Regulation of Met receptor tyrosine kinase signalling and biology

by

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ABSTRACT

Growth factor receptor tyrosine kinases (RTKs) are critical initiators of signal transduction pathways necessary for cell growth, differentiation, migration and survival. Many of these signals are coordinated through scaffold proteins that are phosphorylated upon their recruitment to the activated receptor complex. This provides binding sites for multiple proteins to activate and generate distinct biological responses. The amplitude and duration of a signal is regulated via dephosphorylation and degradation of target proteins. Signal regulation in this manner acts to promote the formation and disassembly of multi-protein complexes and diversify and localize signals downstream from RTKs.

The Met RTK and its ligand, hepatocyte growth factor (HGF), are positive regulators of epithelial morphogenesis, scatter, and survival. However little was known regarding the proteins responsible for attenuating Met receptor activation. In Chapter II, I demonstrated that the Met receptor was hyperphopshorylated in PTP1B-null mice in response to Fas-induced liver damage. Inhibition of Met signaling with PHA665752, removed protection from liver failure in PTP1B-null hepatocytes, demonstrating that PTP1B was a negative regulator of the Met RTK and its removal promoted cell survival against Fas-induced hepatic failure.

In response to Met receptor stimulation, the Gab1 scaffold protein is the prominent protein recruited and phosphorylated downstream from Met and is critical in mediating Met-dependent biological responses. In chapters III and IV, I identified the serine/threonine kinase Pak4 and the microtubule-bound guanine nucleotide exchange factor GEF-H1 as novel proteins recruited to Gab1 following Met receptor activation. I demonstrate that Gab1 and Pak4 synergize to enhance migration and invasion following

HGF stimulation. Furthermore, the recruitment of Pak4 to Gab1 is important for its subcellular localization to lamellipodia and critical for epithelial cell dispersal and morphogenesis downstream from Met. In addition, GEF-H1 is important in focal adhesion formation and turnover and this correlates with the ability of GEF-H1 to promote epithelial migration and invasion downstream from Met.

Overall, these studies investigate molecular mechanisms regulating Met-dependent signals and demonstrate for the first time that the Met receptor is a substrate for PTP1B and identify Pak4 and GEF-H1 as key integrators of Met dependent cellular migration and invasion.

ABRÉGÉ

Les récepteurs tyrosine kinase aux facteurs de croissance sont des initiateurs critiques des voies de signalisation nécessaires à la croissance, la différentiation, la migration et la survie cellulaire. Beaucoup de ces signaux sont coordonnés par des protéines d'échafaudage qui sont phosphorylées au cours de leur recrutement au complexe de récepteurs activés. Ceci fournit des sites de liaison à de multiples protéines permettant l'activation et la génération de différentes réponses biologiques. L'amplitude et la durée d'un signal est régulée via la déphosphorylation et la dégradation des protéines cibles. De cette façon, la régulation du signal agit pour promouvoir la formation et le désassemblage de complexes protéiques et pour diversifier et localiser les signaux en aval des récepteurs tyrosine kinase.

Le récepteur Met et son ligand HGF (Hepatocyte Growth Factor) sont des régulateurs de la morphogenèse, la dispersion et la survie des cellules épithéliales. Toutefois, peu d'informations sont disponibles sur les protéines responsables de l'extinction des signaux issus du récepteur Met. Dans le chapitre II, je démontre que le récepteur Met est hyperphosphorylé dans les souris knock-out pour PTP1B en réponse aux dommages induits par Fas. L'inhibition par le composé PHA665752 de la signalisation par Met, relève la protection contre les crises hépatiques dans les souris KO pour PTP1B. Ceci démontre que PTP1B est un régulateur négatif de Met et son retrait permet la survie cellulaire contre les crises hépatiques induites par Fas.

En réponse à la stimulation du récepteur Met, la protéine d'échafaudage Gab1 est la plus importante des protéines recrutées et phosphorylées en aval de Met et cette protéine est critique dans la médiation des réponses biologiques dépendantes de Met.

Dans les chapitres III et IV, j'ai identifié la kinase Ser/Thr Pak4 et le facteur d'échange de guanine lié aux microtubules (GEF-H1) en tant que nouvelles protéines recrutées à Gab1 suite à l'activation de Met. Je démontre que Gab1 et Pak4 agissent de façon synergique pour promouvoir la migration et l'invasion suite à la stimulation par HGF. De plus, le recrutement de Pak4 à Gab1 est important pour sa localisation cellulaire dans les lamellipodes et est critique pour la dispersion et la morphogenèse des cellules épithéliales en aval de Met. En outre, GEF-H1 est important pour la formation et le roulement des points d'adhésion focaux ce qui est en corrélation avec la capacité de GEF-H1 de promouvoir la migration et l'invasion épithéliale en aval de Met.

Ces études ont pour but d'investiguer les mécanismes moléculaires régulant les signaux dépendants de Met et démontrent pour la première fois que le récepteur Met est un substrat pour PTP1B. Finalement, Pak4 et GEF-H1 sont identifiés comme des intégrateurs clés de la migration et l'invasion cellulaire dépendante de Met.

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PREFACE

This is a manuscript-based thesis. It contains two published manuscripts and one manuscript in preparation. The thesis is divided into five chapters:

1) a general introduction and literature review

2 to 4) manuscripts, each with their own preface, abstract, introduction, materials and methods, results and discussion

5) a general discussion of all the results

CONTRIBUTIONS OF AUTHORS

CHAPTER 2 - Protein Tyrosine Phosphatase 1B Deficiency Protects Against Fas-Induced Hepatic Failure

This manuscript was a collaborative effort between myself and Dr. Veena Sangwan (former post-doctoral student, Dr. Morag Park's lab). Dr. Alan Chang (former Ph.D. student, Dr. Michel Tremblay's lab) provided helpful comments/suggestions in the initial planning of this project to Dr. Sangwan.

All figures presented in this manuscript were assembled by me.

Injections of the agonistic Fas antibody, Jo-2, into the hepatic portal vein, cardiac punctures, and liver perfusions were performed by Anie Monast (animal technician), myself and Dr. Sangwan.

Tissue samples were collected and protein lystates were prepared equally by myself and Dr. Sangwan.

Hepatocytes were prepared equally by myself and Dr. Sangwan.

Immunoprecipitations and western analysis were performed equally by myself and Dr. Sangwan.

Dr. Nadia Dube (Ph.D. student, Dr. Michel Tremblay's lab) maintained PTP1B-null and WT lines used in all experiments.

Dr. Julie Desbarats (Professor, Dept. of Physiology) performed FACs analysis seen in Figure 2.2B.

I contributed the following figures to this manuscript.

I. I performed all TUNEL positive staining (Figure 2.1C and 2.1D).
II. I performed all *in vitro* assays to quantitate the percentage of apoptosis, as measured by Annexin V positive staining, seen in WT and PTP1B-null hepatocytes following:
    a. Jo-2, FasL and TNFa treatment(Figure 2.4A and 2.4B)
    b. Pretreatment with the inhibitors UO126 (MEK1/2), SN50 (NFkB), or HGF prior to Jo-2 treatment (Figure 2.5)
    c. Pretreatment with inhibitor Genistein prior to Jo-2 treatment (Figure 2.6A)
    d. Pretreatment with Met receptor inhibitor, PHA665752 prior to Jo-2 treatment (Figure 2.7C)

III. I isolated and performed in vitro assay on thymocytes of WT and PTP1B-null mice (Figure 2.4C)

CHAPTER 3 - Pak4, a novel Gab1 binding partner, modulates cell migration and invasion by the Met receptor.

Pak4 was identified as a Gab1-associated protein following a proteomic analysis I performed to identify novel Gab1-binding proteins following activation of the Met receptor tyrosine kinase. Although this data was not presented in this manuscript, I generated the Gab1-TAP tag fusion construct and stable cell lines that were used to obtain this data. Stimulations, pulldown assays and SDS-PAGE experiments were performed by myself. Dr. Kurt Dejgaard (McGill Life Science Complex) performed the mass spectrometry analysis.

All constructs used and experiments performed in this manuscript, unless otherwise stated, were generated/performed by me.

Monica Naujokas (senior technician, Dr. Morag Park's lab) performed collagen assays (Figure 3.9F, Supplemental Figure 3.6) and helped with the establishing the MDCK cell lines expressing GFP-Pak4 and mCherry-Pak4.

All figures presented in this manuscript were compiled and assembled by me.

CHAPTER 4 - GEF-H1, a Gab1 binding protein and a mediator of cellular migration, downstream of the Met receptor

All constructs used and experiments performed in this manuscript, unless otherwise stated, were generated/performed by me (Figure 4.2, 4.3, 4.4, 4.5 and 4.6).

Patrick De Koninck (Ph.D. student, Dr. Morag Park's lab) performed transfections, immunoprecipitations and western analysis (Figure 4.1).

Monica Naujokas helped with establishing the MDCK cells lines expressing GFP-GEFH1.

Dongmei Zuo (research assistant, Dr. Morag Park's lab) performed immunofluorescence staining and captured confocal images (Figures 4.6). I plated the cells and performed the HGF stimulation.

All figures presented in this manuscript were compiled and assembled by me.

PUBLICATIONS ARISING FROM THIS WORK

First-Author Publications

1) Paliouras GN*, Sangwan V*, Cheng A*, Dube N, Tremblay ML and Park M. Protein tyrosine phosphatase 1B deficiency protects against Fas induced hepatic failure. *Journal of Biological Chemistry*. 2006. 281(1):221-8. Accepted and published online Oct 17, 2005

2) Paliouras GN, Naujokas M and Park M. Pak4, a novel Gab1 binding partner, modulates cell migration and invasion by the Met receptor. *Moleclular and Cellular Biology.* 2009. 29(11):3018-32. Accepted and published online Mar 16, 2009

3) Paliouras GN, DeKoninck P, Zuo D, Naujokas M and Park M. GEF-H1, a Gab1-binding protein and mediator of cellular migration, downstream of the Met receptor. *Manuscript in preparation.*

Other Publication

Sangwan V, **Paliouras GN***, Abella JV*, Dubé N, Monast A, Tremblay ML, Park M. Regulation of the Met receptor tyrosine kinase by the protein tyrosine phosphatases PTP1B and TCPTP. *Journal of Biological Chemistry.* 2008. 283(49):34374-83. Accepted and published online Sep 26, 2008

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Chapter I

1 Literature Review

The ability of cells to regulate cell growth, differentiation, survival and migration are necessary for their normal functioning and tissue homeostasis. Deregulation of these processes can lead to the development of human disease, such as cancer. Transmembrane receptor tyrosine kinases (RTKs) play a critical role in recognizing changes in the extracellular environment and transducing this information via signaling pathways involved in tissue homeostasis. Their activity is tightly controlled and the intracellular signals generated by these receptors help decide cell fate. One means of regulating these signals involves the reversible phosphorylation of proteins. Phosphorylation and dephosphorylation, catalyzed by kinases and phosphatases, play a role in regulating the delicate balance between the positive and negative signals needed to maintain a homeostatic environment. Many enzymes and receptors are switched "on" or "off" by phosphorylation and dephosphorylation, therefore examining the consequence of this action on RTKs will provide further knowledge in understanding the nature of human disease.

1.1 Met receptor

1.1.1 Discovery of the Met receptor

The Met receptor tyrosine kinase was initially discovered as an oncogenic variant, generated following chromosomal rearrangement. Treatment of human osteosarcoma (HOS) cell line with the chemical carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

(MNNG) lead to genomic rearrangement and a dominant transforming gene was identified using DNA transfection assays (Cooper, Park et al. 1984). Southern blot and karyotype analysis of DNA was used to determine the chromosomal location of the transforming gene, which was mapped to chromosome 7 (7q11.4-7qter) (Cooper, Park et al. 1984). The human homologue of the avian erythroblastosis virus (AEV) transforming gene, *erbB*, is localized to region 7pter-7q22 of chromosome 7, suggesting *erbB* could be the transforming gene in MNNG-treated HOS cells. However, the MNNG-HOS transforming gene failed to hybridize to probes prepared from *erbB* (Cooper, Park et al. 1984), suggesting that another gene may be responsible for the transforming activity. Sequencing of a cDNA fragment derived from the MNNG-HOS transforming gene was found to be highly homologous to tyrosine kinase oncogenes and growth factor receptors (Dean, Park et al. 1985). By *in situ* chromosomal hybridization, the localization of the transforming gene was further refined to 7q21-q31, a region that was distinct from other kinases (Dean, Park et al. 1985). The MNNG-HOS transforming gene was found to be a chromosomal rearrangement between 2 loci, the translocated promoter region (TPR), from chromosome 1q25, which encodes the leucine zipper dimerization motif from the Tpr protein, and a second loci from chromosome 7q31, which contributes the intracellular kinase domain (Park, Dean et al. 1986). The second loci of the MNNG-HOS transforming gene was given the three-letter abbreviation 'MET', named after the "methyl" group from the name of the chemical carcinogen, MNNG. The discovery of Tpr-Met was the first description of an activated gene fusion product from cells treated with a chemical carcinogen (Park, Dean et al. 1986) and would be the prototype of receptor tyrosine kinase-derived oncogenes generated from chromosomal translocation. Tpr-Met is constitutively activated in the absence of ligand as a result of the dimerization formed

between the Tpr leucine zipper motifs (Rodrigues and Park 1993) and is a paradigm for RTK-derived oncogenes generated following chromosomal translocation. To date, over 25 RTK-derived oncoproteins have been identified in human tumors that possess a protein dimerization domain fused to a cytoplasmic kinase domain derived from an RTK (Rodrigues and Park 1994; Lamorte and Park 2001).

1.1.2 Met receptor Structure

The Met receptor is the prototype receptor for a subfamily of receptor tyrosine kinases (RTKs), which include the mammalian Ron and the avian Sea receptors (Gaudino, Follenzi et al. 1994; Wahl, Hsu et al. 1999). The Met receptor is synthesized as a 170 kDa single-chain precursor that is glycosylated to a 190 kDa protein (Giordano, Di Renzo et al. 1989; Giordano, Ponzetto et al. 1989). At the plasma membrane, the 190 kDa Met precursor is cleaved by a furin protease to yield a mature disulfide-linked heterodimer, comprised of an extracellular 45 kDa α-subunit and a 145 kDa β-subunit (Figure 1.1). The latter spans the plasma membrane and contains a cytoplasmic region with a tyrosine kinase domain (Gonzatti-Haces, Seth et al. 1988; Giordano, Di Renzo et al. 1989; Komada, Hatsuzawa et al. 1993). The extracellular portion of Met, specifically the α-subunit and the amino-terminal region of the β-subunit, is responsible for binding of its ligand, HGF/SF (hepatocyte growth factor/scatter factor). This β-subunit is composed of a Sema domain, a PSI domain and four immunoglobulin-like domains (Gherardi, Youles et al. 2003). The β-subunit spans the plasma membrane and contains a cytoplasmic region with a juxtamembrane domain, a kinase domain and a C-terminal multifunctional phosphotyrosine docking site required for biological activity mediated downstream from Met. Following the binding of HGF, the Met receptor is activated via

transphosphorylation of tyrosine 1234 and 1235 within the kinase domain of the receptor, which are critical for the activation of the Met receptor (Longati, Bardelli et al. 1994; Rodrigues and Park 1994). This results in the phosphorylation of tyrosine residues 1349, 1356 and 1365 within the carboxy-terminus of the Met receptor (Ponzetto, Bardelli et al. 1994) and tyrosine 1003 within the juxtamembrane region (Peschard, Fournier et al. 2001). Tyrosines 1003, 1349, 1356 and 1365 serve as specific docking sites for the recruitment of additional signalling proteins to the receptor and are responsible for the activation of a diverse set of cellular processes including cellular proliferation, scatter, migration, invasion and survival.

1.1.3 Hepatocyte growth factor

HGF was originally identified in serum as a potent mitogen for cultured hepatocytes and induced liver regeneration after partial hepatectomy or hepatic injury (Nakamura, Nawa et al. 1984; Nakamura, Teramoto et al. 1986; Zarnegar and Michalopoulos 1989). HGF was also independently identified as scatter factor (SF), which was produced by fibroblasts and induced the dissociation and scattering of tight epithelial colonies (Stoker, Gherardi et al. 1987). HGF and SF were subsequently found to encode the same gene, thus representing the same molecule (Gherardi and Stoker 1990; Weidner, Arakaki et al. 1991) and were identified to be the ligand for the Met receptor (Bottaro, Rubin et al. 1991; Naldini, Weidner et al. 1991).

HGF is derived from a single-chain, inert glycoprotein precursor, which is secreted from mesenchymal cells and then cleaved to produce a biologically active form by extracellular proteases. Mature HGF has 38% overall similarity with plasminogen and is a heterodimer consisting of a 62-kDa α–chain and a 32-kDa β-chain connected through

a disulphide bond. The α-chain contains a N-terminal hairpin loop followed by four kringle domains (K1-K4) (Lokker, Mark et al. 1992; Okigaki, Komada et al. 1992), whereas the β-chain contains an enzymatically inactive serine protease domain (Lokker, Mark et al. 1992; Donate, Gherardi et al. 1994). The K1 portion mediates receptor binding, where K1 is thought to engage two MET molecules leading to receptor clustering and activation (Lokker, Presta et al. 1994; Gherardi, Sandin et al. 2006). The β-chain, although not required for receptor binding, contributes to receptor activation (Lokker, Mark et al. 1992).

1.1.4 Regulation of Met receptor tyrosine kinase signalling

1.1.4.1 Met receptor endocytosis/ubiquitination

A pathway that acts to terminate Met signalling involves receptor internalization and subsequent degradation in the lysosome, although a role for the proteasome has also been proposed in Met degradation (Hammond, Urbe et al. 2001). Stimulation of the Met receptor with HGF/SF induces tyrosine phosphorylation and leads to Met receptor ubiquitination and enhanced degradation through the endocytic pathway (Jeffers, Taylor et al. 1997; Hammond, Urbe et al. 2001; Abella, Peschard et al. 2005). Furthermore, Met phosphorylates regulators of the endocytic pathway and the ubiquitination machinery, such as Cbl (Cas-Br-M (murine) ecotropic retroviral transforming sequence), Eps15 (epidermal growth factor receptor pathway substrate 15) and HRS (hepatocyte growth factor-regulated tyrosine kinase substrate), enhancing ubiquitination and degradation of the Met receptor (Peschard, Fournier et al. 2001; Hammond, Carter et al. 2003; Abella, Peschard et al. 2005; Parachoniak and Park 2009). In addition, activation of protein kinase C (PKC) has also been shown to regulate Met trafficking and down-regulation

(Kermorgant, Zicha et al. 2003; Kermorgant, Zicha et al. 2004). There are likely multiple mechanisms involved in Met receptor degradation, and multiple studies are underway to elucidate these.

