy showed prolonged event-free survival compared to all other treatment arms (Figure 2.7B). Treatment with Crizotinib alone decreased overall tumour volume, though not reaching statistical significance (Figure 2.7C). Strikingly, whereas BGJ398 alone did not affect tumour burden, combination therapy with Crizotinib abrogated tumour penetrance. These results indicate that A1129 tumour-initiation is combinatorially dependent on Met and FGFR1 signaling, and that co-inhibition of both receptors can target A1129 TICs *in vivo*.

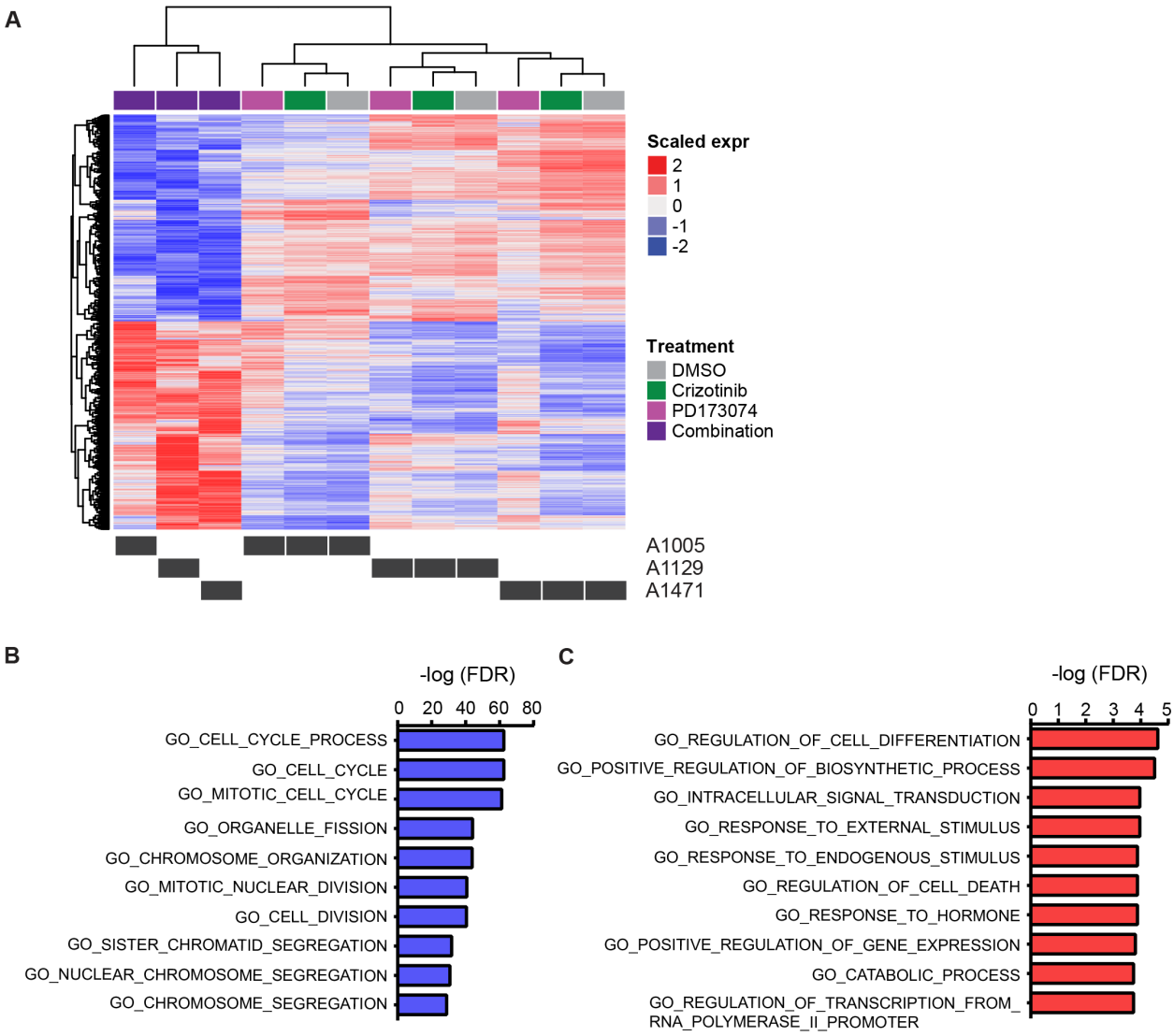


Figure 2.6 Loss of Met and FGFR1 signaling induces a distinct signature of differentiation and cell death in MMTV-*Met*^{mt}*; Trp53fl/+;Cre* spindloid tumourspheres. (A) Tumourspheres generated from 3 independent MMTV-*Met*^{mt}*; Trp53fl/+;Cre* spindloid tumour cell lines were treated for 24h with DMSO, Crizotinib, PD173074, or a combination of both agents. RNA was extracted from treated tumourspheres and sequenced, and the data subjected to unsupervised hierarchical clustering. (B) GSEA was applied to genes that decreased in expression in combination compared to DMSO-treated samples (FDR<0.05, log2fold change ≤1.5) using online tool (http://software.broadinstitute.org/gsea/msigdb/index.jsp). The top 10 down-regulated biological pathways are shown. (C) GSEA was applied to genes that increased in expression in combination compared to DMSO-treated samples (FDR<0.05, log2fold change ≥1.5). The top 10 up-regulated biological pathways are shown.

Top genes with decreased expression combo vs DMSO

Gene	Fold change at 24h (log_2)	Biological process
Ccnd1	-3.99	Cell cycle and DNA replication (*also EMT/stem)
Pbk	-2.61	
Aurkb	-2.58	
Ccnd2	-2.53	
*Mybl2**	-2.51	
Odc1	-2.42	
*Hmga2**	-2.28	
E2f8	-2.26	
Top2a	-2.15	
*Aurka**	-2.11	
Kiaa0101	-2.08	
Pola1	-2.06	
Rrm2	-2.05	
Etv5	-3.99	Migration and invasion
Etv4	-3.03	
Fosl1	-2.69	
Epha2	-2.63	
Kcnn4	-2.37	
Syne3	-2.03	
Dusp6	-3.83	Negative regulation of MAPK
Dusp5	-2.42	
Spred2	-2.06	
Egr3	-2.50	Response to stimulus
Glp1r	-2.06	
Fam78b	-2.09	

Table 2.2 Top genes with decreased expression in combination-treated vs DMSO-treated MMTV-*Met^{mt}; Trp53fl/+;Cre* spindloid tumourspheres.

2.7 Dual inhibition of Met and FGFR signaling impairs progression of established A1129 tumours *in vivo*

We next evaluated the potential of Met and FGFR1 co-inhibition as a therapeutic strategy. To this end, female athymic mice were orthotopically injected with A1129 tumour cells and randomized into 4 treatment groups (n=10/group). Tumours were allowed to reach 50-100mm^3 before treatment with inhibitors was initiated (Figure 2.8A). Combination treatment was unable to induce

Top genes with increased expression combo vs DMSO

Gene	Fold change at 24h (log_2)	Biological process
Ephb6	2.66	Apoptosis and autophagy
Tp53inp1	2.59	
Bmf	2.36	
Tp53inp2	2.27	
Fbxo32	2.07	
Plekhf1	2.02	
Gdpd2	4.01	Differentiation
Atp1b1	2.44	
Ogn	2.25	
Dtx4	2.15	
Dlk2	2.13	
Ikzf2	2.03	
Kalrn	2.45	GEF
St5	2.10	
Rab3il1	2.04	
Nrep	2.88	Migration and invasion
Lamb2	2.06	
Pik3r3	2.04	
Dclk1	2.05	Stem
Foxc1	2.04	
Pdk2	2.05	Metabolism
Me3	2.03	
Msrb3	2.07	ROS response
Fam46a	2.07	RNA binding
Fam214a	2.22	

Table 2.3 Top genes with increased expression in combination-treated vs DMSO-treated MMTV-*Met^{mt}; Trp53fl/+;Cre* spindloid tumourspheres.

regression of established tumours, but did impair progression more effectively than any other treatment arm (Figure 2.8B). Crizotinib alone reduced tumour growth rate to a lesser extent than combination treatment, while BGJ398 once again had no effect when administered on its own. Western blot analysis confirmed the successful targeting of Met and FGFR1 signaling in vivo, and showed re-expression of the tight junction protein Claudin 1 upon efficient co-inhibition of the two receptors (Figure 2.8C). These findings demonstrate that a combination of MET and FGFR1

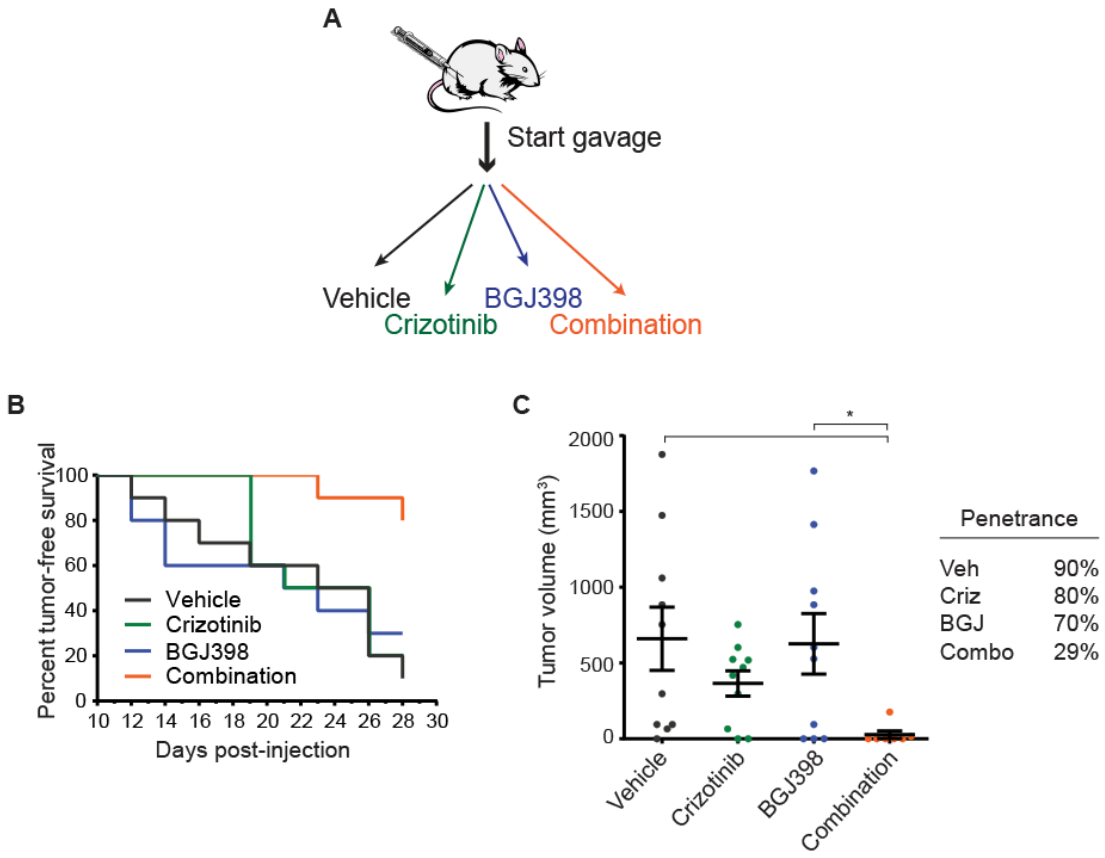


Figure 2.7 Targeting Met and FGFR1 in combination *in vivo* abrogates tumour-initiating potential of MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid cells. (A) Female athymic mice were injected with A1129 cells in the 4th mammary fad pad and treated daily with vehicle control, Crizotinib alone (50mg/kg), BGJ398 alone (30mg/kg), or the two agents in combination by oral gavage. (B) Kaplan-Meier curves of the percentage of tumour-free A1129-injected mice in the indicated treatment groups. Mice were sacrificed after 28 days and their tumors collected. (C) Tumour volumes as measured by calipers on day of sacrifice (left) and percent penetrance in each treatment group (right). *p< 0.05. Error bars indicate SEM.

signaling inhibition disrupts the EMT program and deters A1129 tumour progression. Taken together, our findings thus far support that Met and FGFR1 signaling cooperate to regulate TICs in murine mammary tumours with spindloid pathology, and that co-inhibition of both receptors suppresses tumour initiation and impairs progression *in vivo* by targeting TICs.

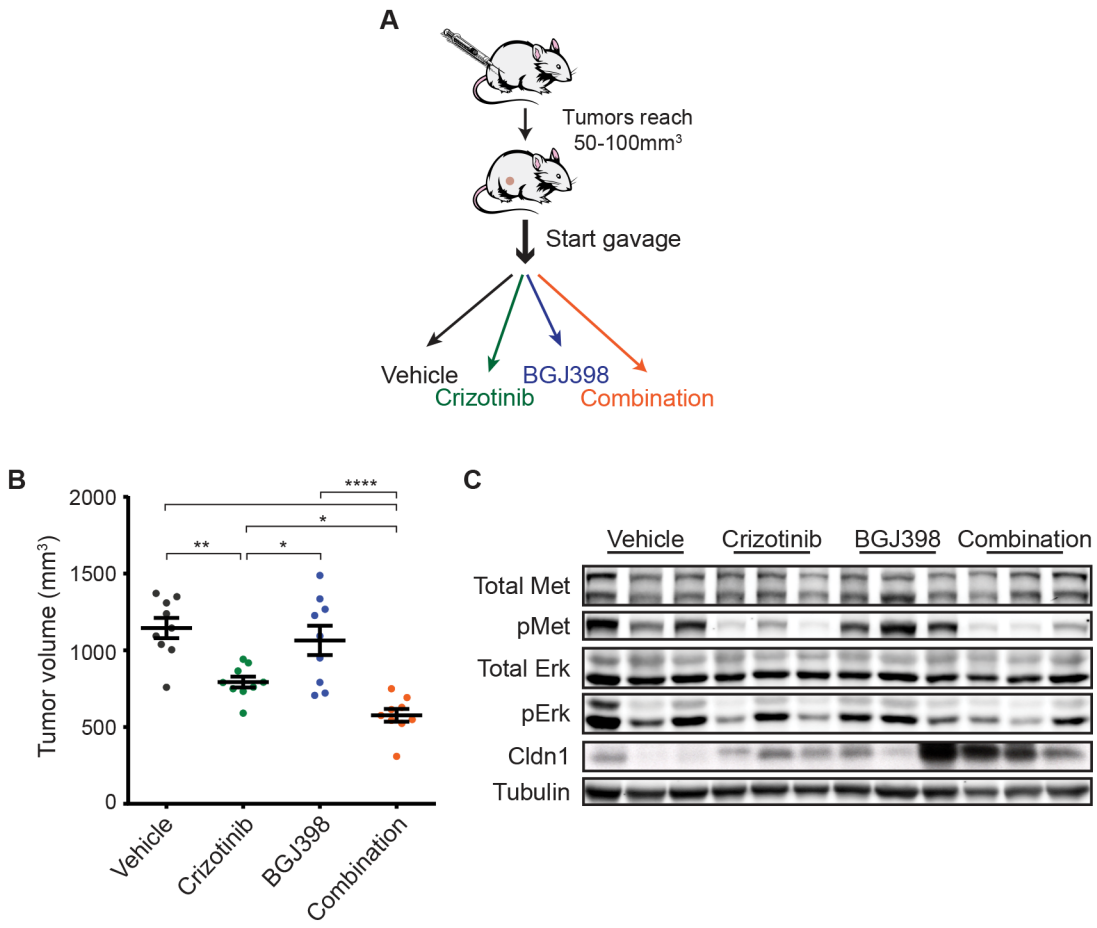


Figure 2.8 Targeting Met and FGFR1 in combination *in vivo* hinders progression of established MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid tumours. (A) Female athymic mice were injected with A1129 cells in the 4th mammary fat pad and tumours were allowed to reach 50-100mm^3 in volume before randomization into treatment groups. (B) Tumour volumes as measured by calipers after 11 days of drug treatment. (C) Tumour lysates were prepared from 3 mice per treatment group and Western blot analysis was performed for the indicated proteins and phospho-proteins. *p< 0.05; **p≤ 0.01; ****p≤ 0.0001. Error bars indicate SEM.

2.8 MET and FGFR1 are highly co-expressed in human Basal B breast cancer cell lines

We next sought to establish if TICs derived from human breast cancers are equally as dependent on MET and FGFR1 signaling as our mouse model mammary tumours. Among the cell lines used to interrogate human breast cancer biology, “Basal B” breast cancer cell lines are the most

genetically representative of claudin-low breast cancer (Grigoriadis et al. 2012; Neve et al. 2006). Utilizing gene expression data from the publicly available database Cancer Cell Line Encyclopedia (CCLE), we found that Basal B cell lines exhibited the highest co-expression of MET and FGFR1 compared to Luminal, HER2, and Basal A cell lines (corresponding to ER+, HER2 amplified, and basal-like breast cancers respectively) (Figure 2.9A). FGFR2 and FGFR3 were not significantly correlated with any subgroup of cell lines (Figure 2.9B and C), whereas FGFR4 expression was elevated in HER2 and Luminal cell lines (Figure 2.9D). Western blot analysis verified that MET and FGFR1 co-expression at the protein level were preferentially found in Basal B cell lines (Figure 2.10A), consistent with our findings in MMTV-*Met;Trp53fl/+;Cre* tumours, which represent the claudin-low (Basal B) subtype.

2.9 Dual inhibition of MET and FGFR signaling targets TICs in Basal B breast cancer cell lines

Recent studies have demonstrated compensatory signaling between MET and FGFRs as a mechanism of resistance in human cancer cell lines (Kentsis et al. 2012; S. M. Kim et al. 2016; Kotani et al. 2015; Wilson et al. 2012; Harbinski et al. 2012). Moreover, FGFR signaling is enriched in Basal B cell lines, several of which display autocrine bFGF signaling (Sharpe et al. 2011). However, to our knowledge, the potential for cooperative signaling between MET and FGFRs in breast cancer TICs has not been directly studied.

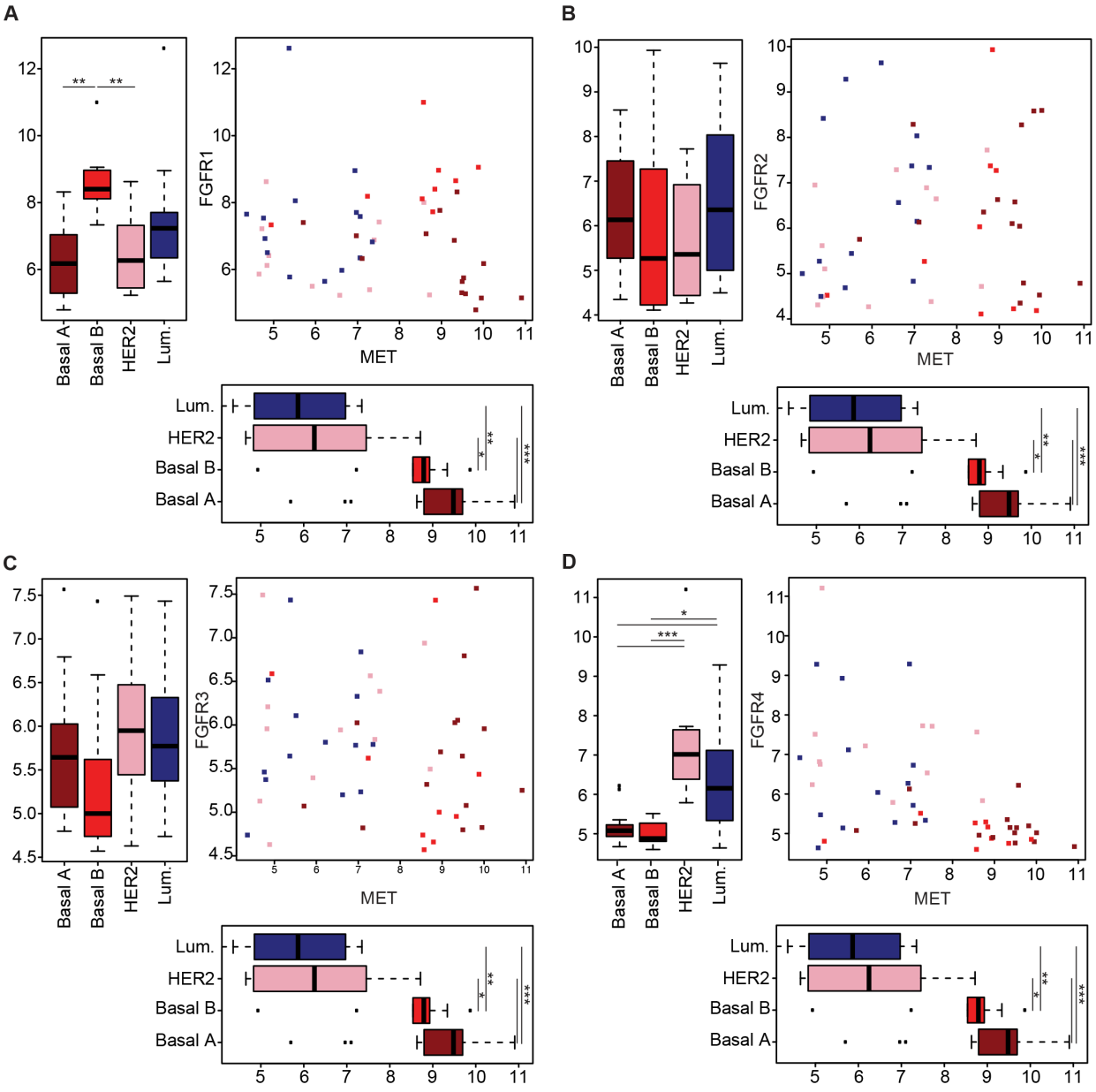


Figure 2.9 Human Basal B breast cancer cell lines are enriched for co-expression of *MET* and *FGFR1*. (A-D) mRNA z-scores of MET, FGFR1, FGFR2, FGFR3, and FGFR4 were acquired from the Cancer Cell Line Encyclopedia, and co-expression of MET and each FGFR was compared among human breast cancer cell lines. *p< 0.05; **p≤ 0.01; ***p≤ 0.001.

To ascertain whether dual inhibition of MET and FGFR signaling results in reduced tumourigenicity, a panel of TNBC cell lines (4 Basal A and 4 Basal B) were cultured as tumourspheres in the presence of DMSO, Crizotinib alone, PD173074 alone, or both inhibitors in combination. In addition to bFGF, a regular component of tumoursphere medium, tumourspheres were also cultured in the presence of HGF to ensure activated MET signaling. After 7 days in culture, tumoursphere proliferation was measured. Tumourspheres from all 4 Basal B cell lines exhibited a greater decrease in proliferation under conditions of MET and FGFR co-inhibition compared to all other conditions (Figure 2.10B). In contrast, Basal A tumourspheres varied in their sensitivity.

In human breast cancer, TICs are enriched within a population of cells characterized by the cell surface marker profile $CD44^{+}CD24^{-/low}$ (Al-Hajj et al. 2003). Reflecting their strong overlap with mesenchymal/stem gene expression signatures, Basal B cell lines typically have high percentages of $CD44^{+}CD24^{-/low}$ cells (Blick et al. 2010). To investigate whether the $CD44^{+}CD24^{-/low}$ phenotype is regulated by MET or FGFR signaling, Basal B cell lines Hs578t and BT549 were treated with Crizotinib and/or PD173074 for 2h before HGF and/or bFGF were added to media. After 72h, the cells were analyzed by flow cytometry. In both cell lines, FGFR inhibition led to TIC depletion, which was rescued upon activation of MET signaling (Figure 2.11A and B). MET inhibition alone did not affect the $CD44^{+}CD24^{-/low}$ population.

We interrogated the downstream signaling changes that occur in Basal B tumourspheres upon MET and FGFR inhibition by Western blot analysis. Established tumourspheres were treated with Crizotinib and/or PD173074 for 2h and then stimulated with HGF for 30min. Consistent with our observations in murine claudin-low breast cancer, co-inhibition of MET and FGFR signaling prevented activation of FRS2, resulting in loss of ERK phosphorylation (Figure 2.12A). Given that

genetic alterations that lead to constitutively active MET signaling are rare in human breast cancer, we wished to directly test whether ligand-stimulated activation of MET can phosphorylate FRS2. To this end, cells were pre-treated with PD173074 for 2h to inhibit FGFR-dependent activation of FRS2 then stimulated with HGF for 30min. We observed an increase in FRS2 phosphorylation that correlated with HGF-dependent MET activation (Figure 2.12B), providing to our knowledge the first evidence that non-amplified, wildtype MET can activate FRS2. Taken together, these results suggest that MET and FGFR signaling are key pathways involved in the regulation of human claudin-low TICs.

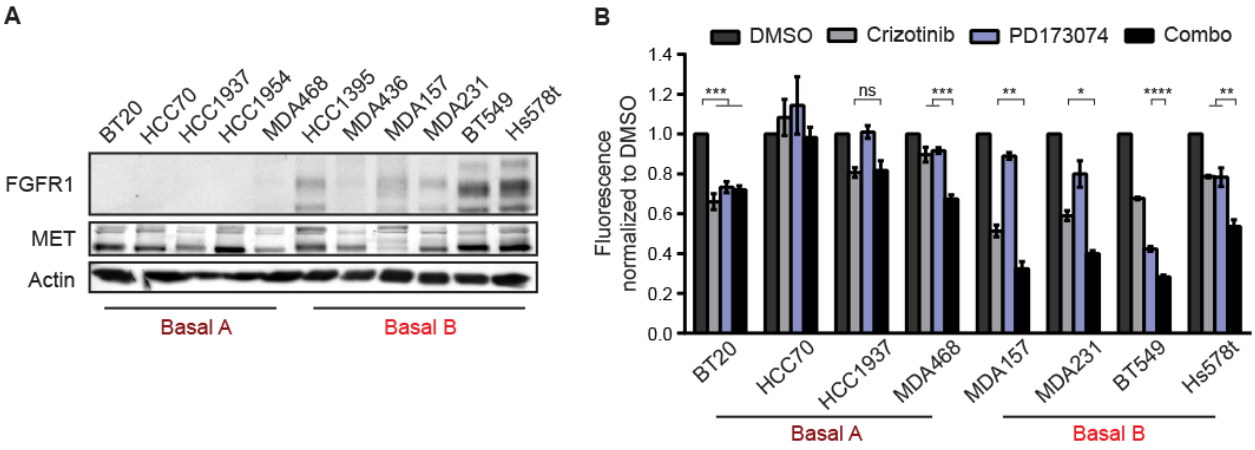


Figure 2.10 Basal B breast cancer cell lines highly co-express MET and FGFR1, and combinatorial targeting of both receptors decreases tumoursphere proliferation. (A) Western blot analysis was performed to validate MET and FGFR1 protein levels across a panel of TNBC cell lines. (B) The indicated TNBC cell lines, comprised of a mix of Basal A and Basal B cell lines, were cultured as tumourspheres in the presence of HGF and bFGF, and treated with 1μM Crizotinib and/or 1μM PD173074. Tumoursphere proliferation was detected by Cyquant proliferation assay. *p< 0.05; **p≤ 0.01; ***p≤ 0.001. Error bars indicate SEM.

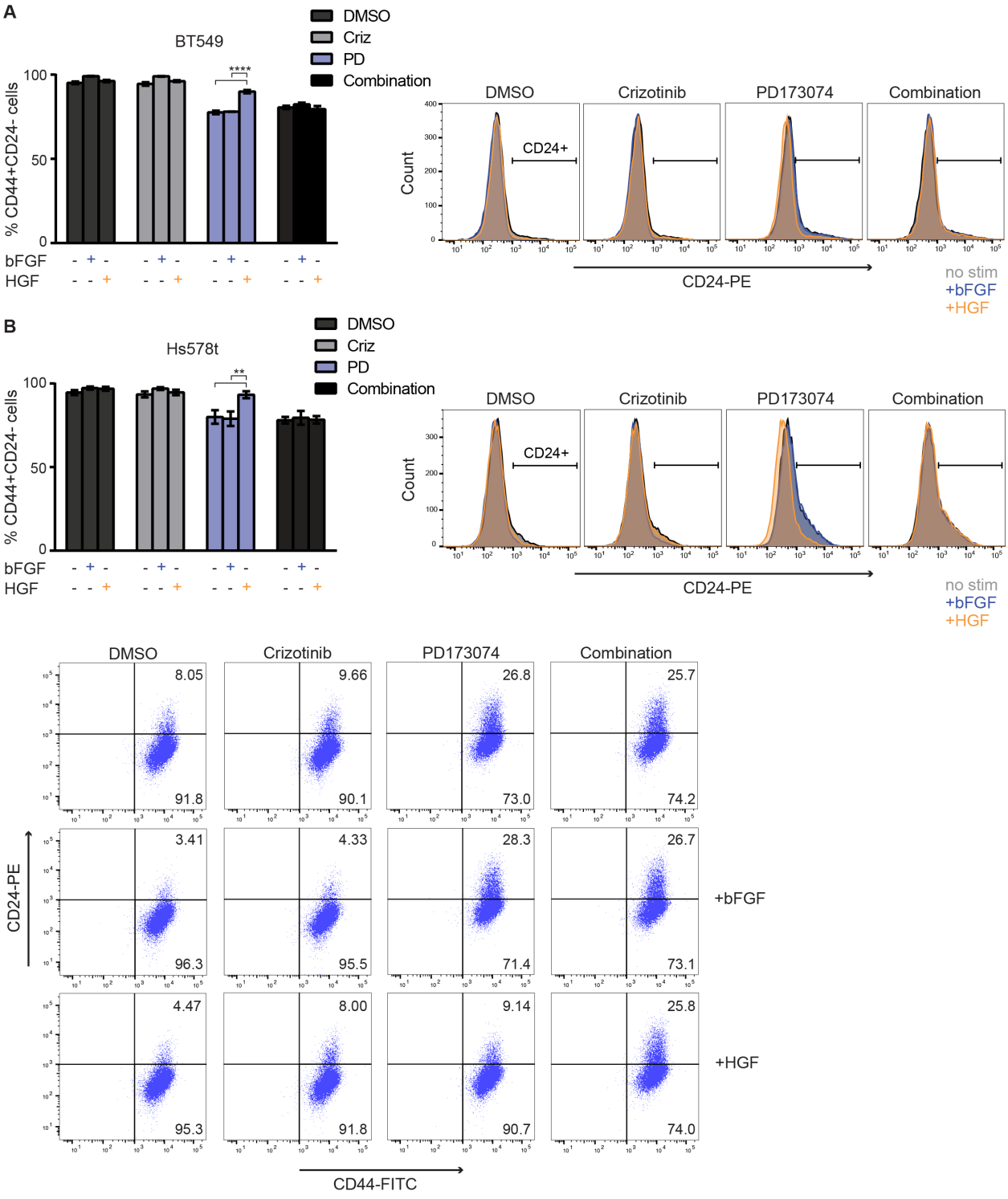


Figure 2.11 Dual targeting of MET and FGFR signaling depletes the TIC population in Basal B cell lines. (A) BT549 and (B) Hs578t cells were treated with Crizotinib and/or PD173074 for 2h, after which HGF or bFGF was added to the media. The proportion of CD44+CD24- cells was determined by flow cytometry 72h hours later **p≤ 0.01; ****p≤ 0.0001. Error bars indicate SEM.