One of the well-described mechanisms of Met receptor degradation involves ubiquitination and degradation of the Met receptor as mediated by the c-Cbl ubiquitin ligase (Peschard, Fournier et al. 2001). Upon Met receptor activation and phosphorylation, c-Cbl is recruited indirectly through Grb2 to tyrosine 1356. In addition, c-Cbl engages Met directly via the c-Cbl phosphotyrosine (TKB) domain and phosphorylated tyrosine 1003 within the juxtamembrane domain of Met, ubiquitinating the receptor and targeting it for degradation (Peschard, Fournier et al. 2001). Substitution of Y1003 with phenylalanine (Y1003F) impaired Cbl-recruitment and ubiquitination of Met and results in the oncogenic activation of the Met receptor through enhanced stability and sustained receptor signalling (Abella, Peschard et al. 2005). Naturally occurring somatic mutations resulting in the deletion of exon 14, which contains the c-Cbl TKB binding site, result in the loss of negative regulation of the Met receptor and oncogenic activation in lung cancer (Ma, Kijima et al. 2003; Kong-Beltran, Seshagiri et al. 2006). The recruitment of the Cbl family of ubiquitin-protein ligases is required for ligand-induced degradation of many RTKs, such as the epidermal growth factor receptor (EGFR), the platelet-derived growth factor receptor (PDGFR), and the colony-stimulating factor-1 receptor (CSF-1R) (Peschard and Park 2007), supporting a role for Cbl as a general mechanism for RTK.

1.1.4.2 Phosphorylation

Activation of the Met receptor results in the phosphorylation of multiple tyrosine residues within the receptor responsible for mediating the recruitment of downstream signalling proteins. The phosphorylation of tyrosine residues 1003, 1349, and 1356 (Ponzetto, Bardelli et al. 1994; Zhu, Naujokas et al. 1994; Fixman, Naujokas et al. 1995; Peschard, Fournier et al. 2001) serve as specific docking sites which are necessary in mediating the biological consequences of the Met receptor (Figure 1.2). Signalling proteins include the adaptor proteins Grb2 (growth factor receptor-bound protein 2) and Shc (Src homology 2 domain containing transforming protein) (Fixman, Fournier et al. 1996), and the scaffold protein Gab1 (growth factor receptor bound protein 2-associated protein 1) (Weidner, Di Cesare et al. 1996; Nguyen, Holgado-Madruga et al. 1997). Once phosphorylated by Met, Gab1 recruits the p85 subunit of PI3K (phosphatidylinositol 3-kinase) (Maroun, Holgado-Madruga et al. 1999; Schaeper, Gehring et al. 2000), PLCγ (phospholipase C gamma) (Gual, Giordano et al. 2000), the tyrosine phosphatases Shp2 (protein tyrosine phosphatase, non-receptor type 11) (Maroun, Naujokas et al. 2000; Schaeper, Gehring et al. 2000), and the Crk (v-crk sarcoma virus CT10 oncogene homolog) adaptor protein (Garcia-Guzman, Dolfi et al. 1999; Lamorte, Kamikura et al. 2000; Schaeper, Gehring et al. 2000) to the Met complex. In addition, activation of Met recruits STAT3 (signal transducer and activator of transcription 3) (Boccaccio, Ando et al. 1998), Src (v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog) (Ponzetto, Bardelli et al. 1994; Rahimi, Hung et al. 1998), FAK (focal adhesion kinase) (Chen and Chen 2006), the p85 subunit of PI3K (Ponzetto, Bardelli et al. 1994), the E3-ligase c-Cbl (Peschard, Fournier et al. 2001), and the lipid kinase SHIP (inositol polyphosphate

phosphatase-like 1) (Stefan, Koch et al. 2001). Met can also form complexes with α3β3 integrin (Liu, Chattopadhyay et al. 2009), Plexin B1 (Giordano, Corso et al. 2002), CD44 (Orian-Rousseau, Chen et al. 2002), as well as the Fas receptor (Wang, DeFrances et al. 2002).

While phosphorylation of tyrosine residues has been shown to be involved in the activation of Met receptor catalytic activity, serine phosphorylation of Met via PKC or other Ca^{2+}-dependent kinase(s) is inhibitory (Gandino, Di Renzo et al. 1990; Gandino, Munaron et al. 1991; Gandino, Longati et al. 1994). Phosphorylation of serine 985 acts as a negative modulator of the tyrosine kinase activity of Met (Gandino, Di Renzo et al. 1990). This was demonstrated when substitution of Ser985 by site-directed mutagenesis resulted in increased tyrosine phosphorylation of the receptor (Gandino, Longati et al. 1994). These data suggest that phosphorylation of Ser985 is an alternative mechanism of Met receptor downregulation (Gandino, Longati et al. 1994), independent of ubiquitination.

1.1.4.3 Met receptor dephosphorylation

1.1.4.3.1 Protein tyrosine Phosphatases (PTPs)

One aspect of cellular signalling rests on the ability of proteins to be reversibly phosphorylated by protein kinases and phosphatases. While kinases function to regulate the amplitude of the signalling response, phosphatases are critical in determining both the rate and duration of the response. Regulation via phosphorylation/dephosphorylation can have multiple consequences on a target protein, such as a conformational change in protein structure, alteration in the intracellular localization of a protein and an increase in the availability of protein docking sites (Hunter 1995).

Overall, there are approximately 100 human PTP-superfamily genes that can be grouped into four classes: the classical receptor PTPs (RPTPs), the classical non-receptor PTP (nrPTPs), the dual specificity PTP (dsPTPs) and the low M_r PTPs (Alonso, Sasin et al. 2004). Two of the families that are most studied with respect to receptor tyrosine kinases are the RPTPs and the nrPTPs (Figure 1.3). RPTPs have been predominantly found to be localized in the plasma membrane and have been implicated in processes that involve cell-cell and cell-matrix contact. Three RPTPs have identified Met as a substrate, DEP-1 (CD148/PTP-eta)(Palka, Park et al. 2003), PTP-S (Villa-Moruzzi, Puntoni et al. 1998) and LAR (leukocyte antigen-related) tyrosine phosphatase (Machide, Hashigasako et al. 2006). nrPTPs are localized to a variety of intracellular compartments including the cytosol, plasma membrane and endoplasmic reticulum (Stoker 2005). nrPTPs that have been shown to associate with Met to date are protein tyrosine phosphatase 1B (PTP1B) (Sangwan, Paliouras et al. 2008), and T cell protein tyrosine phosphatase (TCPTP) (Sangwan, Paliouras et al. 2008).

Receptor tyrosine kinases and downstream signalling can be regulated by PTPs in both a positive and negative manner, altering the perception that PTPs are only negative regulators of signal transduction. For instance, the PTP SHP-2 is essential for growth factor induced activation of the mitogen activated protein kinase (MAPK) pathway via an undetermined mechanism (i.e.: “positive” signalling), whereas dephosphorylation of the insulin receptor or Met RTK by PTP-1B negatively regulates receptor signalling (i.e.: “negative” signalling) (Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000; Maroun, Naujokas et al. 2000; Neel, Gu et al. 2003; Sangwan, Paliouras et al. 2008).

1.1.4.3.2 PTP1B

Protein tyrosine phosphatase (PTP) 1B was the first PTP to be purified (Tonks, Diltz et al. 1988). PTP1B is a 435 amino acid, 50 kDa protein that consists of an N-terminal catalytic domain, a C-terminal hydrophobic stretch, and an intervening region containing two proline-rich motifs (Chernoff, Schievella et al. 1990). PTP1B is widely expressed and its C-terminus contains a 35 amino acid stretch found to be sufficient and necessary to target the protein to the cytoplasmic face of the ER (Frangioni, Beahm et al. 1992; Anderie, Schulz et al. 2007). PTP1B localization and activity can also be regulated by calpain cleavage at the C-terminus, redistributing the protein to the cytoplasm (Frangioni, Oda et al. 1993). The anchoring and cleavage of PTP1B regulates its subcellular localization in a spatial and temporal manner, thus regulating its activity towards substrates (Frangioni, Oda et al. 1993).

PTP1B interacts and dephosphorylates multiple proteins. These include p130Cas (Liu, Hill et al. 1996), p62Dok (Dube, Cheng et al. 2004), Jak2 (Janus activated kinase 2) (Cheng, Uetani et al. 2002; Zabolotny, Bence-Hanulec et al. 2002), Tyk2 (tyrosine kinase 2) (Myers, Andersen et al. 2001), Crk (Takino, Tamura et al. 2003), FAK (Zhang, Lin et al. 2006), STAT5 (signal transducer and activator of transcription 5) (Aoki and Matsuda 2000), β-catenin (Xu, Arregui et al. 2002), and cortactin (Stuible, Dube et al. 2008). PTP1B genetically or biochemically interacts with multiple RTKs, including the insulin, insulin-like growth factor 1 (IGF1), Met, EGF, and PDGFβ receptors (Bourdeau, Dube et al. 2005; Sangwan, Paliouras et al. 2008; Stuible, Doody et al. 2008). The mechanism by which PTP1B interacts with its substrates has been well studied. The phosphotyrosine residue of the substrate interacts with PTP1B recognition motif, thus positioning itself for

dephosphorylation by the catalytic cleft of the phosphatase (Jia, Barford et al. 1995). However, PTP1B has a higher affinity for substrates that possess two adjacent phosphorylated tyrosine residues (Salmeen, Andersen et al. 2000). Biochemical studies have demonstrated that the affinity of PTP1B for a substrate containing adjacent twin tyrosines is 70-fold higher than for a substrate in which one tyrosine is substituted with phenylalanine (Salmeen, Andersen et al. 2000). For example, the Insulin receptor contains twin tyrosine residues (1162 and 1163) within its catalytic kinase domain. The phosphotyrosine at position 1163 of the Insulin receptor increased the peptide affinity for PTP1B by 70-fold relative to the same peptide with an unphosphorylated tyrosine residue at 1163 (Salmeen, Andersen et al. 2000). Therefore, the specificity of PTP1B towards its substrates is affected by the presence of a mono- or bi-phosphotyrosine residue, predicting a hierarchical dephosphorylation cycle. This remains to be determined for the Met receptor, which also possesses twin tyrosine residues within the catalytic domain.

1.1.4.3.3 Met as a substrate of PTPs

The Met receptor is dephosphorylated by several tyrosine phosphatases, PTP-S (Villa-Moruzzi, Puntoni et al. 1998), DEP1 (Palka, Park et al. 2003), LAR (Machide, Hashigasako et al. 2006), PTP1B (Sangwan, Paliouras et al. 2008) and TCPTP (Sangwan, Paliouras et al. 2008). One of the first phosphatases found to associate with the Met receptor was PTP-S (Villa-Moruzzi, Puntoni et al. 1998). PTP-S associates with the Met receptor within the juxtamembrane region and is proposed to dephosphorylate tyrosine residue 1003 (Villa-Moruzzi, Puntoni et al. 1998). However no additional work has since been published on the consequence of Met receptor dephosphorylation via PTP-S, therefore the consequence on Met-dependent biology and signalling is not known.

The conserved protein tyrosine phosphatase DEP-1 has been proposed to negatively regulate Met receptor signalling. DEP-1 is a receptor PTP whose expression is enhanced as cells approach confluence (Ostman, Yang et al. 1994). However, DEP-1 does not target the twin tyrosine residues (1234/1235), but interacts with tyrosine residues 1349 and 1356 within the carboxy-terminal tail of the Met receptor, thus attenuating the association and activation of downstream signalling pathways (Palka, Park et al. 2003). In addition to Met, the adaptor protein Gab1 was also identified as a potential substrate for DEP-1, suggesting that DEP-1 activity can terminate both the recruitment of Gab1 to Met as well as Gab1-dependent signals (Palka, Park et al. 2003).

The receptor PTP LAR (leukocyte antigen-related) targets the Met receptor in confluent cells (Machide, Hashigasako et al. 2006). LAR consists of an extracellular region with three immunoglobulin-like and eight-fibronectin type III structures, as well as a cytoplasmic region containing two tyrosine phosphatase domains (Streuli, Krueger et al. 1988). The interaction between Met and LAR is initiated after cell-cell contact in confluent cells and suppresses the mitogenic response of primary rat hepatocytes, resulting in contact inhibition (Machide, Hashigasako et al. 2006). However, the tyrosine residues that interact with LAR have not been identified.

The Met receptor is also a substrate for PTP1B, which will be described and discussed in Chapter II of this thesis and in the final general discussion.

1.1.5 HGF-Met biology

1.1.5.1 Embryogenesis

HGF and Met are essential for mouse embryogenesis. The knockout models phenocopy each other demonstrating that HGF/SF is the only ligand for Met during

embryonic development (Bladt, Riethmacher et al. 1995; Schmidt, Bladt et al. 1995; Uehara, Minowa et al. 1995). Knockouts of Met or HGF/SF are embryonic lethal at approximately day E14.5 due to defects in placenta and liver development, where a reduction in trophoblast and parenchymal cells were observed, respectively (Bladt, Riethmacher et al. 1995; Schmidt, Bladt et al. 1995; Uehara, Minowa et al. 1995). An additional phenotype that was observed in both the HGF- and Met-knockout mice was a failure of myogenic cell precursors to migrate from the dermomyotome to the tongue, diaphragm, and limb buds, where they differentiate to form skeletal muscles. As a consequence, limb and diaphragm muscles did not form in HGF- and Met-knockout animals (Zhu, Naujokas et al. 1994; Bladt, Riethmacher et al. 1995; Schmidt, Bladt et al. 1995; Uehara, Minowa et al. 1995).

In the adult, Met is expressed by epithelial and endothelial cells in a number of tissues including the liver (Di Renzo, Narsimhan et al. 1991; Tajima, Higuchi et al. 1992), kidney (Di Renzo, Narsimhan et al. 1991; Tajima, Higuchi et al. 1992; Ishibe, Karihaloo et al. 2009), pancreas (Ebert, Yokoyama et al. 1994; Jeffers, Rao et al. 1996), intestine (Di Renzo, Narsimhan et al. 1991), esophagus (Takahashi, Ota et al. 1995), stomach (Takahashi, Ota et al. 1995), lung (Tajima, Higuchi et al. 1992), muscle (Yang, Vogan et al. 1996) and breast (Niranjan, Buluwela et al. 1995). It is also expressed in endothelial cells of the hematopoietic lineage (Bussolino, Di Renzo et al. 1992), as well as in melanocytes (Halaban, Rubin et al. 1992), monocytes (Galimi, Cottone et al. 2001) and neuronal cells (Di Renzo, Bertolotto et al. 1993). Within all of these cell types, Met regulates a diverse set of cellular processes including cell proliferation, scattering, migration, invasion and survival (Birchmeier, Birchmeier et al. 2003).

1.1.5.2 Tissue Repair/Organ regeneration

HGF is a pleiotropic factor that plays an important role in organ regeneration in response to tissue injury. In the adult, expression of MET and HGF is upregulated in response to tissue injury, as observed in the liver, kidney, pancreas, stomach and intestine (Ueda, Takeyama et al. 1996; Michalopoulos and DeFrances 1997; Nakamura, Mizuno et al. 2000; Matsumoto and Nakamura 2001; Rabkin, Fervenza et al. 2001; Jin, Yang et al. 2003). HGF promotes kidney regeneration through its ability to suppress apoptosis and stimulate proliferation and tubulogenesis of kidney epithelial cells (Igawa, Kanda et al. 1991; Yo, Morishita et al. 1998; Matsumoto and Nakamura 2001). The importance of HGF is further highlighted in its ability to reverse the advance of renal failure in laboratory animals (Igawa, Matsumoto et al. 1993; Kawaida, Matsumoto et al. 1994; Miller, Martin et al. 1994). The HGF-Met signalling axis also functions in wound healing of the skin (Chmielowiec, Borowiak et al. 2007). This highlights the importance of the HGF-Met signalling cascade in tissue repair and remodeling.

HGF is a powerful mitogen for epithelial cells. It was originally identified as a potent mitogenic factor for hepatocytes and serum levels of HGF were increased following partial hepatectomy of rats (Nakamura, Nawa et al. 1984; Nakamura, Nishizawa et al. 1989). HGF treatment prevents the onset or progress of cirrhosis and hepatic failure (Michalopoulos and DeFrances 1997; Ueki, Kaneda et al. 1999). The importance of Met in liver regeneration is seen when examining mice that are deficient in Met. Met-deficient mice displayed a defect in liver repair following treatment with carbon tetrachloride (CCl_4) (Huh, Factor et al. 2004) or by partial hepatectomy

(Borowiak, Garratt et al. 2004). Collectively, HGF and Met are important following tissue injury and to limit the extent of damage and promote liver regeneration.

1.1.5.3 MET/HGF in cancer

HGF and Met are deregulated in many human cancers (http://www.vai.org/met/). Deregulation of the HGF-Met signalling axis can occur through multiple mechanisms. Met activation in cancer often has been found to occur through the formation of autocrine and paracrine loops. The Met receptor is expressed in tissues of epithelial and endothelial origin during development and at maturity, while HGF is found in mesenchymal cells (Rosario and Birchmeier 2003). HGF is produced by fibroblasts within the stroma and activates the Met receptor in epithelial cells in a paracrine manner. The co-expression of Met and HGF in an autocrine manner drives sarcomagenesis as seen in human sarcomas, osteosarcoma, and rhabdomyosarcoma (Cortner, Vande Woude et al. 1995). A paracrine loop is also evident in tumors of epithelial origin such as breast, pancreatic, and colorectal cancers (Ghoussoub, Dillon et al. 1998; La Rosa, Uccella et al. 2000; Baldus, Kort et al. 2007). Amplification of the Met receptor has been identified in gastric and colorectal cancers (Di Renzo, Olivero et al. 1995; Nakajima, Sawada et al. 1999). Besides the formation of paracrine and autocrine loops, mutations in the Met receptor have also been linked to disease. In hereditary and sporadic papillary renal cell carcinoma, missense mutations in the kinase domain of Met have been identified (Schmidt, Duh et al. 1997). Somatic mutations have been described in the Met kinase domain in hepatocellular and gastric carcinomas, as well as in gliomas (Park, Dong et al. 1999; Moon, Weil et al. 2000; Park, Oh et al. 2000). Mutations in the juxtamembrane domain of Met have also been found in lung, thyroid and gastric cancers, which result in delayed Met receptor

degradation and sustained signalling (Lee, Han et al. 2000; Ma, Kijima et al. 2003; Wasenius, Hemmer et al. 2005; Kong-Beltran, Seshagiri et al. 2006). These data support a causative role of the Met-HGF signalling axis in tumourigenesis.