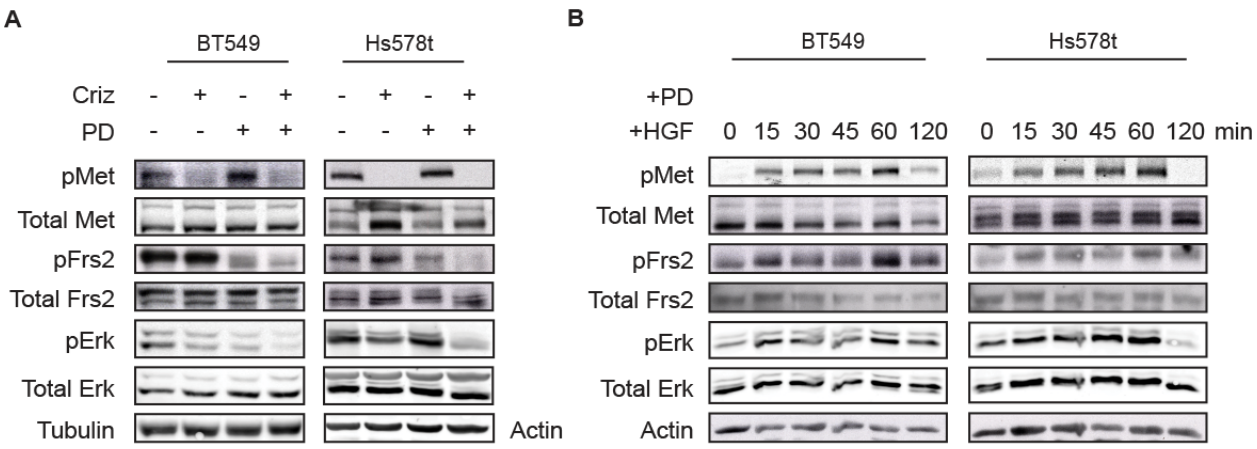


Figure 2.12 HGF-activated MET signaling can signal through FRS2, independently from FGFRs. (A) Protein lysates from BT549 and Hs578t tumourspheres treated with Crizotinib and/or PD173074 for 2h, followed by 30min stimulation with HGF and bFGF, were analyzed by Western blot. (B) BT549 and Hs578t cells were treated with PD173074 for 2h then stimulated with HGF. Protein lysates were collected at the indicated timepoints and analyzed by Western blot.

2.10 MET and FGFR1 co-expressing patient-derived xenografts show depletion in TIC population upon co-inhibition

Our group has generated a panel of PDX lines from TNBC patients (Savage et al. 2017), some of which co-express MET and FGFR1 as shown by Western blot analysis (Figure 2.13A). Immunofluorescence of 2 PDXs (GCRC1886 and 1915) revealed homogeneous expression of MET at moderate levels throughout the tumour epithelium, whereas FGFR1 exhibited heterogeneous but strong expression (Figure 2.13B). A PDX (GCRC1863) with low expression of FGFR1 localized to the stroma is included in the analysis for comparison.

To assess the role of MET and FGFR signaling in putative TICs of PDXs, a GCRC1915 tumour was collected, digested into single cells, and cultured as tumourspheres in media containing DMSO, Crizotinib, PD173074, or both inhibitors in combination. A reduction in SFE was observed in both Crizotinib and combination-treated tumourspheres, with the latter exhibiting a

larger decrease (Figure 2.14A). Serial passaging revealed that MET-FGFR1 co-inhibition had the most deleterious effect on SFE compared to all other conditions.

We next wished to investigate how GCRC1915 would respond to MET and FGFR inhibitors *in vivo*. We injected single cells into mice harboring a knock-in mutation that replaced murine HGF with human HGF (NSG-hHGFki), ensuring that both FGFR and MET signaling pathways can be activated. Once tumours progressed to 50-100mm^3, mice were then orally gavaged with vehicle, Crizotinib, BGJ398, or both agents in combination (n=5/group). Combination treatment resulted in a moderate but significant reduction in tumour burden compared to all other treatment arms (Figure 2.14B). Treated GCRC1915 tumours were collected at end point and digested into single cells for flow cytometry analysis of TIC markers. We observed a depletion in the proportion of the $CD44^+CD24^{-/low}$ TIC population in combination-treated tumours (Figure 2.14C). Single cells from treated tumours were also cultured as tumourspheres, and consistent with TIC depletion, combination treatment resulted in a marked decrease in SFE compared to all other groups (Figure 2.14D). These *in vitro* and *in vivo* findings using a PDX model, strongly support that MET and FGFR signaling can be targeted to deplete TICs in human TNBC.

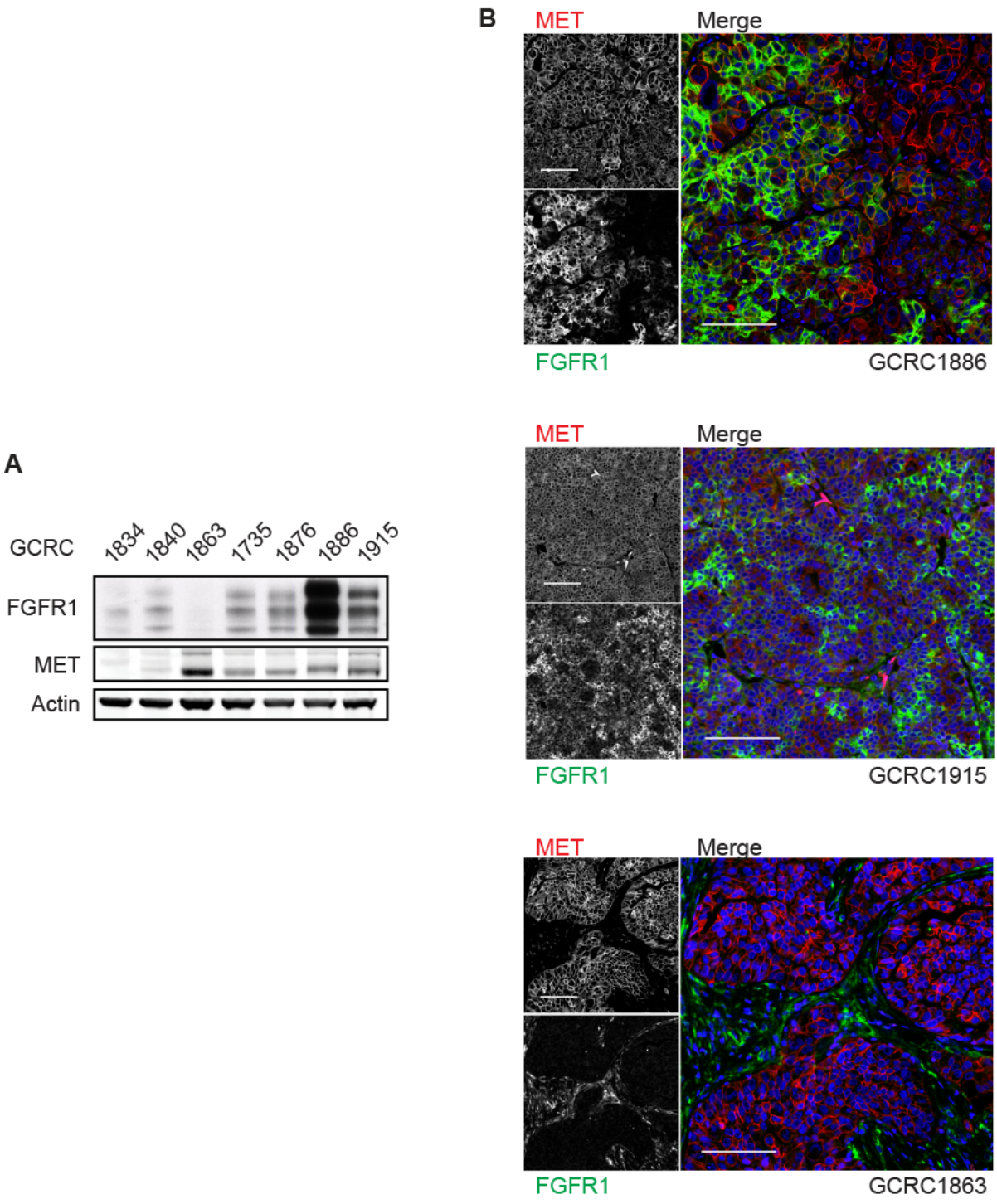
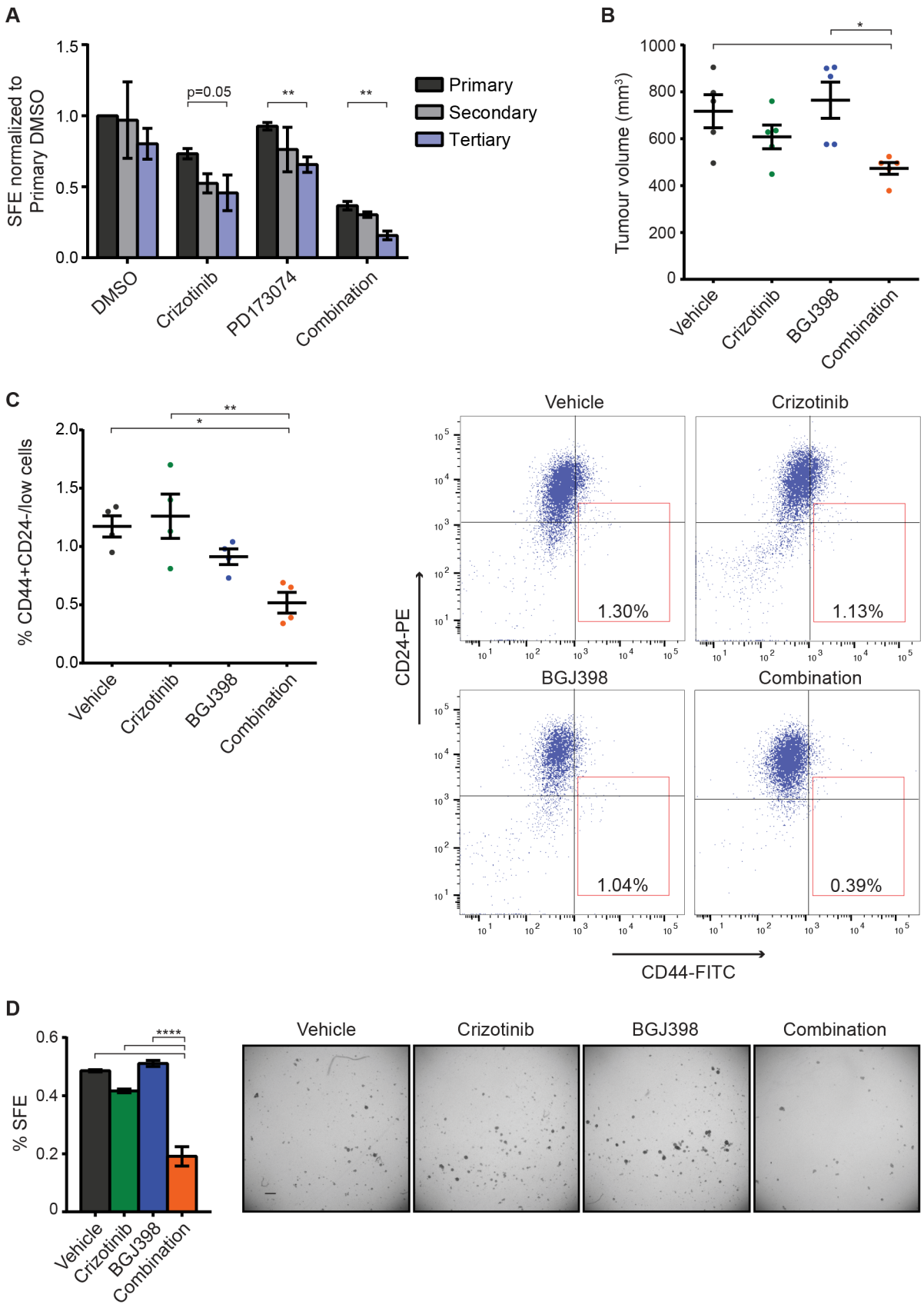


Figure 2.13 A subset of TNBC PDX tumours co-express MET and FGFR1. (A) Protein lysates were generated from mammary PDX tumor fragments and used to determine MET and FGFR1 expression by Western blot analysis. (B) Immunofluorescence was performed on sections of the indicated PDX tumours to validate co-expression of MET and FGFR1. Scale bars: 100µm.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SFE normalized to Primary DMSO
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Primary
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B
Tumour volume (mm³)
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400
200
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Vehicle
Crizotinib
BGJ398
Combination
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% CD44+CD24-/low cells
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Crizotinib
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Figure 2.14 Dual targeting MET and FGFR signaling depletes the TIC population in GCRC1915 PDX *in vitro* and *in vivo*. (A) GCRC1915 PDX was dissociated into single cells and cultured as tumourspheres in the presence of Crizotinib and/or PD173074. Tumourspheres were subsequently serially passaged to evaluate self-renewal capacity. (B) Female NSG-hHGFki mice were orthotopically injected with GCRC1915 cells and tumours were permitted to progress to 50-100mm^3 in volume. Tumour-bearing mice were treated with vehicle control, Crizotinib (50mg/kg), BGJ398 (30mg/kg), or both agents in combination. Tumour volumes as measured by calipers after 17 days of treatment are shown. (C) Treated tumors were dissociated into single cells and evaluated for CD44+CD24-/low populations by flow cytometry, as well as cultured as tumourspheres (D) and counted after 7 days of culture. Scale bar: 200 μm. *p< 0.05; **p≤ 0.01; ****p≤ 0.0001. Error bars indicate SEM.

2.11 Patient tumours enriched for TIC/EMT signatures are also enriched for MET and FGFR1 signaling pathways

Our results thus far indicated that a subset of TNBC tumours respond to pharmacological dual inhibition of MET and FGFR1, both *in vitro* and *in vivo*, and that this combination treatment appeared to target the TIC population. These treatment-responsive tumours tended to correlate with highly mesenchymal breast cancers, including claudin-low breast cancers. We next sought to identify patients who would potentially benefit clinically from combination therapies of MET and FGFR inhibitors. Using single sample gene set enrichment analysis (ssGSEA), we clustered TNBC patients from The Cancer Genome Atlas (TCGA) (Koboldt et al. 2012) dataset of invasive breast carcinomas into TIC/EMT high and TIC/EMT low cohorts using published gene signatures for claudin-low breast cancer (Knight et al. 2018), TIC (Creighton et al. 2009), EMT (Hallmark), and undifferentiated mammary epithelial cells (Dontu 2003) (Figure 2.15A). Accordingly, expression of TIC-related gene *ALDH1A1* was enriched in the TIC/EMT high patients, as were EMT-related genes *SNAI2*, *ZEB1*, *ZEB2*, *TWIST*1, and *VIM* (Figure 2.15B). We next compared the expression of genes encoding FGFR1 and MET, as well as their ligands FGF2 and HGF respectively, between TIC/EMT high and low patients. *FGFR1* and *HGF* were significantly increased in the TIC/EMT

high group, while *FGF2* and *MET* were not significantly different (Figures 2.16A). Despite this, ssGSEA showed that TIC/EMT high patients scored higher for gene signatures associated with both MET (Lai et al. 2014) and FGFR1 (Schwertfeger et al. 2006) signaling activation (Figure 2.16B), further supporting that TNBCs with mesenchymal features were enriched for components of MET and FGFR1 signaling.

2.12 *FGFR1* and *HGF* together predict poor outcome in basal-like breast cancer patients

Since highly mesenchymal TNBCs are associated with elevated expression of both *FGFR1* and *HGF* (Figure 2.16A), we next assessed whether these 2 genes in combination could predict outcome in breast cancer patients. Analysis of publicly available breast cancer expression profiles revealed that high expression of both *FGFR1* and *HGF* was associated with poor relapse-free survival (RFS) specifically in basal-like tumors (Figure 2.17). These data support combination targeting of MET and FGFR1 signaling as a feasible therapeutic option for TNBCs.

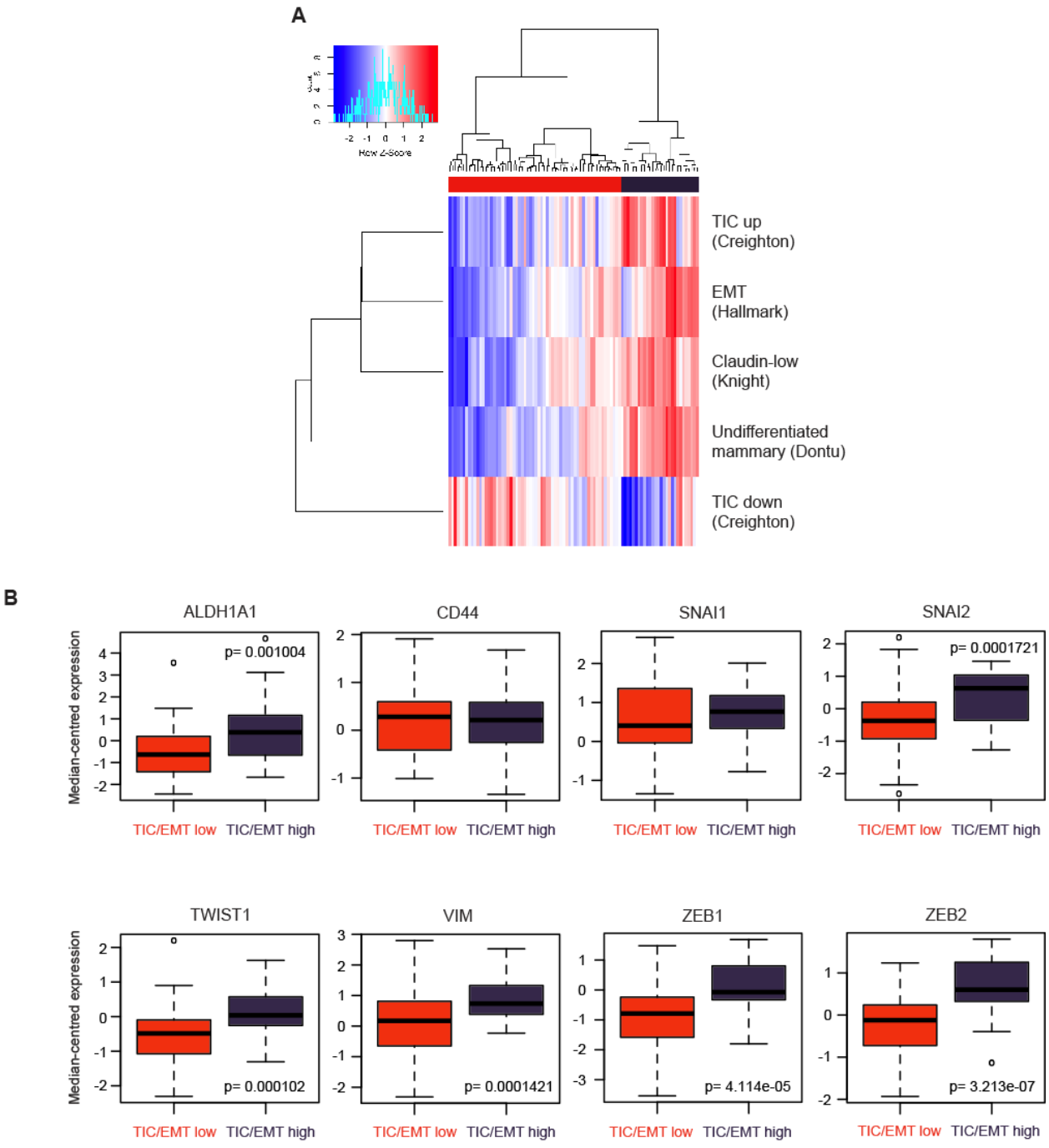

Figure 2.15 TNBCs can be stratified into TIC/EMT high and low patient groups. (A) Gene expression profiles of TNBCs from the TCGA patient cohort were subjected to ssGSEA for the indicated TIC and mesenchymal gene signatures, and stratified into TIC/EMT high and low groups. (B) Box plots of median-centred expression of the indicated genes in TIC/EMT high and low patient groups.

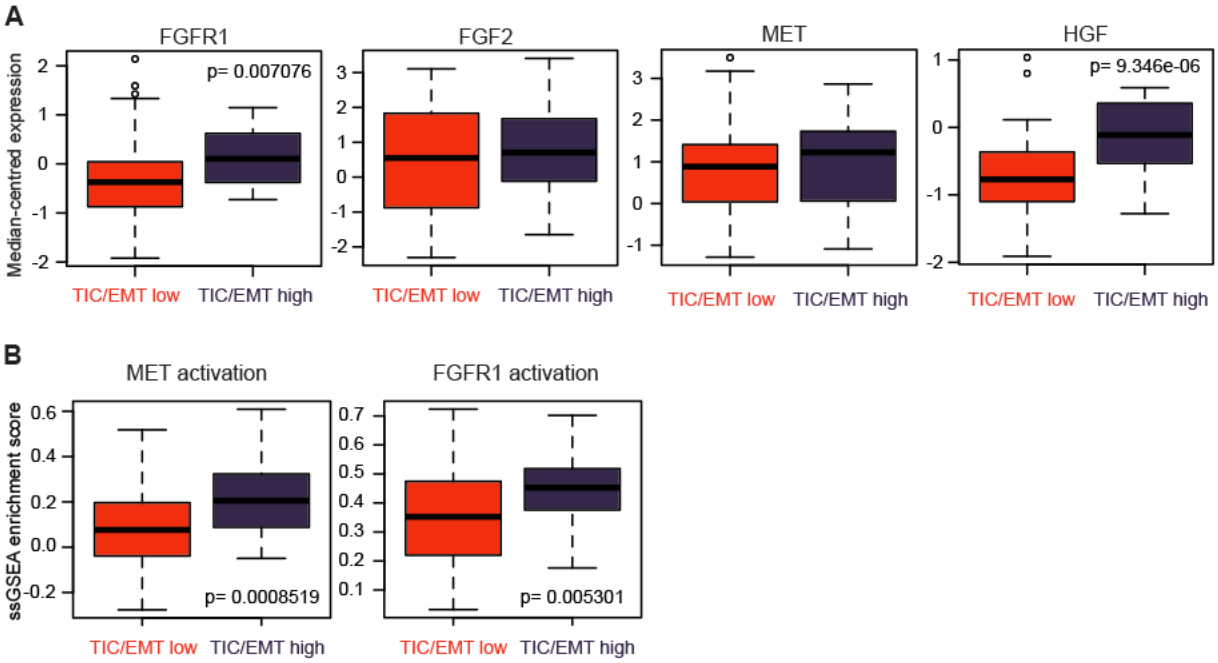

Figure 2.16 Highly mesenchymal TNBCs are enriched for gene signatures associated with MET and FGFR1 signaling activation. (A) Box plots of median-centred expression of the indicated genes in TIC/EMT high and low patient groups. (B) Box plots of ssGSEA enrichment scores for MET and FGFR1 activation gene signatures in TIC/EMT high and low patient groups.

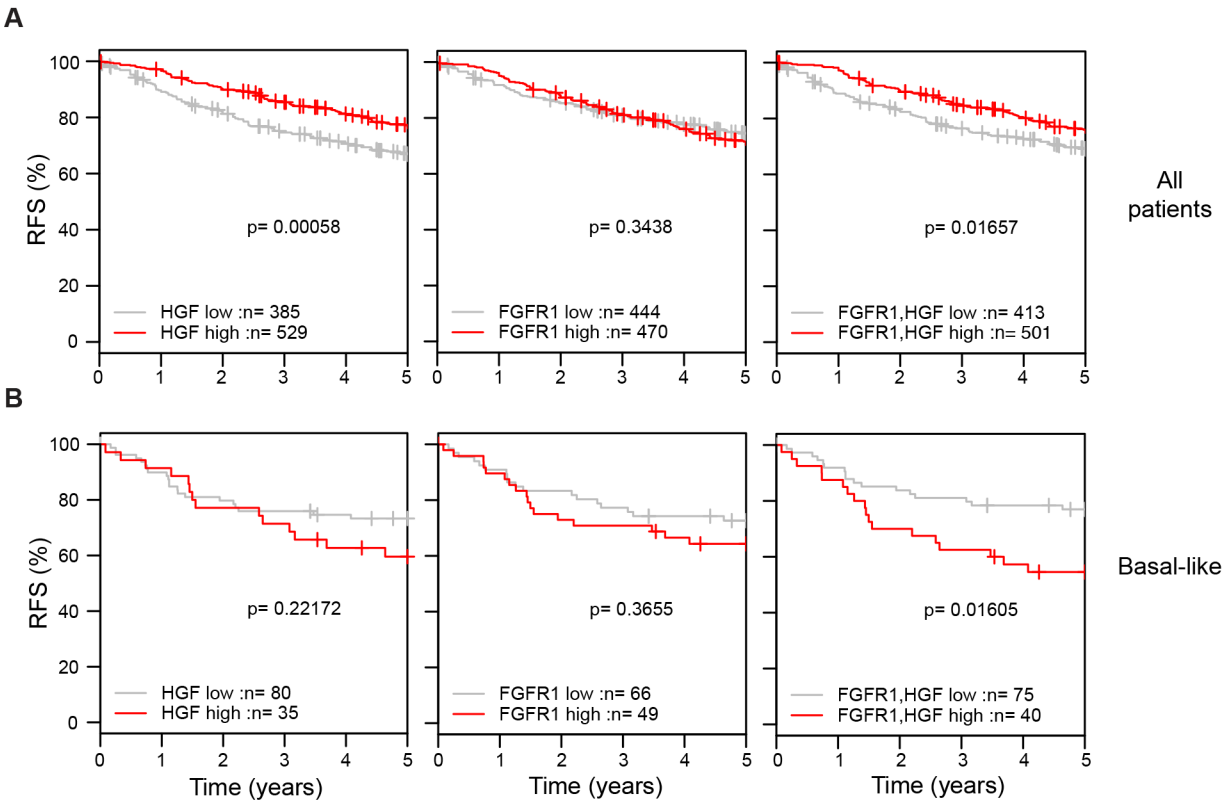

Figure 2.17 Co-expression of HGF and FGFR1 is associated with poor outcome in Basal-like breast cancers. Relapse-free survival (RFS) analysis across (A) all breast subtypes and (B) basal-like breast cancers split by expression HGF and/or FGFR1 expression.

3 DISCUSSION

3.1 Summary

It is well-established that dysregulated MET signaling plays a tumour-promoting role in a wide range of human cancers. In breast cancer, MET is overexpressed in 15-20% of all cases and is associated with poor prognosis (Ponzo & M. Park 2010). Overexpression of MET is found predominantly in basal-like (molecular subtype) and triple negative (histological subtype) breast cancers, subtypes that are enriched with phenotypic and genetic characteristics of mesenchymal cells (as cited in Chapter 1). These characteristics are consistent with the biological functions of MET signaling, raising the possibility that MET contributes to these tumours via its capacity to induce a mesenchymal state. The high frequency of mesenchymal tumours that develop Met-dependent transgenic mouse models of breast cancer, generated by us and others (Ponzo et al. 2009; Knight et al. 2013; Graveel et al. 2009), further supports this hypothesis. A goal of this thesis was to determine whether a MET-promoted EMT program is required for tumourigenesis in mesenchymal basal-like/triple negative breast cancers. To this end, our studies in the MMTV-*Met*^{mt}*;Trp53fl/+;Cre* mouse model of TNBC have uncovered a mechanism of TIC regulation in which a constitutive Met signal is necessary and sufficient to support TICs populations, but loss of the Met signal can be compensated by activation of FGFR1 signaling. In human TNBC cell lines and PDXs, we identified a similar capacity for MET and FGFR1 signaling for co-regulation of TICs. In TNBCs that co-express both receptors, combinatorial inhibition of MET and FGFR1 signaling resulted in a depletion of TIC populations *in vitro* and *in vivo*. To our knowledge, these are the first studies demonstrating a functional role for MET signaling in TNBC TICs.

We have identified a novel signaling network between MET and the scaffold protein FRS2. MET is not known to signal through FRS2, with the exception of studies where MET is amplified and constitutively active and was described as a resistance mechanism for FGFR-directed therapies

(discussed further below). We provide the first evidence that HGF-stimulated activation of MET can promote FRS2 phosphorylation and maintain ERK1/2 phosphorylation in a FRS2-dependent manner in the absence of FGFR signaling. We further demonstrate that FRS2 is required for multiple biological processes downstream of MET, including cell invasion, proliferation, and survival, providing new understanding of the regulation of MET-dependent biology.

3.2 The role of MET in tumourigenesis

3.2.1 MET confers survival signals and initiates a mesenchymal program conducive to maintaining TIC capacity

The functional role that MET plays in tumour initiation and progression is context-dependent. In cases where underlying genetic alterations result in constitutive or enhanced receptor activation, MET is often required for cancer cell proliferation and enhanced metastatic spread. Targeted inhibition of MET in these tumours results in stable disease or overt regression (Bardelli et al. 2013; Lai et al. 2014). More frequently, the wildtype MET protein is overexpressed in tumours, where its transcriptional upregulation and activation are induced by various external stimuli from the tumour microenvironment (Pennacchietti et al. 2003). These same stimuli can also induce upregulation of HGF in the tumour stroma, generating a feedforward loop that sustains MET activation (Boccaccio et al. 1994). In this context, the normal physiological functions of MET, namely inducing an EMT program and conferring survival signals to facilitate invasive growth and migration, are activated to support tumour progression. The link between a MET-dependent mesenchymal program and increased tumourigenicity is supported by studies in glioblastoma, where MET is a functional marker of TICs in the mesenchymal subtype of these tumours (De Bacco et al. 2012). Wildtype MET is expressed by neurospheres derived from mesenchymal

glioblastomas, where it is required to maintain tumourigenic potential, which is further enhanced by HGF stimulation.