1.1.5.4 Cell Scatter and morphogenesis

As previously mentioned, HGF has the ability to induce cell spreading, breakdown of cell-cell junctions and dissociation of a sheet of epithelial cells, resulting in the loss of epithelial cell polarity and the acquisition of a fibroblastic/mesenchymal morphology (Stoker and Perryman 1985; Stoker, Gherardi et al. 1987). The breakdown of cell-cell contacts, reorganization of the actin cytoskeleton, changes in cell adhesion and migration is mediated through multiple pathways downstream from Met. These include Src, members of the small family of small GTPases, Rac1, Cdc42 and RhoA, the serine/threonine kinase Pak1, Gab1, Shp2, and the SH2/SH3 adaptor protein Crk (Royal and Park 1995; Rahimi, Hung et al. 1998; Maroun, Naujokas et al. 2000; Royal, Lamarche-Vane et al. 2000; Lamorte, Royal et al. 2002; Rodrigues, Fathers et al. 2005; Frigault, Naujokas et al. 2008). The regulation of these signalling pathways is critical for normal development and tissue repair, and the deregulation of these contributes to cancer development and metastasis.

The HGF-Met signalling axis is involved in the reorganization of epithelial cells into a branched tubular network, which is a prerequisite for the formation of a variety of mammalian organs such as the mammary gland, liver, lung and kidney (Rosario and Birchmeier 2003). The initial phase of tubulogenesis involves the formation of a polarized, hollow cyst of epithelial cells through the use of cytoskeletal rearrangements to establish cell-cell adherens junctions, tight junctions and cell-matrix contacts (Montesano

1986; Montesano, Soriano et al. 1998; O'Brien, Zegers et al. 2002) (Figure 1.4). The second phase involves changes in the cytoskeletal architecture, polarity and adhesion, resulting in the migration and proliferation of epithelial cells into the extracellular matrix forming a branched tubular network of epithelial sheets encompassing a continuous lumen. The ability of Met to promote tubulogenesis in a three-dimensional matrix is dependent on Gab1 (Weidner, Di Cesare et al. 1996; Maroun, Holgado-Madruga et al. 1999). The most characterized model of epithelial polarity, scatter and morphogenesis involves the use of the Madin-Darby Canine Kidney (MDCK) epithelial cell systems (Montesano, Matsumoto et al. 1991; Royal and Park 1995; Maroun, Holgado-Madruga et al. 1999).

As was described above, many of the signals generated downstream from the Met receptor regulate multiple biological processes. During development, a balance between survival and apoptosis plays a major role in morphogenesis and tissue sculpting. In an adult, this balance is essential for tissue homoeostasis. Conversely, resistance to apoptosis is associated with tumour initiation and progression. The Met RTK plays an important role in the regulation of this balance between apoptosis and survival.

1.2 Apoptosis

Apoptosis comes from an ancient Greek word meaning the “falling off of petals from a flower” or “of leaves from a tree in autumn”. Apoptosis is the process of programmed cell death (PCD) that has been found to occur in multi-cellular organisms. It is defined by multiple morphological changes through a series of biochemical events leading to a characteristic cell morphology and death. These changes include blebbing,

changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Vogt 1842; Kerr 1965; Kerr, Wyllie et al. 1972; Kerr 2002).

1.2.1 Death receptors

Apoptosis is induced by the activation of the Tumor Necrosis Factor (TNF) superfamily of death receptors. The main characteristic of these receptors is the presence of a protein-protein interaction domain, termed the death domain (DD), located in their cytoplasmic tail that binds to adaptor proteins and initiates apoptosis. To date, eight members of the death receptor family have been identified and described. They are the tumor necrosis factor receptor-1 (TNFR1, also known as death receptor 1 [DR1]), Fas receptor (CD95, also known as DR2/APO-1), DR3 (also known as TRAMP/APO-3), DR4 (also known as TRAILR1/APO-1), DR5 (also known as TRAILR2/TRICK2), DR6, ectodysplasin A receptor (EDAR), and nerve growth factor receptor (NGFR) (Lavrik, Golks et al. 2005). The binding of their respective ligands triggers the recruitment of molecules that transduce the apoptotic and/or survival signals. Importantly, two types of death receptor signalling complexes have been distinguished. Firstly, the activation of the Fas receptor, DR4 and DR5 death receptors gives rise to the formation of a Death-inducing signalling complex (DISC), resulting in the activation of the apoptotic cascade downstream from caspase-8 (described in section 1.2.3) (Peter and Krammer 2003). Secondly, activation of the TNFR1, DR3, DR6 and EDAR complex recruit an alternate set of signalling molecules that are capable of promoting both survival and apoptotic signals (Sheikh and Huang 2003; Lavrik, Golks et al. 2005).

1.2.2 Fas receptor /CD95

One of the best-characterized TNF death receptors is the Fas/CD95 receptor. The Fas receptor was isolated from a cDNA library of human T cell lymphoma KT-3 cells (Itoh, Yonehara et al. 1991). The Fas receptor was identified as a 319 amino acid type 1 transmembrane glycoprotein that is expressed in most tissues, including the brain, pancreas and liver (Itoh, Yonehara et al. 1991). It contains 3 extracellular cysteine-repeat domains and an 80 amino acid DD located in the C-terminal region that is functionally conserved among death receptors. The apoptotic signal is initiated upon binding of its ligand, FasL, or by agonistic antibody, Jo-2 (Itoh, Yonehara et al. 1991; Ogasawara, Watanabe-Fukunaga et al. 1993). FasL is a trimeric type 2 transmembrane protein that is primarily expressed by activated $CD4^+$ and $CD8^+$ T cells. It is cleaved and released from its membrane site through the action of metalloproteinases (Suda, Takahashi et al. 1993; Tanaka, Suda et al. 1995). The association of FasL and Fas leads to the oligomerization of the receptor and the assembly of a multi-protein DISC complex and initiation of apoptosis (Papoff, Hausler et al. 1999; Siegel, Frederiksen et al. 2000; Scott, Stec et al. 2009).

1.2.3 Death Inducing Signalling Complex (DISC)

Activation of the Fas receptor leads to the recruitment of a multi-protein DISC complex and the subsequent activation of the apoptotic cascade. The first step in the formation of the DISC complex involves the recruitment of the Fas-associated DD (FADD) adaptor protein to the DD domain of the Fas receptor in response to receptor oligomerization (Kischkel, Hellbardt et al. 1995; Scott, Stec et al. 2009). FADD contains a protein-protein interaction domain at its N-terminus, termed the death effector domain

(DED), which associates with caspases. Caspases also contain DED domains, and the DED domain of FADD and the DED domains of caspases have been shown to interact, thus bringing the caspases in a complex with DISC (Figure 1.5). For example, two initiator caspases have been found to associate with the DISC complex, Caspase-8 and Caspase-10 (Boldin, Goncharov et al. 1996; Muzio, Chinnaiyan et al. 1996; Kischkel, Lawrence et al. 2001; Wang, Chun et al. 2001). However, Caspase-8 is the principle caspase found to be associated with the activated DISC complex. This was shown when cells deficient in Caspase-10 expression retained the ability to undergo apoptosis while Caspase-8-deficient cells were resistant to Fas-mediated apoptosis, even when Caspase-10 was overexpressed (Sprick, Rieser et al. 2002). Caspase-8 is primarily located in the cytoplasm and mitochondria in an inactive form and is recruited to the DISC complex upon binding of FADD. The association of Caspase-8 with FADD is believed to lead to its autoproteolytic cleavage and activation to an active heterotetramer, containing two p18 and two p10 subunits (Medema, Scaffidi et al. 1997) (Figure 1.5). Following Caspase-8 activation, downstream caspases are subsequently cleaved and activated. These include Caspases-3, -7, and -6. These caspases mediate the cleavage of proteins and induce the major morphological changes observed in apoptosis (Fernandes-Alnemri, Litwack et al. 1995; Nicholson, Ali et al. 1995; Duan, Chinnaiyan et al. 1996; Earnshaw, Martins et al. 1999).

Two separate pathways of Fas-mediated apoptosis are generated at the DISC complex. These pathways are dependent on the amount of active Caspase-8. In type I cells (termed the extrinsic pathway), an abundant amount of active Caspase-8 can directly cleave and activate Caspase-3 and initiate the apoptotic cascade. In type II cells (termed the intrinsic pathway), a reduced DISC complex formation is seen and apoptosis occurs

through an amplification loop via the mitochondria (Hengartner 2000). Apoptosis in these cells is dependent on cleavage of the BH3-only pro-apoptotic Bcl-2 homologue protein Bid. Truncated Bid (tBid) migrates to the mitochondria and induces the release of cytochrome c into the cytosol (Luo, Budihardjo et al. 1998). The multi-protein complex referred to as the apoptosome is formed, composed of APAF-1 (apoptotic peptidase activating factor 1), cytochrome c and dATP. This permits the recruitment and activation of Caspase-9, which subsequently cleaves effector caspases and initiates apoptosis (Bao and Shi 2007). Therefore, the mechanism of apoptosis is very dependent on cell type. For instance, hepatocytes are considered to be type II cells, as the apoptotic cascade is inhibited in Bid-null mice (Yin, Wang et al. 1999). In Chapter II, I examined the consequence of Fas-induced liver damage in PTP1B-null mice.

1.2.4 Regulation of DISC complex – FLIP

FLICE-like inhibitory protein (FLIP) was initially identified as a viral cell death inhibitor (v-FLIP) expressed by γ-herpes viruses, capable of blocking Fas receptor apoptosis by associating with the receptor in the DISC complex (Thome, Schneider et al. 1997). Two major isoforms of FLIP have been characterized, a short ($FLIP_S$) and a long ($FLIP_L$) isoform (Irmler, Thome et al. 1997). $FLIP_S$ and $FLIP_L$ are catalytically inactive and inhibit caspase-8 activation at the DISC complex (Scaffidi, Schmitz et al. 1999). FLIP expression levels are regulated through the NFκB transcription factor (Kreuz, Siegmund et al. 2001).

1.2.5 Met and Survival/Anti-Apoptotic signals

The PI3K signalling pathway has been shown to regulate survival or anti-apoptotic signals downstream from Met (Birchmeier, Birchmeier et al. 2003; Duronio 2008). The PI3K pathway is involved in several biological functions downstream from Met including epithelial morphogenesis and cell scatter (Derman, Cunha et al. 1995; Royal and Park 1995; Maroun, Holgado-Madruga et al. 1999). Recruitment of PI3K signals to Met can occur either directly through the association of p85 to Met or indirectly through its association to Gab1 (Maroun, Holgado-Madruga et al. 1999). Additionally, the survival responses induced by HGF correlate with an increase in expression of anti-apoptotic factors Bcl-xL, Bcl-2, TRAF2 and cIAP2 (Fan, Wang et al. 1998; Nakagami, Morishita et al. 2002; Fan, Gao et al. 2005). Furthermore, PI3K activates the serine-threonine kinase Akt, whereby Akt phosphorylates the pro-apoptotic protein BAD, leading to its subsequent inactivation and inhibition of apoptosis (Liu 1999). Thus a link between the Met-HGF signalling pathway and cellular survival has been described.

1.2.6 Met and Apoptosis

One model that has been successfully used to examine the role of Met in survival is through the induction of liver damage via intraperitoneal injection of the agonistic Fas antibody, Jo-2. Injection of Jo-2 results in massive hepatic failure as soon as 6 hours post-injection (Ogasawara, Watanabe-Fukunaga et al. 1993). However, HGF treatment protects against Fas-induced apoptosis in primary hepatocytes and against Jo-2-induced hepatic failure in mice (Kosai, Matsumoto et al. 1998). To further examine the importance of Met in protection against Fas-induced liver damage, hepatocytes derived from Met receptor-deficient mice were found to be hypersensitive to Fas-induced

apoptosis and liver regeneration of the liver following partial hepatectomy was impaired in these animals (Borowiak, Garratt et al. 2004; Huh, Factor et al. 2004). The Met receptor has been shown to interact with the Fas receptor, sequestering the receptor and preventing the interaction of the Fas ligand (FasL) with Fas (Wang, DeFrances et al. 2002). This results in an increase in resistance to Fas-mediated apoptosis. However, stimulation with HGF dissociates the complex, sensitizing cells to Fas-mediated apoptosis. This suggests a contradiction regarding what has already been shown in the literature. However the authors Wang *et.al* propose that the pro-apoptotic response to HGF could be a consequence of HGF concentration and FasL expression (Wang, DeFrances et al. 2002).

The initiation and activation of the apoptotic cascade resembles the initiation and activation of growth factor receptors. Ligand binding induces receptor dimerization/clustering and receptor activation, recruitment of downstream signalling or adaptor/scaffold molecules and the initiation of signal transduction pathways. Downstream of RTKs, adaptor/scaffold proteins play an integral role in recruiting the necessary signals to an activated receptor. In the next section, I will describe the Gab family of adaptor/scaffold proteins, in particular Gab1, and the important role of Gab1 in Met-dependent signalling and biology.

1.3 Adaptor/Scaffold proteins

Adaptor/Scaffold proteins are multi-modular proteins without enzymatic activity that mediate interactions with other signalling molecules. They establish and coordinate diverse signalling pathways. Specifically, they serve as a signalling platform by recruiting multiple enzymes and proteins to propagate and amplify the signal (e.g.: insulin receptor substrate and Gab families of docking proteins) (Gu and Neel 2003). These then provide spatial regulation of signals by targeting associated proteins to specific subcellular compartments (Pawson 2007).

1.3.1 Gab family

The Gab family of adaptor/scaffold proteins consists of Gab1, Gab2 and Gab3 in mammals (Holgado-Madruga, Emlet et al. 1996; Gu, Pratt et al. 1998; Wolf, Jenkins et al. 2002), DOS (Daughter of Sevenless) in *Drosophila melanogaster* (Herbst, Carroll et al. 1996; Raabe, Riesgo-Escovar et al. 1996), and SOC-1 (Suppressor of Clear-1) in *Caenorhabditis elegans* (Schutzman, Borland et al. 2001) (Figure 1.6). The overall sequence identity among Gab family members is only 40-50%, however they exhibit a conserved architecture, containing an N-terminal pleckstrin homology (PH) domain, followed by a poorly conserved C-terminus containing a proline-rich region and multiple tyrosine phosphorylation sites. Phosphorylation of tyrosine residues serve as potential binding sites for Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains (Liu and Rohrschneider 2002; Gu and Neel 2003). Gab proteins become tyrosine phosphorylated upon recruitment to multiple RTK and non-TK receptors. In this manner, they assemble multi-protein signalling complexes and serve to amplify and propagate the

signal initiated at the receptor. They have been shown to be critical for normal growth, development, and differentiation (Liu and Rohrschneider 2002; Gu and Neel 2003).

1.3.1.1 Gab1

Gab1 (Grb2-associated binder 1) was originally identified as a Grb2-binding protein from a human glial tumor expression library (Holgado-Madruga, Emlet et al. 1996) and is localized to chromosome 4q31.1 in human and 8C3 in mouse (Yamada, Nishida et al. 2001). Gab1 was found to be tyrosine phosphorylated following EGF and insulin stimulation (Holgado-Madruga, Emlet et al. 1996). Importantly, it was also identified in a yeast-two hybrid screen as a direct binder of the Met RTK (Weidner, Di Cesare et al. 1996) and subsequently identified as the major tyrosine phosphorylated protein downstream from the Met receptor (Nguyen, Holgado-Madruga et al. 1997). Gab1 is a critical modulator of the morphogenic responses induced by the activated Met receptor and is important for oncogenic transformation downstream from both the Met and EGF receptors (Fixman, Naujokas et al. 1995; Maroun, Holgado-Madruga et al. 1999; Maroun, Moscatello et al. 1999; Gual, Giordano et al. 2000; Lamorte, Kamikura et al. 2000; Maroun, Naujokas et al. 2000; Schaeper, Gehring et al. 2000; Lamorte, Rodrigues et al. 2002; Lock, Maroun et al. 2002; Yamasaki, Nishida et al. 2003; Holgado-Madruga and Wong 2004; Mood, Saucier et al. 2006). As will be described in greater detail later, the interaction between Gab1 and the Met receptor is sustained compared to the EGF receptor due to the ability of Gab1 to associate with Met through both a direct and indirect mechanism. Furthermore, a Gab1 knockout mouse closely phenocopies a Met/HGF knockout mouse, highlighting a crucial role of Gab1 in the HGF-Met signalling axis.

Gab1 is tyrosine phosphorylated downstream of a broad range of additional growth factor receptors, cytokine receptors, G-protein coupled receptors and antigen receptors, linking these membrane-associated proteins to intracellular signalling pathways (Liu and Rohrschneider 2002; Gu and Neel 2003). As mentioned, Gab1 phosphotyrosine residues become potential binding sites for SH2 and PTB domains. In addition, Gab1 also contains several proline rich domains capable of interacting with SH3 domain-containing proteins (Holgado-Madruga, Emlet et al. 1996; Nguyen, Holgado-Madruga et al. 1997; Lock, Royal et al. 2000; Liu and Rohrschneider 2002; Gu and Neel 2003). The proteins found to associate with Gab1 include SHP-2, the p85 subunit of PI3K, phospholipase Cγ, Crk, and Grb2 (Garcia-Guzman, Dolfi et al. 1999; Lecoq-Lafon, Verdier et al. 1999; Maroun, Holgado-Madruga et al. 1999; Maroun, Moscatello et al. 1999; Roshan, Kjelsberg et al. 1999; Gual, Giordano et al. 2000; Lamorte, Kamikura et al. 2000; Maroun, Naujokas et al. 2000; Schaeper, Gehring et al. 2000; Lamorte, Rodrigues et al. 2002).