3.2.2 MET regulates both TIC propagation and function in the *MMTV-Met^{mt};Trp53fl/+;Cre* spindloid model

We established that MMTV-*Met^{mt};Trp53fl/+;Cre* mouse mammary tumors with mesenchymal characteristics (spindloid) were highly enriched in TIC populations. Spindloid tumour cells were more proliferative and formed more tumourspheres *in vitro* compared to non-spindloid adenocarcinoma cells from the same model (Figure 2.1A-B), suggesting that an EMT phenotype conferred tumourigenic properties. This was supported by *in vivo* limiting dilution experiments, where spindloid MMTV-*Met^{mt};Trp53fl/+;Cre* cells formed tumours more readily than non-spindloid cells (Table 2.1). Based on these *in vivo* results, we calculated the TIC frequency in spindloid tumours to be approximately four times higher than that in non-spindloid tumours.

An alternative explanation for the difference in tumourigenicity between spindloid and non-spindloid MMTV-*Met^{mt};Trp53fl/+;Cre* mammary tumours is differing cells of origin. The transformation of a mammary stem cell or an early progenitor cell would likely result in tumour cells that are both more mesenchymal and more tumourigenic, compared to the transformation of a more committed luminal progenitor cell (Figure 1.8). However, the observation that all *Trp53fl/+*;*Cre* spindloid tumours also exhibited *Met* amplification supports the hypothesis that a strong Met signal is important for an EMT phenotype and the associated increase in tumourigenicity.

As discussed earlier, tumours harbouring genetic alterations in *MET* are typically dependent on aberrant signaling through the Met receptor to maintain the transformed phenotype.

Given that our group had previously shown the proliferative capacity of MMTV-*Met*^{mt}*;Trp53fl/+;Cre* spindloid cells to be dependent on constitutive Met kinase activity, we hypothesized that Met signaling would be required to maintain TIC populations as well. Targeted Met inhibition revealed that spindloid TICs required Met activity for survival and proliferation. Our *in vitro* data strongly suggest that these TICs are also dependent on Met for self-renewal, however additional studies are needed to provide definitive evidence. For example, serial transplantations *in vivo* could be performed to functionally test the ability of Met-inhibited TICs to form new tumours.

We discovered that spindloid TICs could be rescued by activation of FGFR1 signaling in the absence of a Met signal (Figure 2.2; Figure 2.3A-B). Maintenance of Met-FGFR1 dependent TICs required phosphorylation of the downstream scaffold protein Frs2, and subsequently of Erk1/2, which was lost only when both RTKs were simultaneously inhibited (Figure 2.3C). We further showed that in mice injected with MMTV-*Met*^{mt}*;Trp53fl/+;Cre* spindloid cells, tumour initiation was prevented upon combination therapy with Met and FGFR1 inhibitors (Figure 2.7). Combination treatment of established tumours resulted in impaired tumour progression and induced re-expression of Claudin 1 (Figure 2.8), indicating a loss of the mesenchymal phenotype.

To determine whether a Met-dependent EMT program played a role in maintaining TICs, we needed a better understanding of the transcriptional events that occurred upon Met and FGFR1 inhibition. To address this, we performed RNA sequencing on Met and/or FGFR1 inhibitor-treated MMTV-*Met*^{mt}*;Trp53fl/+;Cre* spindloid tumourspheres, which are enriched for TICs. Consistent with our hypothesis and observations from functional studies, TICs treated with a combination of Met and FGFR1 inhibitors showed elevated expression of genes associated with differentiation and cell death, and decreased expression of genes associated with cell proliferation and migration

(Figure 2.6; Table 2.2; Table 2.3). Interestingly, gene expression changes induced by inhibition of FGFR1 alone partially resembled those detected when combination therapy was used. This was not observed when Met was inhibited. These results suggest that in tumoursphere culture conditions, activated FGFR1 pathways provide the predominant signal supporting gene expression associated with maintenance of TIC populations. This is in keeping with other data, where bFGF is included in both stem cell and tumoursphere media to promote stem characteristics by preventing differentiation (Dvorak et al. 2005; Amit et al. 2000; Xu et al. 2001). The ability of Met to independently phosphorylate Frs2 (Figure 2.3C) suggests that it can also promote TICs by activating pathways typically associated with FGFRs. Frs2 is a key scaffold protein downstream of FGFRs, the loss of which is embryonic lethal in mice due to failure to maintain self-renewing trophoblast stem cells (Gotoh et al. 2005). Hence, the mesenchymal program invoked by Met could compensate for the absence of FGFR1-dependent stem (Dvorak et al. 2005; Amit et al. 2000; Xu et al. 2001) maintenance pathways through Frs2. Our findings support an important new function for Met in promoting tumourigenesis.

The genes that exhibited the highest increase in expression upon dual inhibition of Met and FGFR1 in MMTV-*Met*^{mt}*;Trp53fl/+;Cre* spindloid TICs also included several that are typically associated with metastasis and stemness (Table 2.3). Although this appears counterintuitive, as our results would predict that simultaneous inhibition of Met and FGFR1 signaling would impair stemness programs and hence metastasis. However, the induction of cell death genes and loss of cell cycle genes observed following MET and FGFR1 dual inhibition likely reflects a scenario whereby combination treatment eradicates a population of more differentiated, amplifying progenitors. This would then result in a relative enrichment of stem-like, slow-cycling cells that survive the initial acute loss of proliferative signal. In support of this, serial passaging of these

combination-treated tumourspheres resulted in the progressive loss of sphere-forming efficiency, indicating loss of self-renewal capacity over time (Figure 2.3B). Overall, these data support that in the MMTV-*Met*^{mt}*;Trp53fl/+;Cre* model of claudin-low breast cancer, an amplified and constitutively active Met receptor provides both a proliferative signal and a mesenchymal program that supports the propagation of TICs (Figure 3.1).

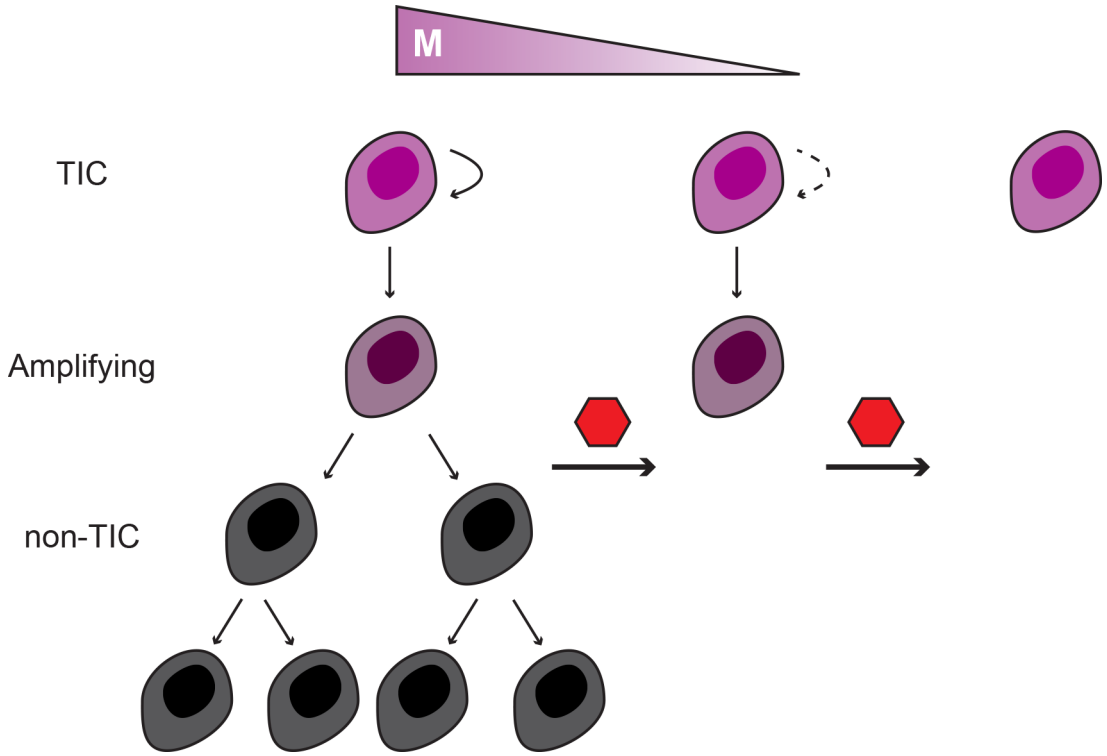

Figure 3.1 Inhibition of the mesenchymal program impairs proliferation and self-renewal. Dual inhibition of Met and FGFR1 (symbolized by red stop sign), resulting in loss of the mesenchymal phenotype, abrogates proliferation of amplifying progenitor cells and impairs TIC self-renewal capacity over time.

3.2.3 MET contributes to tumourigenesis in human models of breast cancer

While the MMTV-*Met^{mt};Trp53fl/+;Cre* mouse model closely resembles claudin-low breast cancer, genomic amplification of *MET* is not a frequent event in human breast cancer (Gonzalez-Angulo et al. 2013). MET is broadly overexpressed without genetic alteration across TNBC cell lines, whereas FGFR1 is selectively expressed in the more mesenchymal, claudin-low associated Basal B cell lines (Figure 2.9A). Co-expression of MET and FGFR1 in human claudin-low breast cancer cell lines mirrors our observations in our mouse model, where FGFR1 expression is selectively upregulated in spindloid, claudin-low tumours (Figure 2.4). Basal B cell lines also express FGF2, exhibit autocrine FGFR signaling, and are sensitive to FGFR inhibitors (Sharpe et al. 2011). We found that upon FGFR inhibition, Basal B cell lines underwent depletion of their $CD44^{+}CD24^{-/low}$ TIC populations, a process that can be prevented by HGF stimulation of MET (Figure 2.11). Importantly, we demonstrated that phosphorylation of FRS2 can occur via HGF-dependent activation of MET (Figure 2.12) (discussed further below). These results mirrored our findings in the MMTV-*Met^{mt};Trp53fl/+;Cre* mouse model, and support the hypothesis that upon activation, MET can play a role in tumourigenesis by either compensating for or independently activating stem-promoting pathways typically mediated through the FGFR1-FRS2 axis.

Recently, we demonstrated that a subset of TNBC PDXs contained EGFR-expressing TICs and were responsive to Gefitinib treatment *in vivo* (Savage et al. 2017). All responders harboured *BRCA1* mutations. In these PDXs and patient tumours, high EGFR expression was observed in a subset of cells that displayed enhanced ALDH activity and sphere-forming efficiency. In the present work, we have identified additional TNBC PDXs that co-expressed MET and FGFR1 (Figure 2.13), where combined inhibition of MET and FGFR signaling resulted in only moderate reduction in overall tumor burden, but was accompanied by a depletion of sphere-forming and

$CD44^{+}CD24^{-/low}$ populations (Figure 2.14). These results are consistent with the idea that breast TICs are heterogeneous, and can exist in a more epithelial-like and proliferative state (ALDH+) or a more mesenchymal-like and quiescent state (CD44+ $CD24^{-/low}$) (S. Liu et al. 2014). In the absence of activating genetic alterations, MET is predominantly associated with potentiating invasion and migration, and FGFRs with regulating stemness. Our data suggest that these pathways cooperate to promote a population of mesenchymal-like TICs in TNBCs. These TICs do not appear to be significant drivers of bulk proliferation, but rather populate a pool of cells expressing an elevated mesenchymal program and enriched for self-renewal. Like other potential therapies targeting EMT-like TIC populations (Bhola et al. 2013; Tam et al. 2013), the clinical benefits of inhibiting these receptors are likely maximized if paired with tumour de-bulking agents.

3.2.4 MET as a therapeutic target in TNBC

TNBC is a highly heterogeneous group of cancers, and the inability to effectively stratify these patients remains a major challenge to treatment overall. Using several existing gene signatures associated with TIC/EMT phenotypes, we stratified TNBC patients from the TCGA dataset (Figure 2.15) and determined that TIC/EMT-enriched tumours were elevated in gene signatures associated with both MET and FGFR1 signaling pathway activation (Figure 2.16B). While the TIC/EMT high patient group was enriched for expression of *FGFR1* compared to the TIC/EMT low group, this difference was not observed for *MET* (Figure 2.16A). This is likely due to the fact that TNBC as a group are already associated with higher levels of MET expression compared to other breast cancer subgroups. Interestingly, *HGF* expression was elevated in the TIC/EMT high patient group, suggesting that ligand-dependent activation of MET may be a key determinant in the functional role of MET in these tumors. This is consistent with the lack of *MET* genetic lesions

in breast cancer and therefore a requirement for HGF stimulation in order for MET to actualize its tumour-promoting capacity. In support of this, we have found that cancer cell lines expressing non-amplified MET exhibits shorter tumour latency when injected into mice engineered to express human HGF when compared to wildtype mice (unpublished data). However, once established these tumours do not overtly respond to MET inhibition, supporting the idea that HGF-dependent MET pathways contribute to tumour initiation and subsequently to progression via processes independent of proliferation.

As a broader implication of these results, in general only tumours harbouring *MET* activating mutation or amplification are predicted to show overt response to MET kinase inhibitors. Indeed, important lessons can be gained from failed MET inhibitor clinical trials with unselected patients. In one such trial involving the MET inhibitor, Tivantinib, a post hoc analysis identified a subgroup of patients with high *MET* copy number gains who exhibited better overall survival when treated (Scagliotti et al. 2015). In this context, tumour shrinkage is unlikely to be an appropriate measure of therapeutic efficacy in cancers overexpressing a wildtype MET. Importantly in TNBC, anti-MET therapy could be beneficial for targeting mesenchymal TICs, diminishing metastatic capacity, or sensitizing cancer cells to radiation therapy. Immunotherapy, which has shown promising results in TNBC, is an interesting therapy to potentially deploy in combination with MET inhibition. A clinical trial (NCT02323126) combining a checkpoint inhibitor with small molecule MET inhibitor INC280 is currently ongoing in NSCLC.

3.3 MET and FGFR signaling

3.3.1 Compensatory signaling between MET and FGFR in cancer

The importance of MET and FGFR1 signaling among various human cancers is emerging. In FGFR1-amplified lung cancer cell lines, multiple mechanisms of MET signaling-mediated resistance to FGFR-targeting drugs have been identified (S. M. Kim et al. 2016; Kotani et al. 2015; Adachi et al. 2017; Malchers et al. 2017). Similarly, FGFR1-dependent resistance to MET inhibition in various cancers has been identified (Kentsis et al. 2012; Jo et al. 2015; Varkaris et al. 2016). In most cases, the cell lines studied harboured MET or FGFR1 amplifications, prompting their selection for treatment with pharmacologic agents against either of the RTKs. Our results here provide evidence of an association between MET and FGFR1 in a non-amplified, wildtype and ligand-dependent state, whereby MET and FGFR1 promote and regulate TICs by converging on pathways that promote mesenchymal and stem programs. Cellular plasticity is central to both the EMT process and the stem cell state, and underlies the link between these two programs and TICs (Ye & Weinberg 2015; Puisieux et al. 2014).

3.3.2 MET and FGFR signaling in TNBC/basal-like breast cancer

The established role of FGFR signaling in stem cell biology, in combination with autocrine bFGF expression in TNBC cell lines (Sharpe et al. 2011) and in TNBC PDXs (data not shown), supports FGFR signaling as required to maintain a stem/mesenchymal phenotype in at least a subset of TNBC. Our finding that treatment with FGFR inhibitor alone in claudin-low cell lines resulted in depletion of the $CD44^{+}CD24^{-/low}$ TIC compartment (Figure 2.11) further supports these data. Importantly, HGF stimulation of these TNBCs abrogated TIC depletion, supporting a scenario whereby MET signaling activated by stromally-produced HGF or by stress plays a supportive

and/or compensatory role to FGFR signaling. In this context, MET activation performs the dual functions of inducing an EMT-like program as well as activating pathways that promote stemness, maintaining cellular plasticity that favours TICs and tumourigenesis (Figure 3.2). Here, the tumour-promoting signals provided by MET reflect physiological functions of MET during embryogenesis, including inductions of a mesenchymal cell state and cell survival signals, rather than cell proliferation.