In vivo, Gab1 is critical for embryonic development, as Gab1-deficient mice die *in utero* between embryonic days E13.5 and E18.5, displaying defects of the heart, skin, muscle, placenta and a reduced size of the liver (Sachs, Brohmann et al. 2000). The muscle defect is due to a decreased ability of myogenic precursor cells to migrate into the limb anlage (Sachs, Brohmann et al. 2000). This results in a virtual absence of the extensor muscle groups of the forelimbs and diaphragm and a reduced number of hindlimb muscles (Sachs, Brohmann et al. 2000). This phenotype is similar to those observed in mice deficient in HGF, Met, PDGF and EGF (Bladt, Riethmacher et al. 1995; Schmidt, Bladt et al. 1995; Threadgill, Dlugosz et al. 1995; Uehara, Minowa et al. 1995; Ohlsson, Falck et al. 1999; Itoh, Yoshida et al. 2000; Sachs, Brohmann et al. 2000).

Recently, a novel genetic interaction was identified between the chemokine receptor CXCR4 (chemokine (C-X-C motif) receptor 4) and Gab1 implicating Gab1 in a new signalling pathway (Vasyutina, Stebler et al. 2005). CXCR4 mutant mice have a reduction in the migration of muscle progenitor cells to the tongue and dorsal limbs, whereas in $CXCR4^{-/-}$;$Gab1^{-/-}$ mice, migration of the myogenic precursors to the tongue is abrogated (Vasyutina, Stebler et al. 2005). Altogether, these genetic studies show that Gab1 is important in mediating the migration of myogenic precursor cells during development, supporting a role of Gab1 in the regulation of actin cytoskeletal dynamics needed for cell migration.

1.3.1.2 Gab2

Gab2 (Grb2-associated binder 2) was cloned as a phosphorylated protein bound to Shp2 and to the p85 subunit of PI3K in IL-3 stimulated hematopoietic cells (Gu, Pratt et al. 1998). It is widely expressed in multiple tissues such as the heart, testis and lung, with lower expression in the brain and liver. Gab2 is localized on chromosome 11q13.4-11q13.5 in human and 7E2 in mouse (Yamada, Nishida et al. 2001). Gab2-knockout mice are viable, however, $Gab2^{-/-}$ mast cells are defective in response to stimulation of high affinity immunoglobulin-epsilon (IgE) receptor FcεRI (Gu, Saito et al. 2001). Accordingly, Gab2 knockout mice experience passive cutaneous and systemic anaphylaxis. Mast cells from these animals undergo cytokine gene expression and degranulation, as well as show development defects (Gu, Saito et al. 2001; Nishida, Wang et al. 2002). Gab2 is crucial for the differentiation of hematopoietic cells, such as the differentiation of macrophages induced by M-CSF, and megakaryocytes from the human K562 chronic myelogenous leukemia cell line (Liu, Jenkins et al. 2001; Dorsey, Cunnick

et al. 2002). Gab2 also is important in osteoclastogenesis and bone homeostasis, as Gab2 acts downstream of receptor activator of NFκB (RANK) and mediates RANK-induced activation of NFκB, Akt and Jnk (c-jun N-terminal kinase) (Wada, Nakashima et al. 2005).

Gab2 is tyrosine phosphorylated in response to multiple stimuli including IL-2, IL-3, erythropoietin, thrombopoietin, stem cell factor, Flt-3 ligand, B-cell receptor, T-cell receptor, Bcr-Abl, EGF, heregulin, and HGF (Gu, Pratt et al. 1998; Nishida, Yoshida et al. 1999; Wickrema, Uddin et al. 1999; Zhao, Yu et al. 1999; Bouscary, Lecoq-Lafon et al. 2001; Lock, Maroun et al. 2002; Lynch and Daly 2002). Following tyrosine phosphorylation, all Gab proteins recruit Shp2 and the p85 subunit of PI3K, leading to potentiation of the Ras/Erk and PI3K/Akt pathways (Gu and Neel 2003). Gab2 is regulated by a negative feedback loop whereby Akt and Erk attenuate Gab2 signalling by phosphorylation of S159 and S623, respectively (Lynch and Daly 2002; Arnaud, Crouin et al. 2004).

A role for Gab2 in cancer has been found. The Gab2 locus is amplified in 10-15 % of human breast cancers (Schwab 1998; Yamada, Nishida et al. 2001). Furthermore, Gab2 is over-expressed in mammary tumors and promotes the growth and metastasis of ErbB2-induced mouse mammary tumors (Daly, Gu et al. 2002; Bentires-Alj, Gil et al. 2006; Ke, Wu et al. 2007). Gab2 is required for cell proliferation, anchorage-independent cell growth, and constitutive PI3K and Erk activation in the chronic myeloid leukemia (CML), Bcr-Abl and Tel-Abl cell-based models (Sattler, Mohi et al. 2002; Scherr, Chaturvedi et al. 2006). Furthermore, Gab2 is hyperphosphorylated in CML models that are resistant to imatinib (Wu, Meng et al. 2008).

1.3.1.3 Gab3

Gab3 (Grb2-associated binder 3) was isolated based on sequence similarity to Gab1 (Wolf, Jenkins et al. 2002). It was found to be tyrosine phosphorylated following M-CSF stimulation, and together with Gab2, plays a role in macrophage differentiation (Wolf, Jenkins et al. 2002). The association of Gab3 with Mona, a Grb2-like adaptor protein appears to play a special role in mediating the M-CSFR differentiation signal (Bourgin, Bourette et al. 2002). Gab3 is expressed primarily in cells of the hematopoietic cell lineage such as lymphocytes and macrophages, albeit at low levels. Gab3-knockout mice are viable and healthy and do not exhibit any detectable defects in normal mouse development, hematopoiesis or immune system function (Seiffert, Custodio et al. 2003).

1.3.1.4 DOS (Daughter of Sevenless)

Sevenless, the gene encoding the EGFR ortholog, is essential for *Drosophila melanogaster* eye development (Michael, Bowtell et al. 1990). One important component of this pathway is the SH2 domain containing protein tyrosine phosphatase Corkscrew (CSW, mammalian Shp2). Herbst and colleagues used a trapping mutant of CSW, csw^{C583S}, to identify potential substrates of the phosphatase (Herbst, Carroll et al. 1996). Using this approach, a 115 kDa tyrosine-phosphorylated substrate was trapped, purified and identified as the product of the DOS gene (Herbst, Carroll et al. 1996). Independently, Raabe and colleagues identified DOS in a screen for mutations that suppress *sevenless* signalling (Raabe, Riesgo-Escovar et al. 1996). DOS has also been shown to be required for signalling downstream from the receptor tyrosine kinases Torso and DER (EGFR and PDGFR orthologues, respectively) (Bausenwein, Schmidt et al. 2000).

1.3.1.5 SOC-1 (suppressor of clear-1)

Soc-1, the Gab ortholog in nematodes, was identified by screening *Caenorhabditis elegans* for mutations that would suppress the clear phenotype associated with a constitutive activation of the EGL-15 (mammalian FGFR) signalling pathway. Soc-1 functions in conjunction with the *C. elegans* SH2 homologue, PTP2 (mammalian Shp2), to mediate EGL-15 (homologue of mammalian FGFR) signal transduction cascade(Schutzman, Borland et al. 2001).

1.3.2 Functional Motifs and domains in Gab1

Structure-function analysis of members of the Gab family of adaptor proteins have identified a common architecture that is critical in mediating the biological responses downstream of activated receptor complexes. These include motifs or domains that are found to mediate the interaction with multiple signalling proteins or that are necessary for Gab1's subcellular localization.

1.3.2.1 Pleckstrin Homology (PH) domain

As mentioned, Gab1 contains an N-terminal pleckstrin homology (PH) domain. PH domains are present in a wide range of proteins involved in intracellular signalling or regulation of the cytoskeleton. There are more than 250 proteins in humans and more than 30 in yeast that contain PH domains (Lemmon 2008). The PH domain specifically binds phosphatidylinositol lipids within biological membranes (such as phosphatidylinositol (3,4,5)-trisphosphate, PIP3, and phosphatidylinositol (4,5)-bisphosphate, PIP2), targeting the proteins to the appropriate cellular compartment and/or enabling them to interact with other components of signal transduction pathways.

The importance of this domain has been shown in several biological systems. The Gab1 and DOS PH domains bind specifically to the PI3K product, PIP3, and are necessary and sufficient for their localization at the sites of cell-cell contact (Isakoff, Cardozo et al. 1998; Maroun, Holgado-Madruga et al. 1999; Maroun, Moscatello et al. 1999; Rodrigues, Falasca et al. 2000). Gab1 mutants with a deletion of the PH domain, or mutation at a conserved phospholipid binding site (W26A/C, R29A/C) within the PH domain, were unable to mediate Met-receptor induced epithelial morphogenesis (Maroun, Holgado-Madruga et al. 1999; Maroun, Moscatello et al. 1999). Likewise, PH domain mutants of DOS failed to function in *sevenless* signalling and rescue the lethal phenotype of DOS loss-of-function mutant flies (Bausenwein, Schmidt et al. 2000), while the PH domain mutant of SOC-1 was unable to rescue the suppressor of clear phenotype in the EGL-15 signalling pathway. These data indicate the importance of Gab1 localization in mediating biological outcomes.

1.3.2.2 Proline-rich domain of Gab1

The central region of Gab proteins is rich in proline, and contains multiple PXXP motifs, which are potential binding sites for SH3 domain containing proteins. The SH3 domain-containing adaptor proteins Grb2 and Grb2-like Gads/Mona, bind to the proline rich domain in Gab1. All Gab family members associate constitutively with Grb2 (Holgado-Madruga, Emlet et al. 1996; Gu, Pratt et al. 1998; Wolf, Jenkins et al. 2002) and contain two conserved binding sites for the Grb2 C-terminal SH3 domain (Lock, Royal et al. 2000). One is a canonical PXXP-containing motif, whereas the other is atypical, with a P-X-X-X-R-X-X-K-P consensus sequence (Lock, Royal et al. 2000). The recruitment of Gab1 to a number of activated receptor complexes appears to depend on an

indirect mechanism involving Grb2. The C-terminal SH3 domain of Grb2 constitutively associates with Gab proteins, and the SH2 domain of Grb2 binds to specific tyrosine residues within a given receptor tyrosine kinase (Lock, Maroun et al. 2002). Additionally, mutation of the Grb2 binding site of either Gab1 or the EGF receptor abolishes the association between Gab1 and the EGF receptor (Lock, Royal et al. 2000; Rodrigues, Falasca et al. 2000). Furthermore, fibroblast cells isolated from mice expressing a null- or hypomorphic-Grb2 show an absence of Gab1 tyrosine phosphorylation in response to EGF (Saxton, Cheng et al. 2001). These results provide evidence for a Grb2-dependent mechanism of recruitment of Gab1 to the EGF receptor. The recruitment of Gab proteins to activated receptors may also occur through a fourth protein in cases where the receptor does not contain a Grb2 binding site. For example, recruitment of Gab2 to the IL-3 receptor beta common chain (βc) occurs through Shc recruitment to the IL-3 receptor and the ability of Grb2 to bind tyrosine phosphorylated Shc (Gu, Maeda et al. 2000). Furthermore, the adaptor protein FRS2 contains five Grb2 binding sites and acts as an intermediate to recruit Gab1 to the FGF receptor through a FRS2-Grb2-Gab1 interaction (Ong, Hadari et al. 2001). Therefore, the proline rich domains within Gab proteins are important in mediating the recruitment of Gab1 to multiple receptors indirectly through Grb2.

1.3.2.3 Gab1 recruitment to Met

Gab1, and its recruitment to Met, is unique as it can interact through both a Grb2-dependent and a Grb2-independent mechanism (Nguyen, Holgado-Madruga et al. 1997; Lock, Royal et al. 2000; Lock, Maroun et al. 2002; Lock, Frigault et al. 2003). Gab1 was found to interact directly with Met using a yeast-two hybrid screen and the region

identified within Gab1 to mediate this interaction is situated within the proline-rich region of Gab1 (coined the Met Binding Domain – MBD) (Weidner, Di Cesare et al. 1996). Furthermore, a 13 amino acid sequence within the MBD, GMQVPPPAHMGFR, known as the Met binding Motif (MBM), was later identified as being sufficient to mediate the direct association between Gab1 and Met (Schaeper, Gehring et al. 2000; Lock, Maroun et al. 2002). In contrast to the interaction of an SH2 domain with a phosphorylated tyrosine residue within a linear peptide motif, the structural integrity of the Met receptor, and residues upstream of tyrosine 1349 (located in the C-terminal lobe of the kinase domain) are required for the direct interaction between Gab1 and Met (Lock, Frigault et al. 2003). The recruitment of Gab1 to Met can also be mediated indirectly by the Grb2 adaptor protein (Lock, Royal et al. 2000; Schaeper, Gehring et al. 2000; Lewitzky, Kardinal et al. 2001; Lock, Maroun et al. 2002). Grb2 interacts with the Met receptor through the association of the Grb2 SH2 domain with tyrosine 1356 of Met (Ponzetto, Bardelli et al. 1994; Fixman, Fournier et al. 1996; Bardelli, Longati et al. 1997; Fixman, Holgado-Madruga et al. 1997). The ability of Gab1 to associate with the Met receptor via two different mechanisms creates a more stable interaction between the two proteins and allows for prolonged Gab1 phosphorylation. The prolonged Gab1 phosphorylation correlates with sustained activation of the Erk MAPK and protein kinase B (PKB)/Akt pathways (Maroun, Holgado-Madruga et al. 1999; Maroun, Naujokas et al. 2000; Rosario and Birchmeier 2003). This may be evolutionarily important in the Met-HGF signalling pathways since this is not the case between Gab1 and other RTKs.

1.3.2.4 Regulation of Gab1 activity

Gab1 is phosphorylated on tyrosine, serine and threonine residues downstream of numerous transmembrane receptors, including the Met, EGF, insulin and TrkA, as well as cytokine receptors for IL-3, IL-6, interferon α, interferon γ, B-cell, T-cell and G-protein-coupled receptors (Holgado-Madruga, Emlet et al. 1996; Nguyen, Holgado-Madruga et al. 1997; Ingham, Holgado-Madruga et al. 1998; Lecoq-Lafon, Verdier et al. 1999; Nishida, Yoshida et al. 1999; Badache and Hynes 2001; Miyakawa, Rojnuckarin et al. 2001). This allows for the recruitment of multiple signalling molecules to a given receptor and allows for signal transduction.

In addition to being directly phosphorylated by RTKs and other transmembrane receptors, tyrosine phosphorylation of Gab1 occurs downstream of Src family kinases (SFK). Gab1 tyrosine phosphorylation is significantly decreased in cells derived from Src/Yes/Fyn-null mouse embryos when compared with wild-type (WT) cells upon HGF stimulation (Chan, Chen et al. 2003). The expression of WT Src enhanced HGF-induced phosphorylation of Gab1 in this system and, in contrast, expression of the kinase-deficient Src mutant or treatment of cells with the specific Src inhibitor PP1, suppressed phosphorylation. Src mediated phosphorylation of Gab1 also contributed to the activation of Erk and Akt (Chan, Chen et al. 2003).

Phosphorylation of Gab1 is important in the activation and regulation of signalling pathways. Gab1 is serine/threonine phosphorylated by Erk1/2 downstream of several receptor tyrosine kinases. Erk-mediated Ser/Thr phosphorylation of Gab1 residues T312, S384, S455, T477, S581 and S598 has differential effects on the signalling pathways downstream of RTKs (Lehr, Kotzka et al. 2004). For example, Erk-mediated

phosphorylation has a negative effect on Gab1 signalling downstream of the EGF and insulin receptor through inhibition of Akt/PKB signalling (Yu, Liu et al. 2002). In contrast, Erk-mediated phosphorylation of Gab1 downstream of the Met receptor enhances Akt activation (Yu, Roshan et al. 2001). The protein phosphatase Shp2 has been found to negatively regulate Gab1 activity by de-phosphorylating specific p85 binding sites (pTyr 448, 473 and 590) within Gab1 and attenuating PI3K signalling downstream of the EGF receptor (Zhang, Tsiaras et al. 2002). Therefore it appears there is both a positive and negative feedback mechanism that regulates Gab1-mediated PI3K signalling and the interplay between the Erk and Akt pathways provides a complexity and specificity downstream of different RTKs.

1.3.3 Gab1 protein complexes and their biological significance

1.3.3.1 Gab-Shp2 complex

One protein that has been found to associate with all members of the Gab family is the tyrosine phosphatase Shp2 and its homologues (Cunnick, Mei et al. 2001). Tyrosine phosphorylation of one or two tyrosine residues located in the C-terminus of Gab family members, including DOS and SOC-1, provide a docking site for Shp2. Shp2 is composed of a C-terminal phosphatase domain and two tandem SH2 domains at the N-terminus, which bind to one or two YXXV/I/L-containing motifs within Gab (Raabe, Riesgo-Escovar et al. 1996; Gu, Pratt et al. 1998; Lehr, Kotzka et al. 1999; Nishida, Yoshida et al. 1999; Schutzman, Borland et al. 2001; Wolf, Jenkins et al. 2002).

The functional/biological significance of the Gab-Shp2 interaction has been extensively studied. In mammalian cells, Gab1 mutant protein containing a tyrosine to phenylalanine substitution in the Shp2 SH2 domain was unable to mediate Met receptor-

induced epithelial morphogenesis (Maroun, Naujokas et al. 2000; Schaeper, Gehring et al. 2000). Similarly, a Gab2 mutant that was unable to bind to Shp2 blocked M-CSF-induced macrophage differentiation (Liu, Jenkins et al. 2001). In *Drosophila*, *sevenless* signalling is impaired as a Shp2-binding mutant of DOS fails to rescue the lethality associated with the *dos* loss of function mutation (Herbst, Carroll et al. 1996; Bausenwein, Schmidt et al. 2000). In *C. elegans*, a mutant SOC-1 that was unable to bind to Shp2 failed to function in EGL-15 signalling (Schutzman, Borland et al. 2001). Importantly, mutations of other tyrosine residues within DOS or SOC-1 had no effect on the biological functions, illustrating the importance of the Gab-Shp2 interaction *in vivo* (Bausenwein, Schmidt et al. 2000; Schutzman, Borland et al. 2001). The *in vivo* significance of the Gab1-Shp2 interaction was recently identified through the generation of knockin mice that carry a point mutation in the Shp2 binding sites of Gab1 (Schaeper, Vogel et al. 2007). Gab1-deficient mice die *in utero* between E13.5 and E18.5, displaying defects in the heart, skin, muscle, placenta and a reduced liver size (Sachs, Brohmann et al. 2000). The defect seen in the muscle is due to a deficiency of myogenic precursor cells migrating into the limb anlage. The Gab1-Shp2 interaction proved to be crucial for this, as Gab1$^{\Delta Shp2/\Delta Shp2}$ mice were embryonic lethal between days E12.5 and E14.5, and had deficiencies in Met receptor-directed placental development and muscle progenitor cell migration to the limbs (Schaeper, Vogel et al. 2007).