In both the murine and human models of TNBC examined in this work, the FRS2 scaffold protein was identified to be critical to facilitate interplay between MET and FGFR signaling to promote TICs. As discussed in Chapter 1, the SHP2-binding sites of FRS2 are critical for activating a sustained ERK signal that support the functions of stem and progenitor cells (Yamamoto et al. 2005; Sato et al. 2010). Interestingly, the phosphatase SHP2 has been found to promote breast TICs and metastasis, and a SHP2 gene signature was enriched in IDC and TNBC compared to DCIS and other breast cancer subtypes, respectively (Aceto et al. 2012). Furthermore, knockdown of SHP2 reduces anchorage-independent growth in breast cancer cells and induces a reversion from a mesenchymal to an epithelial phenotype (X.-D. Zhou & Agazie 2008). Given that MET can activate SHP2 through recruitment to the scaffold protein GAB1 as well as FRS2, SHP2 may be a key mediator of MET and FGFR signaling in promoting TICs. Several allosteric inhibitors of SHP2 have been developed in recent years (Y.-N. P. Chen et al. 2016; J. Xie et al. 2017; Fodor et al. 2018), and these could be a promising approach for disrupting pathways that maintain TICs in TNBC.

Additional support for dual MET and FGFR1 signals in TNBC is provided, whereby co-expression of *HGF* and *FGFR1* predicts poor relapse-free survival specifically among basal-like breast cancers (Figure 2.17). Notably, while EGF/EGFR can participate in compensatory signaling

involving both MET and FGFRs (Wilson et al. 2012; Harbinski et al. 2012), the EGF/EGFR axis, alone or in combination with components of MET and FGFR1 signaling, did not predict poor relapse-free survival in basal-like breast cancers (Figure 3.3). These findings highlight a specific requirement for the tumour-promoting pathways activated by MET and FGFR1 in highly mesenchymal breast tumours.

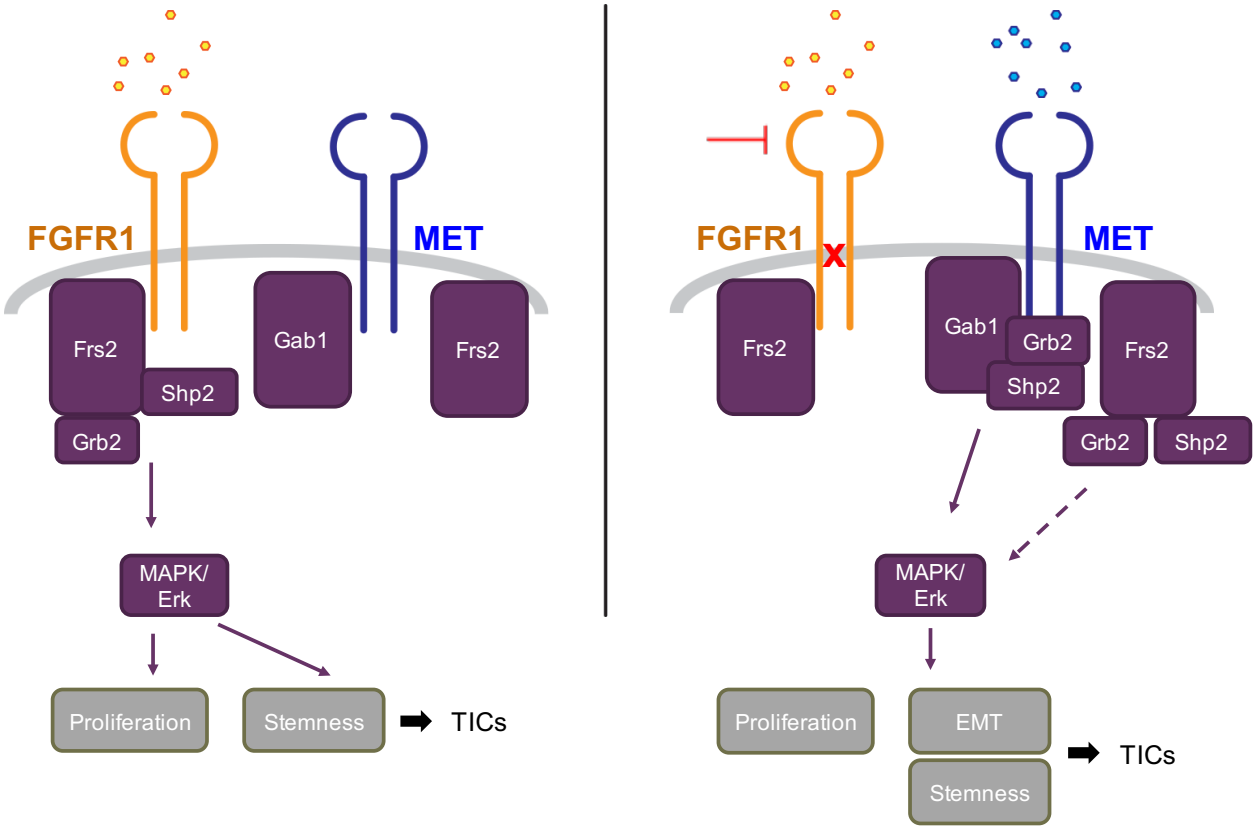

Figure 3.2 HGF-activated MET can compensate for loss of FGFR signaling to maintain TICs. In a wildtype, non-amplified setting, MET activates a mesenchymal pathway that promotes TICs by maintaining a sustained Erk signal in the absence of FGFR signaling. MET-dependent phosphorylation and signaling through FRS2 is denoted with a dotted line because the mechanism is not yet well-characterized.

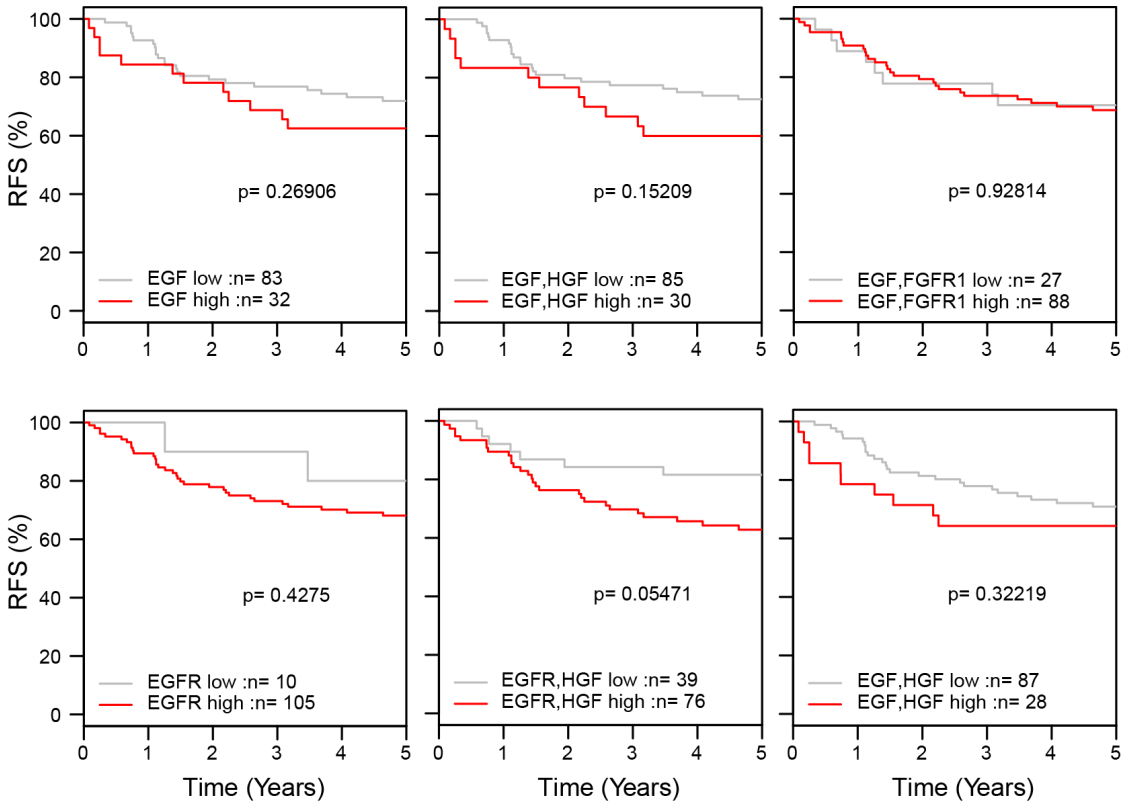

Figure 3.3 EGFR signaling axis is not associated with poor outcome in Basal-like breast cancers. Relapse-free survival (RFS) analysis of basal-like breast cancers split by expression of EGF and EGFR in combination with HGF or FGFR1.

3.4 MET signaling through FRS2

3.4.1 Wildtype MET phosphorylates FRS2 upon HGF stimulation

Previous studies documenting the ability of MET to phosphorylate FRS2 were in the context of amplified or constitutively active MET (Wilson et al. 2012; Harbinski et al. 2012; Adachi et al. 2017). Here, we show that HGF-dependent activation of wildtype MET also results in tyrosine phosphorylation of FRS2. In Basal B breast cancer cell lines that highly co-express MET and FGFR1, HGF-stimulated activation of MET led to FRS2 phosphorylation and promoted ERK1/2 phosphorylation in the absence of FGFR signaling (Figure 2.12). The functional outcome of FRS2

phosphorylation was maintenance of TIC capacity and phenotype in these cell lines (Figure 2.11). Given that wildtype MET was not documented to signal through FRS2, these findings highlight a novel mechanism by which MET can converge with FGFR signaling on a common cellular program to regulate TICs.

Activation of the MET receptor results in prolonged downstream signaling, including a sustained ERK signal, which is crucial for MET-dependent the biological processes. It is known that the key scaffold protein that facilitates MET-dependent signaling networks is GAB1, where *Gab-1-/-* mice phenocopy *Met-/-* and *Hgf-/-* mice (Sachs et al. 2000). Thus, in the absence of genetic lesions in *MET*, it is probable that FRS2 is non-essential but promotes MET-dependent signaling by providing an additional and alternative route to provide sustained ERK activation.

3.4.2 FRS2 promotes diverse biological outputs in MET-amplified cancer cells

A role for FRS2 in MET-dependent processes has not been well-characterized. In this context, we show that shRNA-mediated knockdown of Frs2 in MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid cells did not significantly alter proliferation of adherent cells or tumoursphere formation, supporting that FRS2 is not essential for tumour initiation or growth (Figure 2.5E-F). However, given that Met promotes epithelial cell invasion and migration, we tested the capacity of cells lacking Frs2 to invade through collagen. Loss of Frs2 resulted in a striking decrease in invasive capacity when distance invaded by individual cells plated between two layers of collagen was quantified (Figure 3.4). To test whether the observed loss of *in vitro* invasive capacity translated to the loss of metastatic potential, MMTV-*Met^{mt};Trp53fl/+;Cre* spindloid cells transfected with empty vector or shFrs2 were injected into the 4th mammary fat pad of female

athymic mice. Once established, primary tumours were resected and mice were monitored for metastatic lesions. Consistent with our *in vitro* study results, shFrs2 cells exhibited a reduced capacity for generating both local and distant metastases when compared to empty vector control cells (Table 3.1). These results indicated that in the MMTV-*Met^{mt};Trp53fl/+;Cre* model of claudin-low breast cancer, the scaffold protein Frs2 is important for Met-dependent invasion and migration.

Due to the lack of genetic alterations in *MET* in breast cancer cell lines, and previous data demonstrating FRS2 phosphorylation in MET-amplified cells, we tested the requirement for FRS2 in gastric cancer cell lines (MKN45 and KATO II) where MET amplification, activation, and signaling promote a proliferative response. In MKN45 cells, shRNA-mediated knockdown of FRS2 resulted in the loss of ERK phosphorylation, leading to a corresponding impairment in proliferation (Figure 3.5A). However, these effects were not observed in a second MET-amplified gastric cancer cell line, KATO II, where efficient knockdown of FRS2 did not affect ERK activation nor cell proliferation (Figure 3.5B). However, following MET inhibition in KATO II cells using increasing concentrations of Crizotinib, cells lacking FRS2 showed earlier induction of cleaved PARP and decreased cell viability, supporting a role for FRS2 in survival signals in these cells (Figure 3.6).

These findings provide new evidence that in MET-amplified cancer cells, multiple processes, including cell invasion and migration, proliferation, and survival are dependent on FRS2 in a cell type-dependent manner. These findings expand the current understanding of the oncogenic pathways promoted by amplified MET.

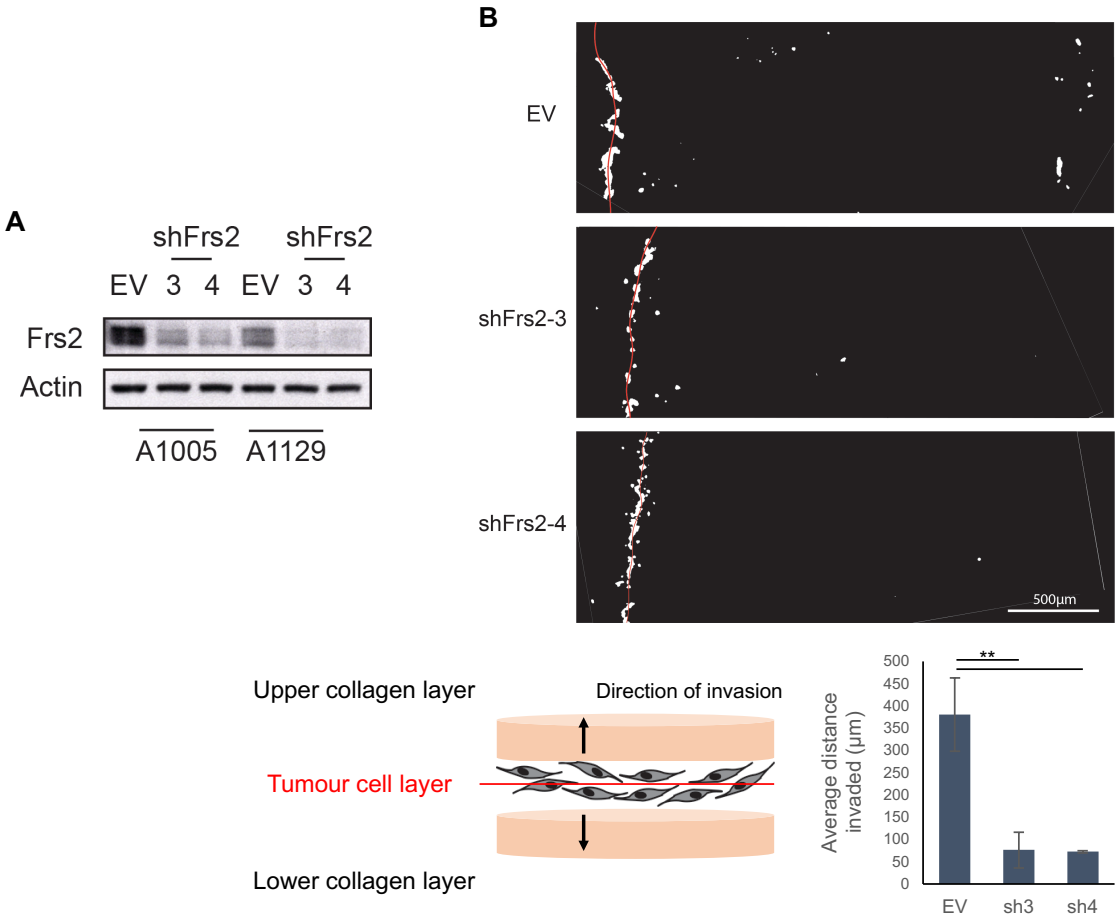

Figure 3.4 Loss of Frs2 in MMTV-*Met*^{mt}*;Trp53fl/+;Cre* spindloid tumour cells impairs cell invasion. (A) A1005 and A1129 cells were transfected with empty vector or shRNA against Frs2, and efficient knockdown of Frs2 was verified by Western blot analysis. (B) A1005 empty vector and shFrs2 cells were seeded in between two layers of collagen and cultured for 10 days before being fixed and embedded in OCT. Frozen blocks were then sectioned and distance invaded by individual cells measured. **p≤ 0.01. Error bars indicate SEM.