Shp2 recruitment to the Gab family of proteins, functions as positive regulators of the MAPK activity downstream from RTKs (Bennett, Tang et al. 1994; Bennett, Hausdorff et al. 1996; Gu, Pratt et al. 1998; Takahashi-Tezuka, Yoshida et al. 1998; Nishida, Yoshida et al. 1999; Cunnick, Dorsey et al. 2000; Itoh, Yoshida et al. 2000; Maroun, Naujokas et al. 2000; Schaeper, Gehring et al. 2000; Liu, Jenkins et al. 2001).

The association of Shp2 to Gab1 results in sustained activation of the MAPK signalling pathway, and this is critical for epithelial morphogenesis downstream of the Met receptor (Khwaja, Lehmann et al. 1998; Maroun, Naujokas et al. 2000; Schaeper, Gehring et al. 2000). However, the mechanism of MAPK activation through the association between Gab proteins and Shp2 has not been defined. One potential mechanism involves the negative regulator of Ras signalling, Ras-GAP, which has recently been found to be a binding partner for Gab1, by associating with tyrosines 307 and 317 of Gab1 (Maroun, Naujokas et al. 2000; Montagner, Yart et al. 2005). Shp2 dephosphorylates the binding sites on Gab1 for Ras-GAP, preventing the recruitment of Ras-GAP, enabling sustained activation of the Ras/MAPK pathway (Montagner, Yart et al. 2005). Mutation of the Shp2 binding site of Gab1 can enhance recruitment and activation of Ras-GAP, thereby inhibiting Ras signalling even further (Montagner, Yart et al. 2005). A similar mechanism has been found in *Drosophila* where the Shp-2 orthologue, Csw, dephosphorylates a tyrosine residue on the Torso RTK that binds to Ras-GAP, thus uncoupling it from the Torso receptor (Cleghon, Feldmann et al. 1998). Another potential mechanism involves the ability of Shp2 to promote SFK activation by regulating the phosphorylation of the Csk regulator PAG/Cbp that controls recruitment of Csk to SFKs (Zhang, Yang et al. 2004). In Shp2-deficient cells, the inhibitory C-terminal tyrosines of SFK are hyperphosphorylated, and the tyrosine phosphorylation of SFK substrates, such as PLCγ, is decreased. Decreased PLCγ phosphorylation leads to defective Ras activation and this may help account for impaired Erk activation in Shp2-deficient cells (Zhang, Yang et al. 2004).

1.3.3.2 Gab-p85

Mammalian Gab proteins contain 3 YXXM motifs, consensus binding sites for the p85 subunit of PI3K, while DOS and SOC-1 contain only one site each (Herbst, Carroll et al. 1996; Holgado-Madruga, Moscatello et al. 1997; Bausenwein, Schmidt et al. 2000). The association between Gab1 and p85 is promoted downstream from multiple growth factor and cytokine receptors (Holgado-Madruga, Moscatello et al. 1997; Ingham, Holgado-Madruga et al. 1998; Laffargue, Raynal et al. 1999; Maroun, Holgado-Madruga et al. 1999; Hayashi, Ichihara et al. 2000; Lehr, Kotzka et al. 2000; Rakhit, Pyne et al. 2000; Rodrigues, Falasca et al. 2000; Zhang and Broxmeyer 2000; Ong, Hadari et al. 2001; Dance, Montagner et al. 2006). The Gab-p85 interaction is predicted to form a positive feedback loop, since PI3K activation leads to the production of PIP3, which recruits Gab1, through its PH domain, to the plasma membrane and promotes further activation of PI3K (Rodrigues, Falasca et al. 2000).

The functional/biological significance of the Gab-p85 interaction has been extensively studied. PI3K activation is required for Met-mediated epithelial morphogenesis, however, mutation of the sites in Gab1 did not affect the ability of Gab1 to rescue Met receptor-mediated morphogenesis in the presence of serum (Derman, Cunha et al. 1995; Royal and Park 1995; Maroun, Holgado-Madruga et al. 1999). The *in vivo* significance of the Gab1-p85 interaction was recently identified through the generation of knockin mice that carry a point mutation in the p85 binding sites of Gab1 (Schaeper, Vogel et al. 2007). Unlike the Met- and Gab1-knockout mice and $Gab1^{\Delta Shp2/\Delta Shp2}$-knockin mice, the $Gab1^{\Delta PI3K/\Delta PI3K}$ mice were viable, limb muscle formation was normal and liver growth was unaffected (Bladt, Riethmacher et al. 1995; Itoh,

Yoshida et al. 2000; Sachs, Brohmann et al. 2000; Schaeper, Vogel et al. 2007). Gab1$^{\Delta PI3K/\Delta PI3K}$ mice were born with open eyes at birth, a phenotype that is associated with perturbed EGFR signalling (Sibilia and Wagner 1995). Mutation of the p85 binding sites in DOS or SOC-1 does not lead to functional abnormalities in *Drosophila* or *C. elegans* respectively (Bausenwein, Schmidt et al. 2000; Schutzman, Borland et al. 2001).

1.3.3.3 Gab-Crk/PLCγ interactions

Gab1 and Gab2 contain multiple YXXP motifs that overlap as binding sites for the SH2 domains of the adaptor protein Crk and PLCγ (Gual, Giordano et al. 2000; Lamorte, Kamikura et al. 2000; Sakkab, Lewitzky et al. 2000; Crouin, Arnaud et al. 2001). Crk and PLCγ have both been shown to be critical for Met receptor-mediated biology, as mutation of their known binding sites within Gab1 abrogates the ability of HGF stimulation to induce epithelial morphogenesis downstream from Met (Gual, Giordano et al. 2000; Lamorte, Royal et al. 2002). Crk functions in multiple cellular processes such as cell spreading, actin reorganization, and cell migration. When overexpressed, Crk promotes lamellipodia formation, cell spreading, and the loss of epithelial adherens junctions in the absence of growth factor stimulation (Lamorte, Royal et al. 2002). Downstream of the Met receptor, association between Gab and Crk is important for anchorage-independent growth (Lamorte, Kamikura et al. 2000), activation of the JNK and MAPK (Garcia-Guzman, Dolfi et al. 1999) and activation of the small GTPases Rap1 and Rac (Lamorte, Royal et al. 2002; Lamorte, Rodrigues et al. 2003).

Crk recruitment to Gab1 is important in promoting the morphogenic program downstream from Met. A well-characterized model of epithelial polarity and morphogenesis involves the use of the Madin-Darby Canine Kidney (MDCK) epithelial

cell systems (Montesano, Matsumoto et al. 1991; Royal and Park 1995; Maroun, Holgado-Madruga et al. 1999). The ability of Met to promote tubulogenesis in three-dimensional matrix is dependent on Gab1 (Weidner, Di Cesare et al. 1996; Maroun, Holgado-Madruga et al. 1999). Unlike the response seen with HGF stimulation, MDCK cells stimulated with EGF are unable to induce the morphogenic program and promote tubulogenesis when grown in a 3D collagen matrix. However, over-expression of Crk is sufficient to promote and activate this program in MDCK cells following EGF stimulation (Lamorte, Rodrigues et al. 2002). The ability to undergo morphogenesis requires remodeling of the actin cytoskeleton.

Many of the known binding partners of Crk are regulators of actin cytoskeletal dynamics. These proteins include p130Cas and paxillin, which are components of focal adhesions (Chodniewicz and Klemke 2004). The Ras superfamily of small GTPases are activated downstream of multiple transmembrane receptors. One subset of the Ras superfamily of small GTPases, known as the Rho family of small GTPases, functions in regulating actin cytoskeletal dynamics. Crk associates with two known activators of Ras GTPases, the guanine exchange factors C3G (Gotoh, Hattori et al. 1995), and DOCK180 (Kiyokawa, Hashimoto et al. 1998), known activators of the GTPases Rap1 and Rac, respectively. Crk functions as a key integrator of actin cytoskeletal dynamics capable of forming large multimeric protein complexes that mediates these responses. For instance, Crk can form a multimolecular complex with Paxillin, GIT2 (an ARF-GAP) and β-Pix (a Rac1 exchange factor) and promote spreading of epithelial colonies, by influencing the recruitment of paxillin to focal complexes, events critical for cell migration and invasion (Lamorte, Rodrigues et al. 2003).

1.4 Ras superfamily of GTPases

Ras is a family of genes that encodes a group of small guanosine triphosphatases (GTPases) actively involved in cellular signal transduction. Ras activation promotes multiple biological outcomes culminating in cell growth, actin reorganization, differentiation and survival. To date, 150 proteins in the Ras superfamily with evolutionarily conserved orthologs found in *Drosophila*, *C. elegans*, *S. cerevisiae*, *S. pombe*, *Dictyostelium* and plants have been identified. Based on structure, sequence and function, the Ras superfamily is divided into nine main families, each of which is further divided into subfamilies: Ras, Rho, Rab, Rap, Arf, Ran, Rheb, Rad and Rit (Wennerberg, Rossman et al. 2005). Miro is a recent contributor to the superfamily (Wennerberg, Rossman et al. 2005). The Ras superfamily of GTPases functions as GDP/GTP-regulated molecular switches that cycle between an inactive and active state. The cycling between GDP and GTP is controlled by two main classes of regulatory proteins; guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs). In the inactive state, GTPases are GDP bound and become activated by exchange of GDP for GTP through the action of a GEF (Ridley 2001). They return to an inactive state through the hydrolysis of GTP to GDP, which is catalyzed by GAPs. More specifically, the Rho and Rab subfamilies have also been found to associate with guanine-nucleotide-dissociation inhibitors (GDIs), which prevent their association to the plasma membrane. The structural differences in both the GDP- and GTP-bound form were first observed in Ras and termed the "switch regions" (Milburn, Tong et al. 1990). The switch regions have similar conformations but pronounced differences corresponding to two regions called switch I (Ras residues 30-38) and switch II (Ras residues 59-67). Switch regions are sites through which regulatory and

effector proteins associate with GTPases. The controlled regulation of Ras GTPase is critical in the maintenance of biological function in the cell.

The HGF-Met signalling axis activates Rho GTPases that play key roles in regulating the organization of the actin cytoskeleton in mammalian cells necessary for cellular migration (Ridley, Comoglio et al. 1995; Royal and Park 1995; Royal, Lamarche-Vane et al. 2000). This occurs through the activation of Rac and Cdc42, which are important for the formation of filopodia and lamellipodia.

1.4.1 The Rho Family of small GTPases.

The Ras homologous (Rho) proteins serve as key regulators of extracellular-stimulus-mediated signalling networks that regulate actin organization, cell cycle progression and gene expression. Twenty members of the Rho family have been identified with RhoA, Rac1 and Cdc42 being the best studied (Ridley 2001). RhoA promotes actin stress fiber formation and focal adhesion assembly; Rac1 promotes lamellipodium formation and membrane ruffling; and Cdc42 promotes actin microspikes and filopodium formation. Consequently, the coordinated activation of each of these Rho GTPases is critical in the regulation of cell polarity, cell shape, cell-cell and cell-matrix interactions needed to generate the protrusion and pulling forces that regulate cellular migration.

1.4.1.1 Cdc42

Cdc42 (cell division cycle 42) was discovered as an essential gene in *Saccharomyces cerevisiae* that was required for budding and establishment of cell polarity (Madaule, Axel et al. 1987; Adams, Johnson et al. 1990; Johnson and Pringle

1990). Cdc42 has been found to regulate signalling pathways that control diverse cellular functions including cell morphology, migration, endocytosis and cell cycle progression. Cdc42-knockout mice are embryonic lethal and die before embryonic day 7.5 (Chen, Ma et al. 2000). Mutant embryos were small in size, disorganized in structure and largely lacked the embryonic primary ectoderm.

Multiple downstream effectors of Cdc42 have been found to regulate Cdc42-dependent biological functions. Some of these downstream effectors include the Wiskott-Aldrich syndrome protein (WASp)-Arp2/3 pathway (Snapper, Takeshima et al. 2001), mammalian diaphanous (mDia) (Peng, Wallar et al. 2003), p21-activated kinase (Pak) (Manser, Leung et al. 1994; Abo, Qu et al. 1998), partitioning-defective-6 (Par6)-Par3/atypical protein kinase C (aPKC) (Goldstein and Macara 2007), IQGAP (IQ motif containing GTPase activating protein)(Osman and Cerione 1998), insulin-receptor substrate p53 (IRSp53) (Krugmann, Jordens et al. 2001), and γCOP (Wu, Erickson et al. 2000). Most studies performed with Cdc42 have involved using either constitutively active or dominant-negative mutant of Cdc42 to deduce its importance in regulating actin cytoskeletal dynamics. The main biological phenotype associated with Cdc42 activation is the formation of filopodia, finger-like, actin-rich protrusions that form on the cell surface, and are thought to function as "feelers" for the new extra-cellular space adjacent to the leading edge (Gupton and Gertler 2007) (described in section 1.5).

1.4.1.2 Rac

The Rac (Ras-related C3 botulinum toxin substrate) subfamily of Rho GTPases (~21 kDa) is a composed of 4 members, Rac1, Rac2, Rac3 and RhoG (Didsbury, Weber et al. 1989; Boureux, Vignal et al. 2007). It is a pleiotropic regulator of many cellular

processes, including the cell cycle, cell-cell adhesion, motility, and epithelial differentiation. Rac has predominantly been found to stimulate lamellipodium and membrane ruffles, and induce membrane extension during phagocytosis (Jaffe and Hall 2005). Knockout mouse studies of Rac isoforms have shown that they have non-redundant functions in the cell. The Rac1 knockout animal is embryonic lethal before embryonic day 9.5 due to severe defects in germ layer formation (Sugihara, Nakatsuji et al. 1998). This is in contrast to Rac2- (Roberts, Kim et al. 1999), Rac3- (Cho, Zhang et al. 2005; Corbetta, Gualdoni et al. 2005) and RhoG- (Vigorito, Bell et al. 2004) knockout mice which are viable. Tissue-specific knockouts in haematopoietic cells (Gu, Filippi et al. 2003), epidermal cells (Benitah, Frye et al. 2005; Chrostek, Wu et al. 2006), myeloid cells (Glogauer, Marchal et al. 2003), Schwann cells (Benninger, Thurnherr et al. 2007; Nodari, Zambroni et al. 2007) and endothelial cells (Tan, Palmby et al. 2008) have been widely used to study Rac1 function, demonstrating a different role for Rac among cell types. In macrophages, Rac1-null macrophages have an elongated morphology and do not spread normally, however they are capable of forming membrane ruffles (not lamellipodia) and can migrate at a similar speed to WT macrophages (Wells, Walmsley et al. 2004). Furthermore, Rac1/Rac2-null macrophages migrate at a similar speed or faster than WT cells suggesting a Rac-independent mechanism for forming membrane protrusions (Wheeler, Wells et al. 2006). In neutrophils, where Rac2 is predominantly expressed, Rac2 has been found to be critical in the formation of lamellipodia and migration (Roberts, Kim et al. 1999) and in FcR-mediated phagocytosis (Williams, Tao et al. 2000). In neurons, there is controversy regarding the impact of the Rac family as it has been found to be important in neurite outgrowth and axonal guidance. This arises from the use of a dominant-negative Rac1 mutant suggesting a role for Rac1 in axon

growth. However, in a neuron-targeted conditional knockout of Rac1, axon growth is normal, although a defect in axon guidance and a failure of axons to cross the midline is described (Luo, Liao et al. 1994; Koh 2006; Chen, Liao et al. 2007).

Rac proteins utilize similar signalling pathways to Cdc42 to regulate actin polymerization during lamellipodia formation. Rac proteins can activate the WAVE-Arp2/3 complex and mDia2 and thus actin nucleation (Suetsugu, Yamazaki et al. 2003; Yamazaki, Fujiwara et al. 2005). Rac also functions to control lamellipodia formation by regulating the availability of free actin barbed ends (and thus actin polymerization) through the removal of barbed-end capping proteins or severing of actin filaments by Pak-LIMK-cofilin pathway and gelsolin (Azuma, Witke et al. 1998; Kiuchi, Ohashi et al. 2007).

The coordinated activation of Cdc42 and Rac1 are important steps in activating the necessary downstream effectors responsibl for generating changes in the actin cytoskeleton. The generation of actin-rich protrusions, in the form of filopodia and lamellipodia, are hallmark structures necessary to produce the protrusive forces needed by the cell for cellular migration.

1.5 Actin-rich membrane protrusions

The protrusive structures present at the leading edge of a migrating cell are called filopodia and lamellipodia. They are formed from the elongation of fast-growing, barbed ends of actin filaments directed towards the plasma membrane. Filopodia are thin (0.1-0.3 μm), finger-like structures filled with tight parallel bundles of filamentous (F)-actin. A lamellipodia is a thin (0.1-0.2 μm) sheet-like protrusion that is filled with a branched

network of actin. The coordinated activity of actin polymerization to generate these membrane protrusions is at the core of cellular motility. The initiation and turnover of these dynamic structures is linked to the assembly/disassembly cycles of actin filaments, which are tightly regulated by the cell.