A1005	EV (n=2)		shFrs2-3 (n=3)			shFrs2-4 (n=2)	
Total metastases	3	4	0	0	2	2	0
Metastatic sites	Right Forelimb Lymph nodes (x2)	Right thigh Lymph nodes (x2) Lung			Lung Lymph node	Lung Pleura	
A1129	**EV (n=2)**		**shFrs2-3 (n=3)**			**shFrs2-4 (n=2)**	
Total metastases	1	1	0	0	0	0	0
Metastatic sites	Right thigh	Right thigh					

Table 3.1 Incidences of metastasis following resection of primary tumour.

3.4.3 Mechanism of MET-dependent FRS2 phosphorylation

Our preliminary investigations into MET-dependent FRS2 phosphorylation raises the question of how these two proteins interact. FRS2 is a member of a subfamily of membrane-linked scaffold proteins, which includes the GAB, insulin receptor substrate (IRS), and downstream of kinase (DOK) proteins. Given that MET signals through GAB1 and, as we have demonstrated, FRS2, it is interesting to speculate whether membrane-linked scaffold proteins are common RTK signaling substrates due to vicinity. This type of interaction likely requires an abundance of the interacting proteins in the plasma membrane. While there is one report of EGFR-dependent phosphorylation of FRS2 in a cell line where EGFR is overexpressed (Y et al. 2003), this is not observed in other cells that have modest levels of EGFRs (Kouhara et al. 1997; Lax et al. 2002). Similarly, the breast cancer cell lines (BT549 and Hs578t) we have used to study HGF-stimulated, MET-dependent FRS2 phosphorylation also overexpress the MET wildtype protein. In cancer cells, aberrant mechanisms may be in place to maintain these scaffold proteins at the plasma membrane, enhancing their accessibility to RTK. Alternatively, MET could be recruited to FRS2 through

GRB2-GAB1. An interesting study using biotin proximity labeling found MET to be in proximity of FGF1-activated FGFR4 (Haugsten et al. 2016), suggesting that MET could be recruited to FGFR4-FRS2 and its associated signaling complex.

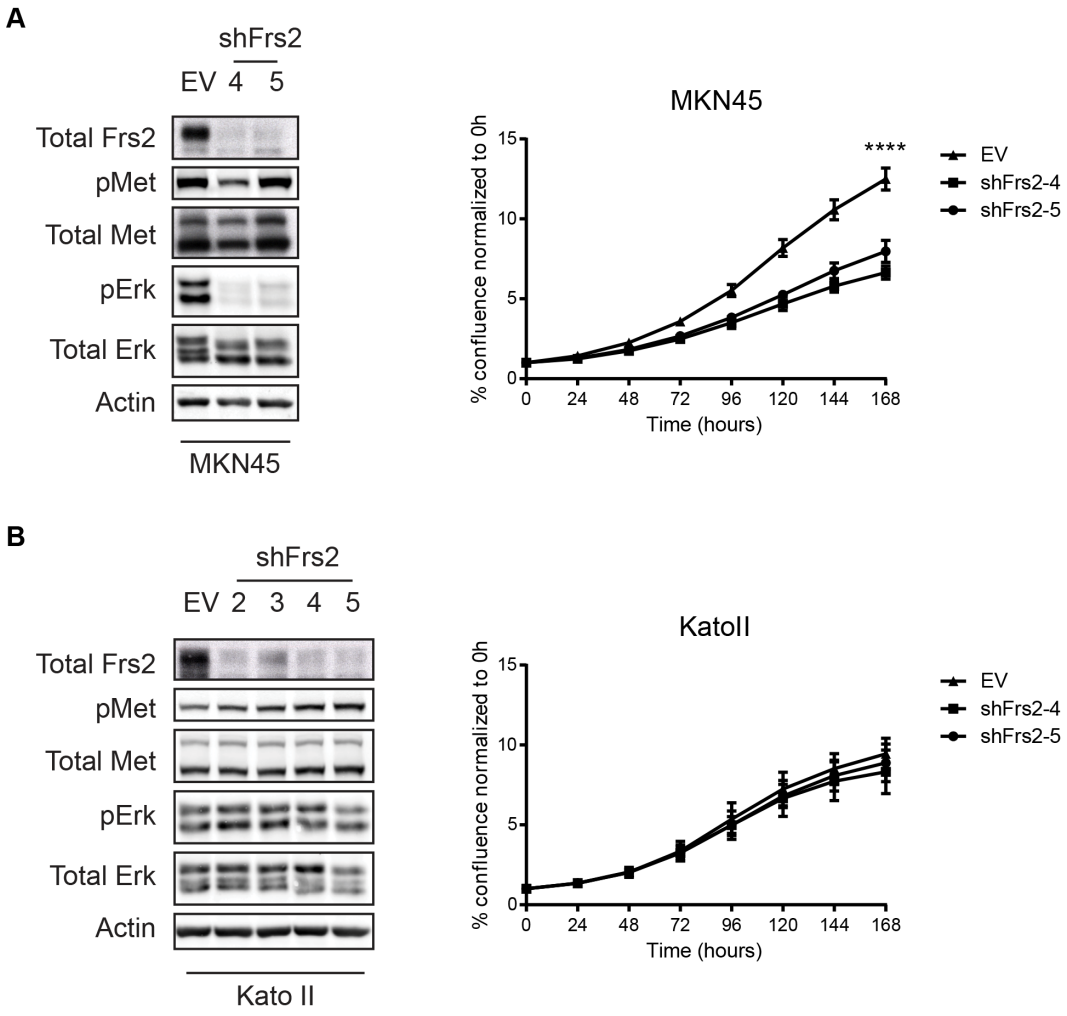

Figure 3.5 Loss of FRS2 in human MET-amplified cells affects proliferation. MKN45 (A) and KATO II (B) cells transfected with empty vector or shRNA against FRS2, and efficient knockdown of FRS2 was verified by Western blot analysis. The effect of FRS2 knockdown on proliferation in adherent condition was assessed for 7 days using Incucyte Live-Cell Analysis System. ****$p \leq 0.0001$. Error bars indicate SEM.

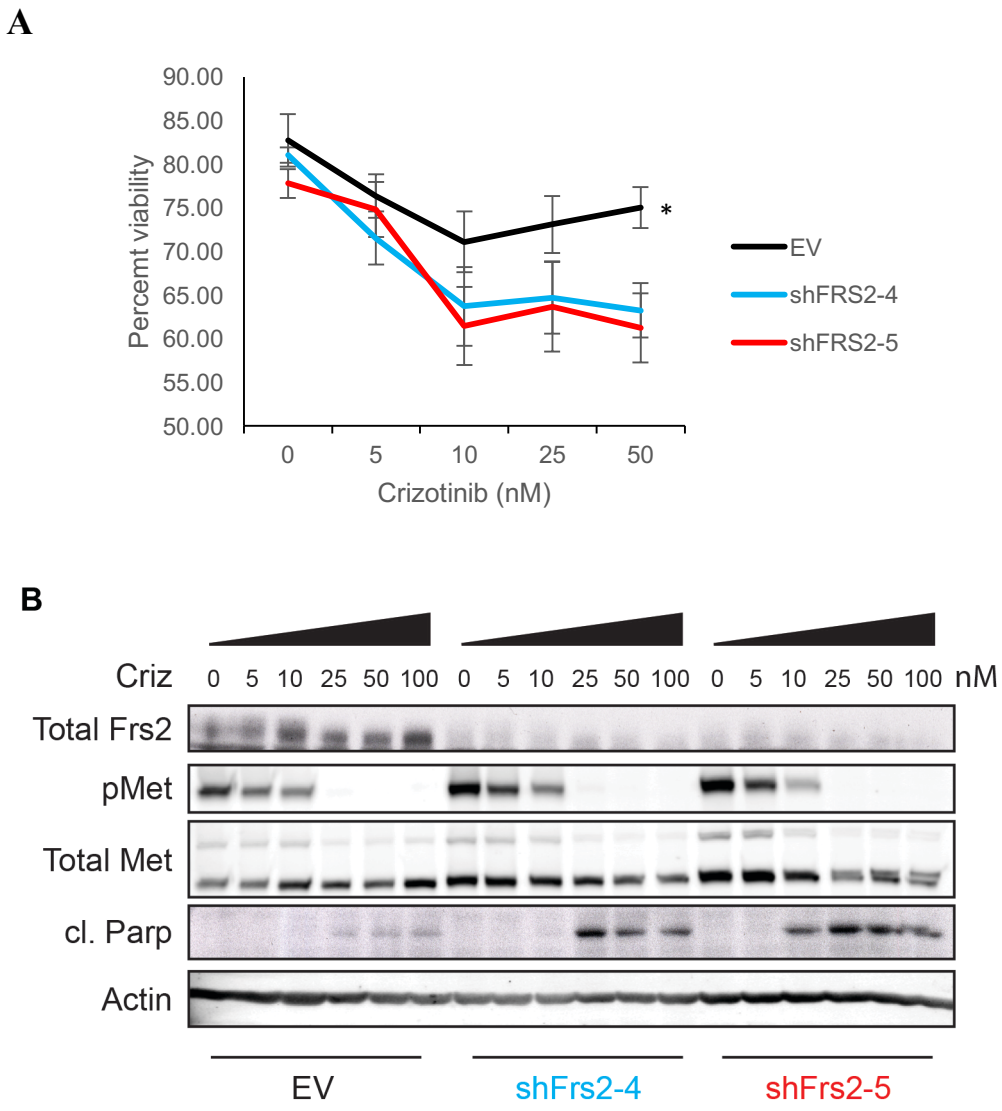

Figure 3.6 Loss of FRS2 in human MET-amplified cells affects survival under stress. (A) KATO II cells transfected with empty vector or shRNA against FRS2 were treated with Crizotinib at the indicated concentrations for 24h. Cells were then collected and assessed for viability by flow cytometry using 7-AAD exclusion. (B) Crizotinib-treated EV and shFRS2 cells were also collected and analyzed by Western blot analysis. *p<0.5. Error bars indicate SEM.

3.5 Conclusions

In summary, we find that MET and FGFR1 are co-expressed in highly mesenchymal TNBCs and that they co-regulate TICs through compensatory signaling interplay that is largely mediated through the scaffold protein FRS2. A MET-dependent mesenchymal program can promote TICs by enhancing survival and self-renewal capacities, as well as proliferation if MET kinase is constitutively active due to genetic alterations. Importantly, we uncover that *HGF* and *FGFR1* co-expression is associated with poor patient outcome in basal-like breast cancers. These findings offer new stratification tools for TNBC patients, as well as potential anti-TIC therapeutic targets that warrant further investigation.

We characterize a requirement for FRS2 in a range of MET-dependent biological functions, including cell invasion, proliferation, and survival, that had not been previously understood. We also demonstrate for the first time, to our knowledge, the ability of non-amplified, wildtype MET to independently phosphorylate FRS2, facilitating the engagement by MET of pathways typically regulated by FGFR signaling that promote a stem program. These findings contribute to and expand the current understanding of MET signaling networks and the regulation of MET-dependent cellular processes.

4 EXPERIMENTAL PROCEDURES

4.1 Cell culture

Primary mouse cell lines were previously established by dissociation of MMTV-*Met*^{mt}, *Trp53fl/+;Cre*, and MMTV-*Met*^{mt}*;Trp53fl/+;Cre* mammary tumours as described (Ponzo et al. 2009; Knight et al. 2013). Cells were cultured in DMEM supplemented with 5% serum, epidermal growth factor (5ng/ml), insulin (5µg/ml), bovine pituitary extract (35µg/ml), and hydrocortisone (1µg/ml). BT20, HCC70, HCC1937, HCC1954, HCC1395, MDA468, MDA436, MDA157, MDA231, BT549, and Hs578T were cultured according to ATCC recommendations. All cells were grown at 37°C and 5% CO_2. Small molecule inhibitors used were Crizotinib (LC Laboratories) against MET, and PD173074 against FGFRs (Selleckchem) at 1µM.

4.2 Proliferation assays

A1005, A1129, A12221, and A1222 cells were trypsinized and collected as single cells at the indicated time points. Cells were mixed 1:1 with trypan blue dye and counted using an automated cell counter (Cellometer, Nexcelom Bioscience). Inclusion of the dye identified dead cells and these were excluded from the cell count.

MKN45 and KATO II cells were plated in 96-well plates at 3000 cells/well. Cells were then cultured in the IncuCyte ZOOM Live Kinetic Imaging System (Essen BioScience) to capture phase-contrast images every 4 hours and to measure confluence over 7 days.

4.3 Tumoursphere formation assays

Single cells were seeded in 6-well ultra-low attachments plates (Corning) in 2 ml serum-free DMEM/F12 supplemented with 1x B27, 10µg/ml insulin (Gibco), 20ng/ml EGF (BP Bioscience), 20ng/ml bFGF (StemRD), 10µg/ml heparin (StemCell Technologies), and 0.5ug/ml

hydrocortisone (Wisent). Tumoursphere number and size were determined after 5-7 days of culture using the software AxioVision (Carl Zeiss). To serially passage, tumourspheres were enzymatically and mechanically dissociated in 0.05% Trypsin-EDTA (Gibco), passed through a 25G needle, and re-seeded as single cells. All experiments were performed in triplicate wells.

4.4 Tumoursphere proliferation assays

Proliferation of tumourspheres was determined using the CyQUANT Cell Proliferation Assay Kit (Invitrogen) according to the manufacturer's instructions with some modifications. Briefly, single cells were seeded in 96-well ultra-low attachments plates (Corning) and cultured for 5-7 days. Plates were collected on the first and last days of culture and centrifuged at 2000 rpm for 20 minutes to pellet the tumourspheres. Plates were then inverted to remove media, frozen at -80°C for a minimum of 24 hours, and thawed at room temperature when ready to quantify. The CyQUANT dye/lysis buffer solution was added to all wells and each plate was analyzed using a VarioSkan plate reader (Thermo Scientific) Proliferation was expressed as a ratio of fluorescence on last day to first day of culture. All experiments were performed in triplicate wells.

4.5 Immunoblotting

Tumour-derived cell lines were lysed in 1% Triton lysis buffer (150 mM NaCl, 50 mM HEPES, 1 mM EDTA, 1.5 mM MgCl2 , 1% Triton X-100, 4% glycerol) containing protease and phosphatase inhibitors. Snap-frozen mammary tumours were powdered under liquid nitrogen and then lysed in 1% Triton lysis buffer as above. Whole cell lysates were resolved by SDS/PAGE and transferred to PVDF membranes. Membranes were blocked with Li-COR Blocking Buffer (Li-COR Biosciences) and probed with primary antibodies overnight at 4°C. After TBST washes,

membranes were incubated with infrared-conjugated (Li-COR Biosciences) or HRP-conjugated (Cell Signaling and GE Healthsciences) secondary antibodies for 1 hour at room temperature for signal detection by Odyssey IR Imaging System (Li-COR Biosciences) or enhanced chemiluminescence (Amersham Biosciences), respectively. Antibodies are detailed below.

4.6 Quantitative RT-PCR

RNA was isolated using the RNAeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. qPCR reactions were performed using SYBR Green I Master on a LightCycler480 (Roche). FGFR primer sequences (Integrated DNA Technologies) used were as follows: FGFR1_F 5'-ATGGTTGACCGTTCTGGAAG-3', FGFR1_R 5'-GGAAGTCGCTCTTCTTGGTG-3', FGFR2_F 5'-GGATCAAGCACGTGGAAAAG-3', FGFR2_R 5'-TATTCCCCAGCATCCATCTC-3', FGFR3_F 5'-ATCGACAAGGACCGTACTGC-3', FGFR3_R 5'-CCCCAGCAGGTTAATGATGT-3'. Hprt and Rpl13a were used as reference genes with the following primers: Hprt_F 5'-GCCCCAAAATGGTTAAGGTT-3', Hprt_R 5'-CAAGGGCATATCCAACAACA-3', Rpl13a_F 5'-AAGGCCAAGATGCACTATCG-3', Rpl13a_R 5'-GAGTCCGTTGGTCTTGAGGA-3'.

4.7 Immunohistochemistry

Tumour tissues were formalin-fixed and paraffin-embedded in a previous study (Knight et al. 2013), and sections were cut at 4μm. Sections were deparaffinised in xylene and re-hydrated in ethanol, followed by antigen retrieval in Tris-EDTA at boiling temperature. Slides were cooled

and blocked with Power Block (BioGenex) and incubated overnight with primary antibody at 4°C. Slides were rinsed in distilled water then incubated in 3% H_2O_2 for 30min. SignalStain Boost (Cell Signaling) was used as secondary antibody and the SignalStain DAB substrate kit (Cell Signaing) was used to detect signal prior to counterstaining with Harris' hematoxylin. Finally, slides were dehydrated, mounted, and scanned using Aperio-XT slide scanner (Aperio). Antibodies and detection reagents used are detailed in Table 4.1.

4.8 Knockdown by shRNA

Knockdown of *FGFR1* and *Frs2* in A1005 and A1129 cells were carried out using shRNAs cloned into pLKO.1 lentiviral vectors (Sigma-Aldrich). The following clones were used for *FGFR1*: TRCN0000023295 and *Frs2*: sh3, TRCN0000097281; sh4, TRCN0000097282. HEK293T cells were transfected using FuGENE HD (Promega) to produce lentivirus. Packaging vectors used were pRSV-Rev, pHCMV-VSVg, and pMDLg/pRRE. Media containing viral particles was collected and passed through a 0.45μm filter. A1005 and A1129 cells were treated with virus in media containing 8μg/ml polybrene. Infected A1005 and A1129 cells with stable knockdown were selected under Puromycin (2μg/ml) and the drug efflux inhibitor Cyclosporin A (2.5μM).

Knockdown of FRS2 in MKN45 and KATO II cells were carried out using shRNAs cloned in pLKO.1 lentiviral vectors (Sigma-Aldrich). The following clones were used for *FRS2*: sh2, TRCN0000061719; sh3, TRCN0000061720; sh4 TRCN0000061721; sh5, TRCN0000061722. Viral particles were produced, and collected as above. Infected MKN45 and KATO II cells with stable knockdown were selected Puromycin (1μg/ml).

4.9 RNA sequencing

A1005, A1129, and A1471 tumourspheres treated with inhibitors as indicated were collected and total RNA was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's instructions. High RNA quality was verified using the Bioanalyzer RNA 6000 Nano assay (Agilent). The samples were sequenced using a NextSeq500 sequencer (Illumina) by the Genomics Core Facility of the Institute of for Research in Immunology and Cancer, Université de Montréal. Reads were mapped to human genome version hg19 using Spliced Transcripts Alignment to a Reference (STAR). Reads counts were normalized using mean-centered and log-transformed. Differentially expressed genes among groups were identified using the R packages DESeq2 (Love et al. 2014) and Lima. After unpaired analysis, only genes with False Discovery Rate (FDR) < 0.05, and log2 fold change ≥1.5 were considered. Hierarchical clustering of differentially expressed genes was used to represent the results (R package ggplot2).

Gene set enrichment analysis was applied to genes that decreased in expression in combination compared to DMSO-treated samples (FDR<0.05, log2fold change ≤1.5) using the MSigDB online tool (http://software.broadinstitute.org/gsea/msigdb/index.jsp) to generate a list of the top 10 down-regulated GO biological processes. The same approach was applied to genes that increased in expression in combination compared to DMSO-treated samples (FDR<0.05, log2fold change ≥1.5) to generate a list of the top 10 up-regulated GO biological processes.

4.10 *In vivo* limiting dilution assay

A1129 and A1221 cells were resuspended in sterile PBS and injected at decreasing numbers (100, 50, 10 cells) into the 4th mammary fat pad of female athymic mice (Taconic Farms). The study

was conducted over 12.5 weeks, during which mice were palpated every 2-3 days monitor for tumour outgrowth. TIC frequency was calculated using the Extreme Limiting Dilution Analysis (ELDA) online software available at http://bioinf.wehi.edu.au/software/elda/ . All experiments were carried out in accordance with the guidelines of the McGill University Animal Ethics Committee and the Canadian Council on Animal Care (Protocol #7514).

4.11 *In vivo* inhibitor treatments

For the tumour initiation study, 100,000 A1129 cells were resuspended in sterile PBS and injected into the 4th mammary fat pad of female athymic mice (Taconic Farms, Inc.). On day of injection, mice were randomized into 4 groups (n=10), with each group receiving vehicle control, Crizotinib (50 mg/kg/d, p.o.), BGJ398 (30mg/kg/d, p.o.), or both drugs in combination. Both inhibitors were purchased from Active Biochemicals. BGJ398 was used instead of PD173074 in *in vivo* studies to inhibit FGFR signaling because it is an orally available drug. For the tumour progression study, mice were injected with A1129 cells as above. Once tumours reached 50-100mm^3, mice were randomized into 4 treatment groups (n=10) and gavaged daily as above. Tumour diameters were measured every 2 days with calipers, and tumour volumes (mm^3) were calculated by the following formula: (length x4.18879)/2 x (width/2)^2.

PDX GCRC1915 tumours were transplanted as fragments into the 4th mammary fat pad of female NSG mice with *HGF*$^{tm1.1(HGF)Aveo}$ "humanized" knock-in allele (NSG- hHGFki). Once tumours reached 50-100mm^3, mice were randomized into 4 treatment groups (n=5) and gavaged daily as above. Tumour growth was monitored and volumes calculated as above. All experiments were carried out in accordance with the guidelines of the McGill University Animal Ethics Committee and the Canadian Council on Animal Care (Protocol #7514).

4.12 *In vivo* metastasis study

A1005 and A1129 cells stably expressing firefly luciferase were previously generated as described (Knight et al. 2013). 200,000 A1005Luc cells and 100,000 A1129Luc cells with stable knockdown of Frs2 were injected into the 4th mammary fat pad of female athymic mice (Taconic Farms, Inc.). Tumours were resected once they reached 50-100mm^3, and the mice were monitored by luciferase activity for metastases. For imaging, mice were injected intraperitoneally with the luciferase substrate D-luciferin (Caliper Life Sciences, Inc.) dissolved in PBS (50µl at 30mg/ml), anaesthetized with isoflurane, and imaged by bioluminescence (Xenogen IVIS 100, Caliper Life Sciences, Inc.). Mice were imaged once a week, and necropsies were performed after sacrifice to identify metastatic lesions. All experiments were carried out in accordance with the guidelines of the McGill University Animal Ethics Committee and the Canadian Council on Animal Care (Protocol #7514).

4.13 Flow cytometry

Single cells were stained with fluorophore-conjugated antibodies in 100µl of PBS with 2% FBS for 30 minutes on ice protected from light. Cells were washed twice then resuspended in PBS with 2% FBS, and viability dye 7-AAD (eBioscience 00-6993) was added to each sample. Multi-colour cell sorting was performed on a FACS CantoII (BD Biosciences) and data analysis was performed using FlowJo (Tree Star Inc.). Antibodies used are detailed in Table 4.1.

4.14 Immunofluorescence

Tumour tissues were formalin-fixed and paraffin-embedded, and sections were cut at 4µm. Sections were deparaffinised in xylene and re-hydrated in ethanol, followed by citrate buffer (pH

6) at boiling temperature. Slides were cooled and blocked with 2% BSA and incubated overnight with primary antibody at 4°C. Slides were rinsed in distilled water, treated with 3% H_2O_2 for 30min, incubated with secondary antibody for 45min at room temperature, and counterstained with 0.5ng/ml DAPI. Tyramide signal amplification (Perkin Elmer) was used according to the manufacturer's instructions. Finally, slides were imaged with an LSM800 confocal microscope and analyzed using Zen software (Zeiss). Antibodies and detection reagents used are detailed in Table 4.1.

4.15 Dissociation of PDX tumours

Tumours were minced using razor blades and transferred to 50ml conical tube containing tissue digestion media (DMEM+2.5 FBS with collagenase IV). Tubes were placed in rotating ovens and to dissociate over 2-3h at 37°C. The suspension was centrifuged to pellet the epithelial and fibroblast cell content. The pellet was then resuspended in warm PBS and the epithelial organoids/cells were allowed to gravity sediment for approximately 5min. The stromal cell-rich supernatant was removed and the epithelial content washed in warm PBS then pelleted. The pellet was then trypsinized in 0.05% Trypsin-EDTA and passed through a 40μm cell strainer to generate a single cell suspension. Murine stromal cells were further removed using a Mouse Cell Depletion Kit (Miltenyi) according to the manufacturer's instructions.

4.16 Collagen invasion assay

Assays were performed as detailed elsewhere (Brekhman & Neufeld 2009) with minor modifications as follows. Type I Human Collagen Solution (Advanced BioMatrix), prepared to 3mg/ml in PBS and 0.01N NaOH with no additional supplements, was used. Cells were seeded in

between two layers of collagen at a density of $5x10^4$ cells/chamber of an 8-well chamber slide (Nunc). Media was refreshed every 48h and cells were grown for a total of 10 days prior to fixation for 1h in 4% PFA. Fixed collagen gels were embedded in OCT and immediately frozen in liquid nitrogen. Frozen collagen blocks were flipped by 90˚, sectioned at 8µm thickness and mounted on microscope slides pre-coated with Poly-L-lysine (Sigma) according to the manufacturer's instructions. Sections were dried and counterstained with DAPI prior to mounting with a glass coverslip. Sections were imaged at 4X magnification using an EVOS Cell Imaging System.

Invasion of individual cells into collagen was quantified using MetaMorph software (Molecular Devices). Briefly, a line was drawn to indicate the plane where cells were seeded and was used to generate an Euclidean distance map. Nuclei identified with the 'granularity' application were transferred onto the Euclidean map to calculate the distance invaded into the collagen from the seeding-line. The mean distance of all nuclei was calculated. Two or three images were analyzed per condition.

4.17 Analysis of publicly availably gene expression data

The R package GSVA (Hänzelmann et al. 2013) was used to analyze data from TNBC patients of the TCGA dataset (Koboldt et al. 2012). In order to enrich for patients with high expression of TIC or EMT gene signatures, ssGSEA was applied using published gene signatures for claudin-low breast cancer (Knight et al. 2013), TIC (Creighton et al. 2009), EMT (Hallmark), and undifferentiated mammary epithelial cells (Dontu 2003). Welch two-sample t-test was used to compare expression of single genes, as well as MET and FGFR1 activation signatures between TIC/EMT high and TIC/EMT low patient cohorts.

The gene expression-based outcome for breast cancer online (GOBO) tool (http://co.bmc.lu.se/gobo/) was used to determine the association of HGF, FGFR1, EGF, and EGFR expression with relapse-free survival across a large set of breast cancers analyzed by Affymetrix U133A arrays (Ringnér et al. 2011).

4.18 Statistical analysis

Prism 7 (GraphPad) was used to perform unpaired two-tailed Student's t-test, and ordinary one-way ANOVA with Tukey's correction for multiple comparisons, unless otherwise indicated. Data represent mean +/- standard error of the mean. Values were considered to be significantly different when p values < 0.05.

Antibody (clone)	Company	Dilution
pMET Y1234/5 (D26) XP	Cell Signaling (3077)	1:1000 (IB)
Met	R&D System (AF527)	1:500 (IB)
MET	In-house (148)	1:1000 (IB)
MET (SP44)	Spring Bioscience (M3442)	1:50 (IF)
pAKT S473 (D7F10) XP	Cell Signaling (9018)	1:500 (IB)
AKT (40D4)	Cell Signaling (2920)	1:1000 (IB)
pERK1/2 T202/Y204	Cell Signaling (9101)	1:1000 (IB)
ERK1/2 (3A7)	Cell Signaling (9107)	1:1000 (IB)
pFRS2 Y196	Cell Signaling (3864)	1:500 (IB)
pFRS2 Y436	Cell Signaling (3861)	1:500 (IB)
FRS2 (H-91)	Santa Cruz (sc-8318)	1:500 (IB)
FGFR1 (D8E4) XP	Cell Signaling (9740)	1:1000 (IB) 1:200 (IHC) 1:200 (IF)
CLAUDIN1 (2H10D10)	Thermo Fisher (37-4900)	1:1000 (IB)
Cl. CASPASE-3 (Asp175)	Cell Signaling (9661)	1:1000 (IB)
Cl. PARP (Asp214) XP	Cell Signaling (5625)	1:1000 (IB)
β-ACTIN (AC-15)	Sigma (A5441)	1:10000 (IB)
α/β-TUBULIN	Cell Signaling (2148)	1:1000 (IB)
7-AAD Viability Stain	eBioscience (00-6993)	1:20 (FC)
CD24-PE	BD Biosciences (555428)	1:10 (FC)
CD44-FITC	BD Biosciences (555478)	1:10 (FC)
IRDye 800CW Goat Anti-Rabbit IgG	Li-COR (926-32211)	1:10000 (IB)
IRDye 680CW Goat Anti-Mouse IgG	Li-COR (926-68070)	1:10000 (IB)
Rabbit IgG, HRP-linked	Cell Signaling (7074)	1:10000 (IB)
Mouse IgG, HRP-linked	GE Healthcare (NA931)	1:10000 (IB)
SignalStain Boost HRP (Rabbit)	Cell Signaling (8114)	Pre-diluted (IHC)
Alexa Fluor 555 Tyramide	Thermo Fisher (B40923)	1:100 (IF)
Alexa Fluor 488 Tyramide	Thermo Fisher (B40922)	1:100 (IF)

Table 4.1 Antibodies and detection reagents.

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