1.5.1 Cell migration

The initial phase of migration requires the establishment of polarity, a front and a back. This ensures a cell will not move in all directions at once and appear spread. Following the exposure of cells to growth factors, they bind to RTKs and local activation and amplification of signalling events occurs. Cdc42 is active towards the front of the cell and is necessary for the formation of filopodia, finger-like, actin-rich protrusions that form on the cell surface, and function as sensors for the new extra-cellular space adjacent to the leading edge (Itoh, Kurokawa et al. 2002; Gupton and Gertler 2007). Cdc42 activation is followed by Rac1 activation leading to additional actin polymerization and the formation of a membrane protrusion in the direction of migration known as a lamellipodium. The membrane protrusions are stabilized by the formation of focal adhesions. This process requires integrin activation, clustering, and the recruitment of structural and signalling components to nascent adhesions, such as paxillin and Fak. Adhesions transmit the propulsive forces and serve as traction points over which the cell moves. The migration cycle is completed as adhesions disassemble and the rear retracts.

1.5.2 Filopodia

Filopodia are protruding F-actin structures composed of parallel bundles of actin filaments that extend in a finger-like manner beyond the cell edge (Lewis and Bridgman

1992). Barbed ends of actin filaments within the filopodium are oriented outwards and the protrusions and retractions of the filopodia are mediated through a dynamic balance between barded-ended polymerization and F-actin retrograde flow (Mallavarapu and Mitchison 1999). They are generally described as "antennae" that probe their microenvironment during cell migration, sensing for chemo-attractants/chemicals, acting as sites of signal transduction and attaching to the extracellular matrix. Filopodia contain receptors for multiple growth factors and extracellular matrix molecules, such as integrins and cadherins, which detect signals in the extracellular microenvironment (Lidke, Lidke et al. 2005; Galbraith, Yamada et al. 2007). Integrin-containing filopodia form initial sites of adhesion. Mature focal adhesion-anchored actin stress fibers are then formed through the recruitment of protein complexes including paxillin and Fak.

Multiple proteins have been shown to be involved in filopodia formation. Cdc42 (described in section 1.4.1.1) is the small GTPase that induces filopodia formation by recruiting downstream effector molecules to the active site of actin polymerization. These downstream effectors include the WASp family proteins, which activate the Arp2/3 complex. The Arp2/3 complex then binds to the side chain of actin filaments and nucleate a new filament branch from the principle filament (Rohatgi, Ma et al. 1999). Additional proteins involved in filopodia formation include Enabled (Ena)/VASP (vasodilator-stimulated phosphoprotein), IRSp53, Fascin and Diaphanous-related formin, Dia2. These proteins nucleate actin filaments, accelerate actin polymerization, and protect barbed ends from capping proteins and bundle F-actin (Bachmann, Fischer et al. 1999; Kureishy, Sapountzi et al. 2002; Zigmond 2004; Barzik, Kotova et al. 2005; Schirenbeck, Bretschneider et al. 2005; Kovar, Harris et al. 2006; Schirenbeck, Arasada

et al. 2006; Goode and Eck 2007; Pasic, Kotova et al. 2008; Scita, Confalonieri et al. 2008).

1.5.3 Lamellipodia

The lamellipodium is considered to be a motile organelle that is indispensable for the movement of rapidly migrating cells. The lamellipodium covers a distal region of 1-5 μm, which consists of a flat, (height of 0.1-0.2 μm) membrane-enclosed, dynamic leaflet of cytoplasm (Abercrombie, Heaysman et al. 1970).

The formation of a lamellipodial edge requires the coordinated activities of both the actin and microtubule cytoskeleton of the cell. Microtubules (MT) are polymers of α- and β-tubulin dimers. MTs are organized in such a manner that the minus ends are anchored to the centrosome, which is positioned between the nucleus and the leading edge. The plus ends radiates towards the leading edge, which allows for an interaction with actin/plasma membrane proteins. MTs thus act as a platform which brings the necessary proteins to the leading edge to establish cortical polarity between the actin-rich cortex and the microtubule plus ends (Siegrist and Doe 2007). Following the establishment of polarity, MTs provide the necessary cues to promote lamellipodia formation. For instance, the activation of Rac1 through the formation of a complex between the microtubule plus end proteins CLIP170, IQGAP and Rac1 (Fukata, Watanabe et al. 2002; Watanabe, Wang et al. 2004). New actin filaments arise downstream of signalling pathways that had been activated downstream via Rac1, leading to the formation of a dendritic branched actin network. Nucleation promoting factors (NPFs) such as WASp and WAVE coordinate the recruitment and stimulation of the Arp2/3 complex (Miki, Suetsugu et al. 1998; Marchand, Kaiser et al. 2001). These

initiate the formation of new actin filaments as a branch on the sides of existing filaments (Machesky, Atkinson et al. 1994). Together with the Arp2/3 pathways, Pak kinases regulate leading edge actin branched networks through ADF/cofilin, actin-binding proteins which disassembles actin filaments (Ridley 2001; Small, Stradal et al. 2002; Wittmann, Bokoch et al. 2003). The branched filament is elongated through the addition of actin monomers to the barded end, generating the lamellipodium. Furthermore, actin is organized into contractile bundles, and together with myosin motors, generates traction within the cell needed to drive the cell in the direction of migration. The contractile actin bundles pull on focal contacts that are attached to the substrate, which are assembled at the cell front and disassembled at the rear of the cell (Webb, Parsons et al. 2002). This coordinated activity is the driving force allowing the cell to move.

1.5.4 Role of Met and Gab1 in cell polarity and actin cytoskeletal rearrangements

As mentioned earlier, the HGF-Met signalling axis is involved in the formation of epithelial organs by the creation of a tubular network of polarized, epithelial sheets that encompass a well-defined continuous lumen to make a functional organ such as the lung, breast, kidney, pancreas liver and testis (Rosario and Birchmeier 2003). This is a process known as epithelial morphogenesis. It is a highly organized process that results in changes in epithelial cell polarity and adhesion (termed epithelial to mesenchymal transition [EMT]), promoting migration, division, and finally, differentiation to form polarized branched hollow tubules with organ-specific functions (Rosario and Birchmeier 2003). This process requires precise control not only of the cellular cytoskeleton, adhesion, cell division, and apoptosis, but also remodeling of the cellular environment

(Weidner, Sachs et al. 1993; Zhu, Naujokas et al. 1994; Matsumoto and Nakamura 1997; Zegers, O'Brien et al. 2003). MDCK cells grown in collagen is a well-established method of studying this process (Montesano, Schaller et al. 1991).

Epithelial morphogenesis, cellular migration and scattering require cellular processes such as actin cytoskeleton rearrangement and the formation of membrane protrusions. As mentioned, the Met receptor is known to promote tubulogenesis, migration and scattering, which requires Gab1 (Maroun, Holgado-Madruga et al. 1999). Uncoupling Gab1 function from Met inhibits the ability of HGF to promote these biological responses (Maroun, Holgado-Madruga et al. 1999). One function of Gab1 is to act as a mediator of the actin cytoskeletal changes downstream from Met. This is supported by data implicating Gab1, and not Gab2, as a promoter of epithelial morphogenesis and cell scatter (Lock, Maroun et al. 2002; Frigault, Naujokas et al. 2008). Our lab has previously shown that a redistribution of Gab1 to the leading cortical edge of the cell occurs during cellular migration, allowing for the initiation of downstream signalling pathways within this subcellular compartment (Frigault, Naujokas et al. 2008). This suggests that Gab1 is localized to HGF-induced membrane protrusions to coordinate their formation and form a signalling complex in order to transduce information from this subcellular compartment (Maroun, Moscatello et al. 1999; Lamorte, Kamikura et al. 2000; Lamorte, Rodrigues et al. 2003; Frigault, Naujokas et al. 2008). For example, the interaction between Gab1 and Crk promotes the localized activation of Rac at the leading edge of the cell (Lamorte, Kamikura et al. 2000; Lamorte, Rodrigues et al. 2002; Frigault, Naujokas et al. 2008). Furthermore, the Gab1-Shp2 complex, which is critical for sustained Erk signalling downstream from Met, is required for the rescue of lamellipodia formation downstream of Met, since a Gab1 protein harboring a tyrosine to phenylalanine

substitution at the sites for Shp2 binding is unable to induce lamellipodia formation in MDCK cells (Frigault, Naujokas et al. 2008). Activated ERK is localized to lamellipodia in a sustained manner and MEK inhibition diminishes lamellipodia formation, further demonstrating the importance of the Gab1-Shp2-MEK-ERK pathway in lamellipodia formation (Frigault, Naujokas et al. 2008). Therefore, the ability of Gab1 to recruit and localize downstream signalling pathways to the leading edge and regulate actin cytoskeletal membrane dynamics is critical for Met biological function.

The Pak family of Ser/Thr kinases are downstream effectors of Rho GTPases that regulate actin cytoskeletal dynamics. Met receptor activation leads to the activation of Pak1 (Royal, Lamarche-Vane et al. 2000). Pak1 is translocated to membrane ruffles following HGF stimulation and inhibition of Pak1 impairs epithelial cell spreading (Royal, Lamarche-Vane et al. 2000). The next part of this introduction will focus on introducing the different members of the Pak family and the regulation of their activity, with an emphasis on Pak4, which was recently identified as a novel Gab1-associated protein (Chapter 3) (Paliouras, Naujokas et al. 2009).

1.6 p21-activated kinase (PAK)

1.6.1 Pak family and structure

Pak kinases are involved in many cellular signalling pathways including cell survival, cell transformation and cytoskeletal reorganization. Paks are a family of serine/threonine kinases that bind to and are stimulated by activated forms of the small GTPases, Cdc42 and Rac. Currently, there are 6 members of the Pak family that can be divided into two additional groups based on biochemical and structural features (Figure

1.7). Group I Paks (Pak1, Pak2 and Pak3) share common structural features. They share a p21-binding domain (PBD) located in the N-terminal region. This domain is at least 88% identical among Group I Paks. Group I Paks also contain a kinase domain at the C-terminus, which is at least 93% identical among these three kinases. Group II Paks share a similar structural composition to Group I Paks, however, they only possess a 57% identity to Group I Paks. The PBD domain of Group II Paks shares at least 60% identity between Pak4-6. The C-terminal kinase domain is only 54% identical between Group II Paks. The differences observed between the PBD domain and the kinase domain of the different groups correlate with differences in signalling and biology observed in Group I and II.

1.6.2 Regulation of activity

Biochemical and structural features define the distinction between Group I and Group II Paks. Group I Paks are downstream effectors of Cdc42 and Rac, whereas Group II Paks are predominantly Cdc42 effector proteins, while Pak5 also associates with Rac1(Dan, Nath et al. 2002). The principal distinguishing feature between the two groups is the presence of an auto-inhibitory domain (AID) that overlaps with the PBD in Group I Paks but is missing in Group II Paks, although Pak5 has been suggested to possess an N-terminal AID (Ching, Leong et al. 2003). The AID maintains Group I Paks in an inactive conformation. When unphosphorylated, Group I Paks homodimerize, the AID binds to the kinase domain, which masks the phosphorylation of activation segment, thereby inhibiting its activity (Buchwald, Hostinova et al. 2001; Parrini, Lei et al. 2002). The binding of Group I Paks to activated GTPases disrupts Pak dimerization, destabilizing the auto-inhibitory structure, allowing auto-phosphorylation and activation of the kinase. In

contrast, Group II Pak family members posses no AID and their association with activated Cdc42 does not enhance their kinase activity (Abo, Qu et al. 1998; Dan, Nath et al. 2002; Schrantz, da Silva Correia et al. 2004). Therefore, rather than stimulating kinase activity, the association with GTP-loaded Cdc42 may target Group II Paks to a specific subcellular localization, such as the Golgi for Pak4 (Abo, Qu et al. 1998) or the mitochondria and nucleus for Pak5 (Cotteret, Jaffer et al. 2003; Wu and Frost 2006). This would suggest that Group II Paks are potentially regulated through different mechanisms compared to Group I Paks.

1.6.3 Discovery of Pak family

The first Pak was discovered by an overlay assay using [γ-^{32}P] GTP-p21 as a substrate for the identification of interactors of Rac1, Cdc42 and RhoA (Manser, Leung et al. 1994). The most prominent binding proteins initially detected in the overlay assay had molecular weights of 68, 65 and 62 kDa, and bound to GTP-loaded Cdc42 and Rac1, but not to RhoA. A GTP-loaded, GST-Cdc42 affinity chromatography column allowed for purification of the 65 kDa protein. In addition, the kinase activity of the 65 kDa protein could only be stimulated by binding of activated GTP-bound Cdc42 and Rac (Manser, Leung et al. 1994). Based on these results, the 65 kDa protein was termed p21-(Cdc42/Rac) activated kinase (Pak). Soon after the initial characterization of p65 Pak, the 68 kDa and 62 kDa proteins were later identified as Pak3 (Bagrodia, Taylor et al. 1995; Manser, Chong et al. 1995) and Pak2 (Teo, Manser et al. 1995), respectively. This provided the first piece of evidence that Cdc42 and Rac act like other traditional GTP-binding proteins to stimulate a target/effector protein. The discovery of Pak1 and subsequently Pak2 and Pak3, highlighted an interesting analogy to the Ras signalling

pathway, where activated Ras directly binds to the serine/threonine protein kinase Raf. Paks show a significant sequence homology to the Ste20 protein kinase, which participates in the pheromone/mating-factor pathway in budding yeast.

1.6.4 Discovery of Group II Paks

New Pak-related proteins, Pak4, Pak5 and Pak6, were later identified as new members of the Pak family of serine/threonine kinases. The first member of the Group II Paks that was discovered was Pak4, and it is the most well characterized to date. Pak4 was discovered from a PCR screen with degenerate primers corresponding to regions of homology between the kinase domains of yeast Ste20 and mammalian human Pak2 from Jurkat cell cDNA (Abo, Qu et al. 1998). Pak4 was found to be expressed in most tissues examined, with highest levels being seen in the prostrate, testis, ovary, small intestines and colon. Multiple biological functions have been identified for Pak4 including actin cytoskeletal reorganization, apoptosis, cellular transformation and oncogenesis (Abo, Qu et al. 1998; Dan, Kelly et al. 2001; Gnesutta, Qu et al. 2001; Callow, Clairvoyant et al. 2002; Gnesutta and Minden 2003; Li and Minden 2005; Ahmed, Shea et al. 2008; Liu, Xiao et al. 2008)

Pak6 was identified through a yeast two-hybrid screen with the purpose of identifying novel androgen receptor interacting proteins (Yang, Li et al. 2001). Pak6 is highly expressed in the testis and brain and Pak6 knockout mice are viable, fertile and display no visible abnormalities or phenotypes (Yang, Li et al. 2001; Lee, Ramos et al. 2002; Nekrasova, Jobes et al. 2008). Pak6 is the first Pak protein that has been identified to associate with the androgen and estrogen alpha nuclear receptors (Yang, Li et al. 2001; Lee, Ramos et al. 2002). Expression of Pak6 and androgen receptor resulted in the

inhibition of both androgen receptor- and estrogen receptor-mediated transcriptional response (Yang, Li et al. 2001; Lee, Ramos et al. 2002).

Pak5 was identified in a screen of the EST database using a BLAST search to identify new members of the Pak family. Pak5 is expressed primarily in the brain and is important in filopodium formation and neurite outgrowth (Dan, Nath et al. 2002). However, unlike Pak4 and Pak6, Pak5 interacts with both Cdc42 and Rac (Dan, Nath et al. 2002; Cotteret, Jaffer et al. 2003). Pak5 is similar to *Drosophila* MBT protein (for "mushroom body tiny") which has a role in development, proliferation, and survival of cells in the mushroom body, a structure of the *Drosophila* brain. Pak5 knockout mice, like Pak6 knockouts, develop normally and are fertile with no defects in neural development (Li and Minden 2003). Furthermore, Pak5/Pak6 double knockout mouse were viable and fertile, however they possessed several locomotor and behavioural defects (Nekrasova, Jobes et al. 2008). These results suggest a functional redundancy between Pak5 and Pak6.

1.6.5 Pak4

1.6.5.1 Pak4 Knockout

To examine the physiological and developmental functions of Pak4, the Pak4 gene was disrupted in mice. Pak4 absence led to embryonic lethality by embryonic day 11.5, due to a defect in the fetal heart (Qu, Li et al. 2003). Defects were also found in the brain, as improper folding occurred in the caudal neural tubes, resulting in the formation of two neural lumens (Qu, Li et al. 2003). In addition, Pak4-knockout mice had severe defects in the development and migration of neurons (Qu, Li et al. 2003). Neuronal progenitors appeared to form normally, however, spinal cord motor neurons and interneurons failed to

differentiate, axonal outgrowth was impaired and failed to migrate to their proper location (Qu, Li et al. 2003). Therefore, these defects in neuronal differentiation reflect the importance of Pak4 in inducing cytoskeletal changes, including the formation of filopodia (Abo, Qu et al. 1998; Qu, Cammarano et al. 2001; Callow, Clairvoyant et al. 2002).

1.6.5.2 Pak4 Biological Functions

1.6.5.2.1 Actin cytoskeleton regulation

One biological function of Pak4 is its role as a mediator of cytoskeletal organization (Figure 1.8). Pak4 was initially identified as a downstream effector of Cdc42 (Abo, Qu et al. 1998). Pak4 alone is unable to induce any changes to the actin cytoskeleton. However, in the presence of an activated Cdc42 (Cdc42V12), Pak4 is targeted to the Golgi compartment, reorganization of the actin cytoskeleton occurs and formation of filopodia ensues. This requires both Pak4 kinase activity and interaction with Cdc42(V12). In contrast to Pak1, 2 and 3, the kinase activity of Pak4 is not regulated by Cdc42 (Abo, Qu et al. 1998). Pak4 appears to have constitutive kinase activity in the absence or presence of Cdc42(V12) and the recruitment to Cdc42, rather than stimulation of its kinase activity, is important for actin polymerization and cytoskeletal changes.

The interaction of Pak4 with Cdc42 mediates the actin cytoskeletal reorganization that is seen downstream from Pak4 (Figure 1.8). In addition to binding Cdc42, Pak4 can also associate with Rho GEFs, such as GEF-H1 and PDZ-RhoGEF (PRG) (Barac, Basile et al. 2004; Callow, Zozulya et al. 2005). GEF-H1 is a microtubule associated GEF and the association of GEF-H1 with Pak4 occurs through the GEF-H1 interaction domain (GID) in Pak4 and the C-terminus of GEF-H1 (Callow, Zozulya et al. 2005). Pak4

phosphorylation of GEF-H1 on Ser885 resulted in the release of GEF-H1 from microtubules into the cytoplasm, leading to the formation of actin-rich lamellipodia in NIH3T3 cells (Callow, Zozulya et al. 2005). This implicated the Pak4-GEF-H1 interaction as an initiator of lamellipodia formation through an unidentified mechanism. PRG`, together with LARG and p115RhoGEF`, links the G(12/13) family of heterotrimeric G proteins to Rho activation. In the case of PRG`, the C-terminal kinase domain of Pak4 interacts with the C-terminal region of PRG (excluding the PH-DG domain) (Barac, Basile et al. 2004). The interaction resulted in the phosphorylation of PRG and abolished its ability to mediate the accumulation of Rho-GTP by Galpha13 and decreased Rho GTP loading *in vivo* (Barac, Basile et al. 2004). Interaction and phosphorylation of GEF-H1 and PRG via Pak4 resulted in a decrease in Rho-dependent activity and stress fiber formation.

Pak4 influences actin polymerization through the LIMK-cofilin/ADF (actin depolymerizing factor) pathway. Cofilin is an actin binding protein that regulates cytoskeletal dynamics by dissembling actin filaments. Cofilin is negatively regulated by LIMK, whose activity is regulated through phosphorylation by effector kinases of the Rho-GTPases, ROCK, Pak1 and Pak4 (Edwards, Sanders et al. 1999; Ohashi, Nagata et al. 2000; Dan, Kelly et al. 2001; Soosairajah, Maiti et al. 2005). LIMK inactivates cofilin by phosphorylating cofilin at Ser3, resulting in the recruitment of the scaffold protein 14-3-3ζ, which negatively regulates the cofilin pathway. The phosphatases Slingshot (SSH), releases this block and activates cofilin. In addition, SSH can also inactivate LIMK1 through dephosphorylation of Thr508. Within this pathway, Pak4 preferentially phosphorylates and activates LIMK1 compared to LIMK2 (Soosairajah, Maiti et al. 2005; Ahmed, Shea et al. 2008) and activates SSH by phosphorylation. Therefore, Pak4 is the

only dual regulator that controls the LIMK-ADF/cofilin pathway through phosphorylation of both the activator SSH and the suppressor LIMK1 (Figure 1.8).

Activation of the Met RTK through the binding of HGF can stimulate the kinase activity of Pak4 (Wells, Abo et al. 2002). In unstimulated MDCK cells, transfection of an activated Pak4 induced a decrease in stress fiber formation. However, when cells were stimulated with HGF, it induced a loss of focal complexes and cell rounding. This response was dependent on Pak4 kinase activity and did not require Cdc42 binding (Wells, Abo et al. 2002). In addition, HGF stimulation led to a relocalization of Pak4 from the cytoplasm to the cell periphery within lamellipodia (Wells, Abo et al. 2002). This occurred in a PI3K-dependent manner, as LY294002, a PI3K inhibitor, inhibited HGF-induced Pak4 kinase activation, relocalization, and cell rounding (Wells, Abo et al. 2002). Pak4 was subsequently identified as an important component of HGF-induced migration and polarity in the PC3 prostate cancer cell line (Ahmed, Shea et al. 2008). Pak4 siRNA knockdown studies resulted in a reduction of phospho-cofilin levels and increased formation of F-actin rich ruffles at the cell periphery. These results pointed to an importance in the HGF-Pak4-LIMK-cofilin pathway in mediating actin cytoskeletal changes (Ahmed, Shea et al. 2008). However, the mechanism and molecular function for this HGF-dependent response is not completely understood.

1.6.5.2.2 Cellular transformation and Cancer

In addition to actin cytoskeletal organization and apoptosis, Pak4 plays a role in oncogenic transformation. NIH3T3 cells that expressed an activated version of Pak4, displayed phenotypes of cells characteristic of oncogenic transformation. Activated Pak4 expressing cells led to the dissolution of stress fibers, loss of focal adhesions, defect in

cell spreading onto fibronectin-coated surfaces and a change in morphology (cell rounding) (Qu, Cammarano et al. 2001). Activated Pak4 expressing cells were capable of anchorage-independent growth when grown in soft agar (Qu, Cammarano et al. 2001; Callow, Clairvoyant et al. 2002). Constitutively active mutants of the Dbl family of GEFs, are potent oncogenes (Cerione and Zheng 1996; Lin, Cerione et al. 1999). Cdc42, Rac, and Rho were all shown to be necessary for oncogenic transformation by oncogenic Dbl, and each GTPase appears to contribute to different aspects of transformation, including anchorage-independent growth, superoxide production, and loss of contact inhibition, respectively (Lin, Cerione et al. 1999). Consistent with this, a dominant-negative Pak4 mutant inhibited focus formation by oncogenic Dbl (Qu, Cammarano et al. 2001) and inhibited anchorage-independent growth of a Ras-driven human colon cancer cell line, HCT116 (Callow, Clairvoyant et al. 2002), highlighting an important role for Pak4 in oncogenic transformation.

Studies are underway to examine the role Pak4 in tumourigenesis. One study examined the ability of WT and activated Pak4 to produce tumors. NIH3T3 cells stably expressing WT and constitutively activated Pak4 were generated and injected subcutaneously into the flanks of nude mice to determine whether they can promote tumor formation (Liu, Xiao et al. 2008). Activated Pak4 formed tumors 16 days post injection (Liu, Xiao et al. 2008). The tumors were morphologically characterized as sarcomas and showed increased proliferation as measure by Ki67 staining of tissue sections and a decreased level of apoptosis compared to control tissue. Furthermore, Pak4-null and WT fibroblasts were infected with oncogenic Ras (RasV12) or activated Cdc42 (Cdc42V12) and injected into mice. Pak4-null fibroblast (RasV12;$Pak4^{-/-}$) were unable to grow as quickly as fibroblast expressing WT Pak4 (RasV12;$Pak4^{+/+}$), which

were sacrificed 6 days later. In contrast, Pak4-null fibroblasts infected with Cdc42V12 (Cdc42V12;Pak4$^{-/-}$) produced no tumors, compared to Cdc42V12 infected WT cells (Cdc42V12;Pak4$^{+/+}$). This is consistent with a role of Pak4 as a Cdc42 effector protein and indicated a direct role for Pak4 downstream to Cdc42 in oncogenic transformation.

Pak4 is overexpressed in 75% of the NCI 60 cell line panel (Callow, Clairvoyant et al. 2002), suggesting an important role for Pak4 in cancer. Moreover, Pak4 is localized to human chromosome 19q13.2, a region that is frequently amplified in colorectal and pancreatic cancers (Mahlamaki, Kauraniemi et al. 2004; Parsons, Wang et al. 2005; Chen, Auletta et al. 2008; Kimmelman, Hezel et al. 2008). In addition to colorectal and pancreatic cancers, Pak4 was also found to be elevated in mammary and esophageal tumours (Liu, Xiao et al. 2008). To date, no activating mutations in Pak genes have been found in cancer. However, in a colorectal tumor sample a mutation was identified in the Pak4 kinase domain (E329K) but it was unknown if it affected the kinase activity of the protein (Parsons, Wang et al. 2005).

Two different studies have examined the impact of Pak4 on the migratory and invasive capacity of tumor derived cell lines. Pak4 interacts directly through its C-terminus with the β5 subunit of the αvβ5 integrin and promotes cell migration in the human breast carcinoma cell line MCF-7 (Zhang, Li et al. 2002). In another study, Pak4 was found to be amplified in pancreas ductal adenocarcinoma (PDAC). shRNA mediated knockdown of Pak4 in cells that showed Pak4 amplification and robust expression, reduced anchorage-independent growth and diminished invasion through boyden transwell assays when compared to control shRNA treated cells (Kimmelman, Hezel et al. 2008). WT Pak4 expression in pancreatic ductal cells showed minimal migratory

enhancement, while activated Pak4 significantly increased both the migratory and invasive capacity.

1.7 Hypothesis and Specific Aims

Activation of the Met RTK, induced by the binding of its ligand, HGF, is an important modulator of cellular proliferation, differentiation, migration, adhesion, apoptosis and survival. Deregulation of signal transduction pathways, including activation of the Met receptor and its downstream substrate Gab1, are involved in tumor growth, invasion and metastasis in many cancers. The aim of my thesis project is to identify and understand the positive and negative signals that regulate Met receptor-dependent signalling and biology. The impact of Met receptor ubiquitination and internalization as a mechanism of regulating Met receptor activation had already been a focus of research in the lab and scientific community. Interestingly, little work has been done to look at the role of protein tyrosine phosphatases as a means of regulating Met receptor signalling. *I hypothesize and will test that PTP1B is an important regulator of Met RTK signaling and biology (Chapter 2).*

Gab1 is a key integrator of signals downstream from the Met receptor and plays an important role in modulating epithelial remodeling and morphogenesis. This occurs through its ability to recruit and localize specific proteins and downstream signalling pathways necessary for stimulating tumourigenesis, cellular migration and invasion. Since Gab1 acts as a scaffold protein integrating signals downstream from RTKs that are deregulated in cancer, I propose that Gab1 is a key modulator of the invasive response downstream of the Met receptor. The functions of known proteins recruited and activated downstream from Gab have been shown to be involved in cellular migration, invasion,

adhesion, apoptosis and proliferation. However, a thorough analysis of proteins associated to Gab1 following RTK activation has not been performed. I identified the serine/threonine kinase Pak4 and the guanine exchange factor, GEF-H1, to be novel Gab1-associated proteins following Met receptor activation. *I hypothesize and will test that Pak4 and GEF-H1 play key roles in modulating epithelial remodeling morphogenesis, cellular migration and invasion downstream of the Met receptor ability following their recruitment to Gab1 (Chapter 3,4).* This thesis will further our understand of the molecular mechanisms regulating Met receptor tyrosine kinase signalling and biology.

1.8 ABBREVIATIONS

ADF	actin depolymerizing factor
AID	autoinhibitory damain
Akt	v-akt murine thymoma viral oncogene homolog
APAF-1	apoptotic peptidase activating factor 1
aPKC	atypical protein kinase C
Bad	BCL2-associated agonist of cell death
Bcl-2	B-cell CLL/lymphoma 2
Bcr-Abl	break point clustering-Abelson
Bid	BH3 interacting domain death agonist
Cbl	Cas-Br-M (murine) ecotropic retroviral transforming sequence
Cdc42	cell division cycle 42
CML	chronic myeloid leukemia
Crk	v-crk sarcoma virus CT10 oncogene homolog
CSF-1R	colony-stimulating factor-1 receptor
CSW	Corkscrew
CXCR4	chemokine (C-X-C motif) receptor 4
DD	death domain
DED	death effector domain
DISC	Death-inducing signalling complex
DOS	Daughter of Sevenless
dsPTP	dual specificity PTP
EDAR	ectodysplasin A receptor

EGFR	epidermal growth factor receptor
Ena/VASP	Enabled/Vasodilator-stimulation phopshoprotein
Eps15	epidermal growth factor receptor pathway substrate 15
Erk	extracellular signal-regulated kinase
FADD	Fas-associated DD
FAK	focal adhesion kinase
FLIP	FLICE-like inhibitory protein
Flt3	fms-related tyrosine kinase 3
FRS2	fibroblast growth factor receptor substrate 2
Gab1	growth factor receptor bound protein 2-associated protein 1
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine exchange factor
GEF-H1	guanine exchange factor-H1
Grb2	growth factor receptor-bound protein 2
GTPase	guanosine triphosphatase
HGF	hepatocyte growth factor
HRS	hepatocyte growth factor-regulated tyrosine kinase substrate
IGF1	insulin-like growth factor 1
IL-2	Interleukin 2
IQGAP	IQ motif containing GTPase activating protein
IRSp53	insulin-receptor substrate p53
Jak2	Janus activated kinase 2
Jnk	c-jun N-terminal kinase

LAR	leukocyte antigen-related
Limk	LIM domain kinase
M-CSF	macrophage colony-stimulating factor
MAPK	mitogen activated protein kinase
mDia	mammalian diaphanous
MT	microtubules
NFκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NGFR	nerve growth factor receptor
NPFs	nucleation promoting factors
nrPTP	the classical non-receptor PTP
Pak	p21-activated kinase
Par	partitioning-defective
PBD	p21-binding domain
PCD	programmed cell death
PDGFR	platelet-derived growth factor receptor
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKB	protein kinase B
PKC	protein kinase C
PLCγ	phospholipase C gamma
PTB	phosphotyrosine binding

PTP	protein tyrosine phosphatases
PTP1B	protein tyrosine phosphatase 1B
Rac	Ras-related C3 botulinum toxin substrate
RANK	receptor activator of NFκB
Rho	Ras homologous
RPTP	receptor PTPs
RTK	receptor tyrosine kinase
SF	scatter factor
SFK	Src family kinase
SH2	Src homology 2
Shc	Src homology 2 domain containing transforming protein
SHIP	inositol polyphosphate phosphatase-like 1
Shp2	protein tyrosine phosphatase, non-receptor type 11
SOC-1	Suppressor of Clear-1
Src	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SSH	slingshot
STAT3	signal transducer and activator of transcription 3
STAT5	signal transducer and activator of transcription 5
TCPTP	T cell protein tyrosine phosphatases
TKB	tyrosine kinase binding
TNF	tumor necrosis factor
TNFR1	tumor necrosis factor receptor-1
TRAF2	TNF receptor-associated factor 2
Tyk2	tyrosine kinase 2

WASp	Wiskott-Aldrich syndrome protein
WT	wild-type

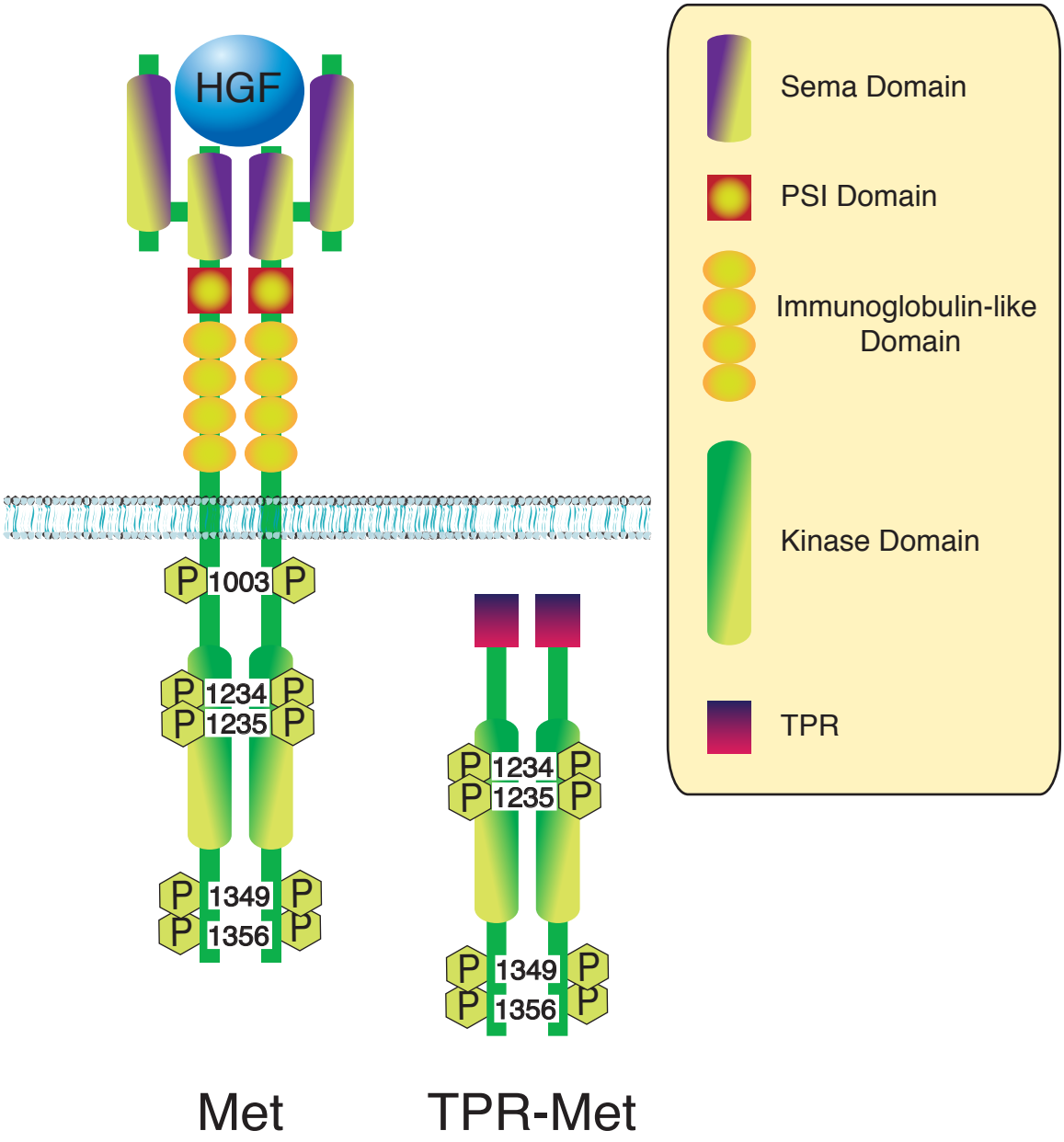


Figure 1.1: The Met receptor tyrosine kinase and Tpr-Met. The Met receptor was originally identified as an activated oncogene, Tpr-Met. Tpr-Met contains only a portion of the Met cytosolic domain fused to a protein–protein dimerization domain, Tpr. The extracellular domain of the Met receptor contains a Sema, plexin/semaphoring/integrin (PSI), and immunoglobulin-like domains. The intracellular domain contains the juxtamembrane and kinase domains. The carboxy-terminal tail of Met (Y1349 and 1356) act as a multisubstrate binding site conserved in Met family members.

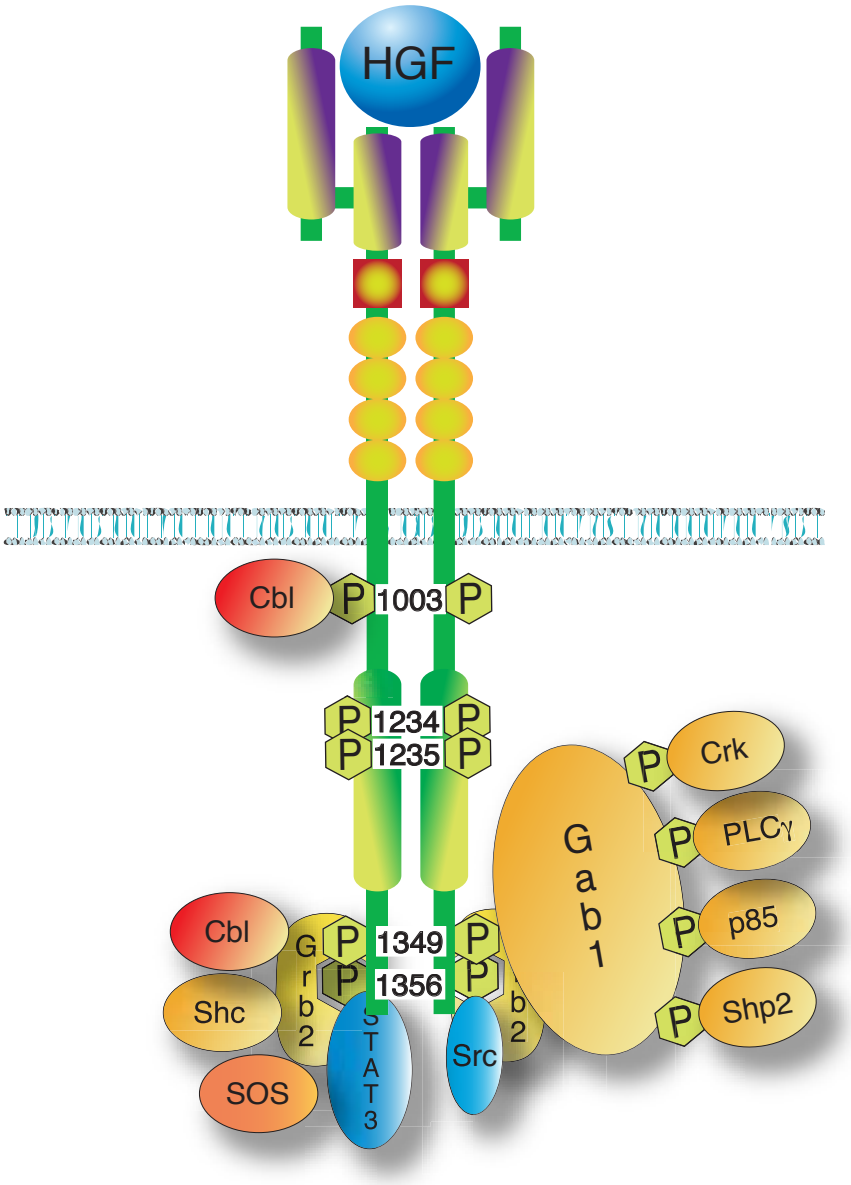


Figure 1.2: The HGF-Met signalling axis. Upon activation of the Met receptor, phosphorylation occurs on tyrosine residues resulting in the recruitment of SH2 domain containing signalling proteins. The major phosphoprotein to interact with Met is the adaptor/scaffold protein Gab1. Once phosphorylated, Gab1 recruits the SHP-2 tyrsosine phosphatase, Crk adaptor protein, PI3'kinase and phospholipase Cγ, activating a number of signalling pathways, which lead to multiple biological responses, including survival, proliferation, actin/cytoskeletal reorganization, migration/invasion and morphogenesis. Association of Cbl to juxtamembrane Y1003 promotes Met receptor ubiquitination.

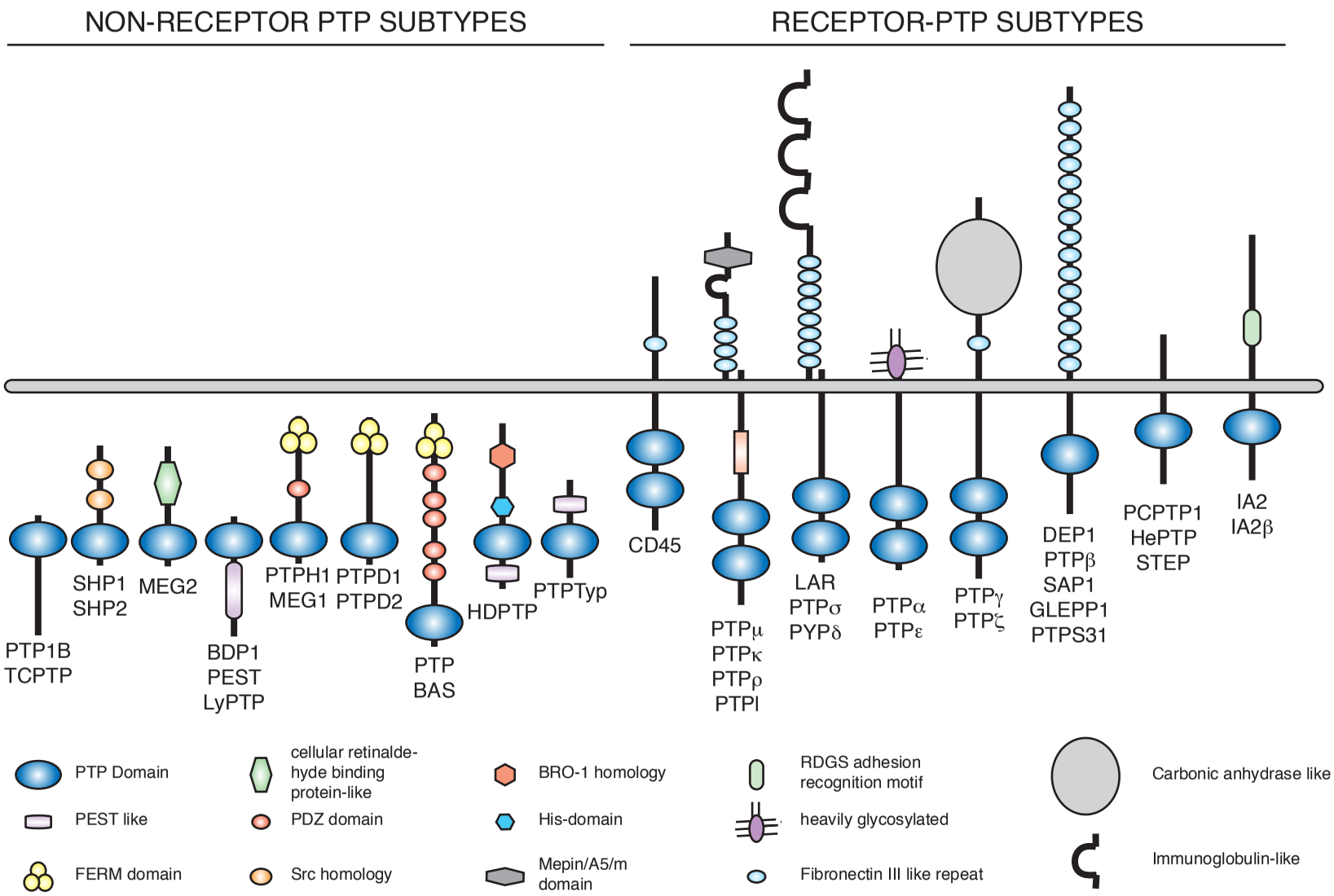


Figure 1.3: Schematic representation of receptor and non-receptor PTP family members.

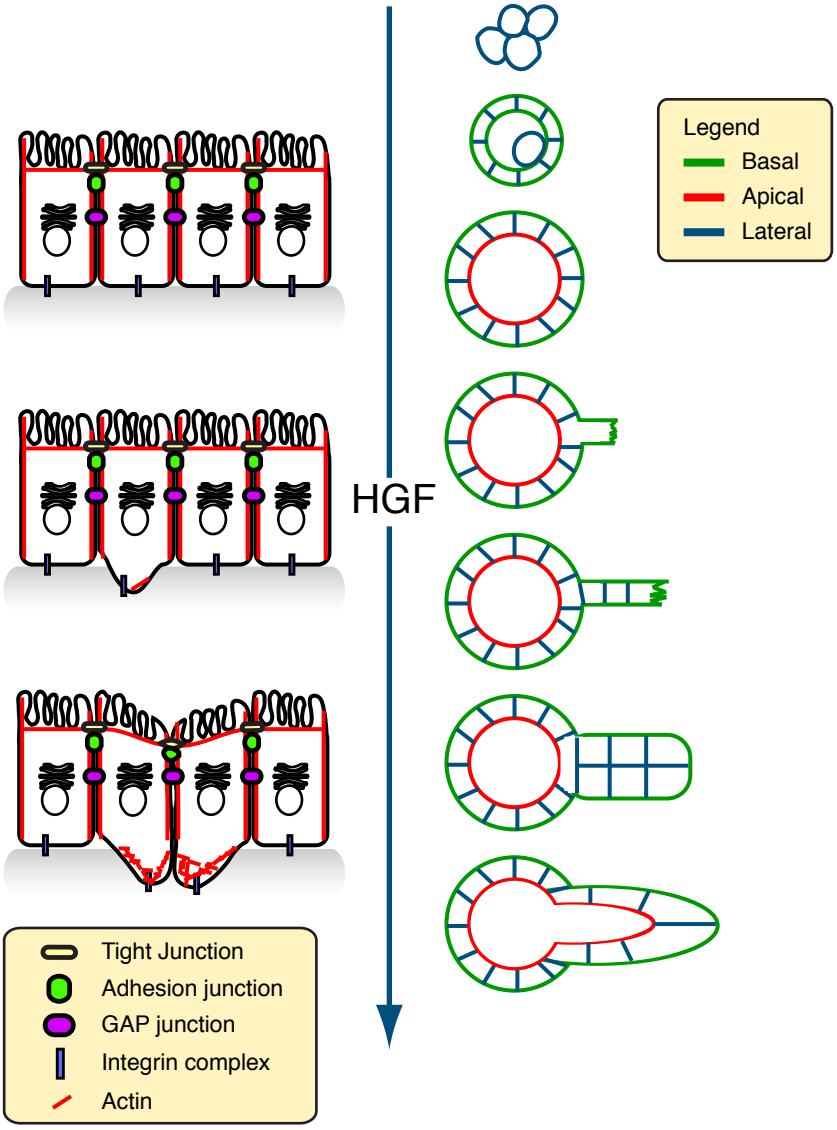


Figure 1.4: Model of epithelial morphogenesis/tubulogenesis. Epithelial morphogenesis results in the generation of a well-polarized monolayer of epithelial cells. The left hand side of this figure demonstrates the organization of a few cells in contact with their neighbors via junctional complexes and with the ECM via integrin complexes. Signals from engagement of these complexes with their ligands or those of the ECM respectively, result in a monolayer of cells with apical, basal and lateral membrane domains. During organogenesis/tubulogensis, HGF is secreted by the surrounding tissues and activates the remodeling of cell-cell contacts and the formation of protrusions into the ECM (bottom, left hand side). The initial phase of morphogenesis/tubulogenesis involves the formation of a polarized, hollow cyst of epithelial cells through the use of cytoskeletal rearrangements to establish cell-cell adherens junctions, tight junctions and cell-matrix contacts. The second phase involves changes in the cytoskeletal architecture, polarity and adhesion, resulting in the migration and proliferation of epithelial cells into the extracellular matrix forming a branched tubular network of epithelial sheets encompassing a continuous lumen.

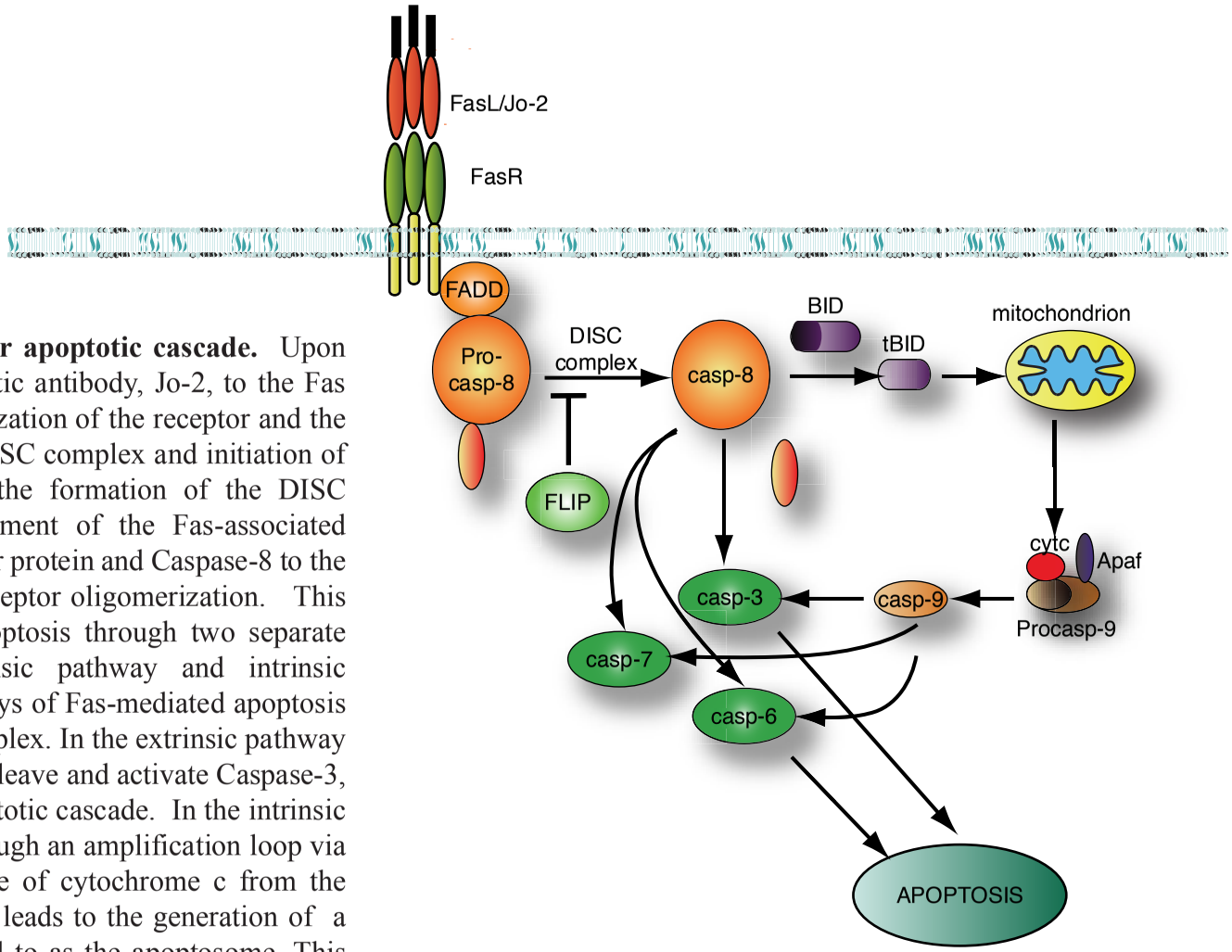


Figure 1.5: The Fas Receptor apoptotic cascade. Upon binding of the FasL or agonistic antibody, Jo-2, to the Fas receptor leads to the oligomerization of the receptor and the assembly of a multi-protein DISC complex and initiation of apoptosis. The first step in the formation of the DISC complex involves the recruitment of the Fas-associated Death Domain (FADD) adaptor protein and Caspase-8 to the Fas receptor in response to receptor oligomerization. This leads to the activation of apoptosis through two separate pathways termed the extrinsic pathway and intrinsic pathway. Two separate pathways of Fas-mediated apoptosis are generated at the DISC complex. In the extrinsic pathway active Caspase-8 can directly cleave and activate Caspase-3, -6, and -7 and initiate the apoptotic cascade. In the intrinsic pathway, apoptosis occurs through an amplification loop via the mitochondria. The release of cytochrome c from the mitochondria into the cytosol, leads to the generation of a multi-protein complex referred to as the apoptosome. This permits the recruitment and activation of Caspase-9, which subsequently cleaves effector caspases and initiates apoptosis.

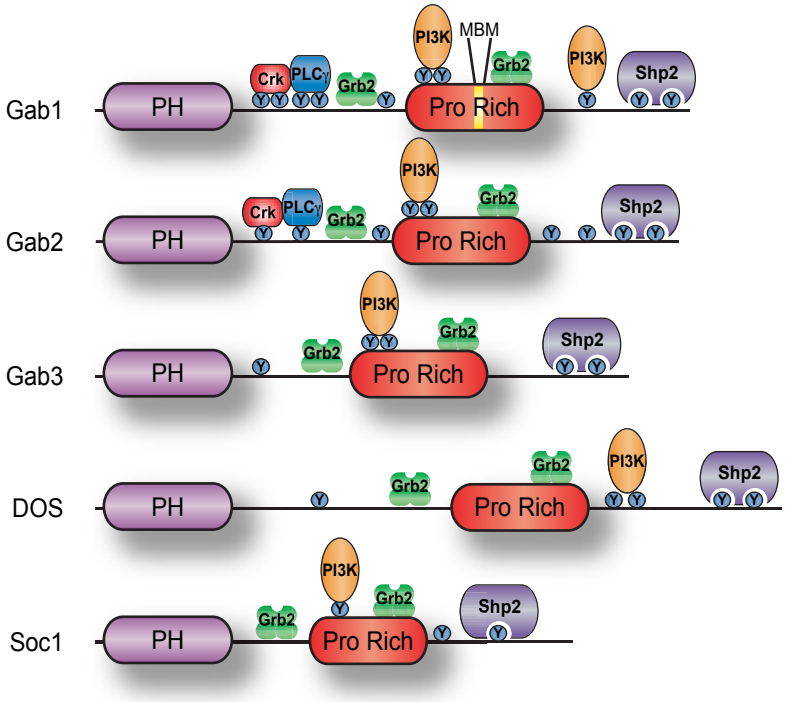


Figure 1.6: Gab family of adaptor/scaffold proteins. The Gab family of adaptor/scaffold proteins is composed of 5 family members. To date mammalian Gab1, Gab2 and Gab3, DOS (Daughter of Sevenless) in *Drosophila melanogaster*, and SOC-1 (Suppressor of Clear-1) in *Caenorhabditis elegans* have been identified. They all contain an amino terminal PH domain and a central proline rich domains. Gab family members recruit a variety of signalling molecules which contain SH2 and/or SH3 domains to organize signaling platforms downstream of cell surface receptors.

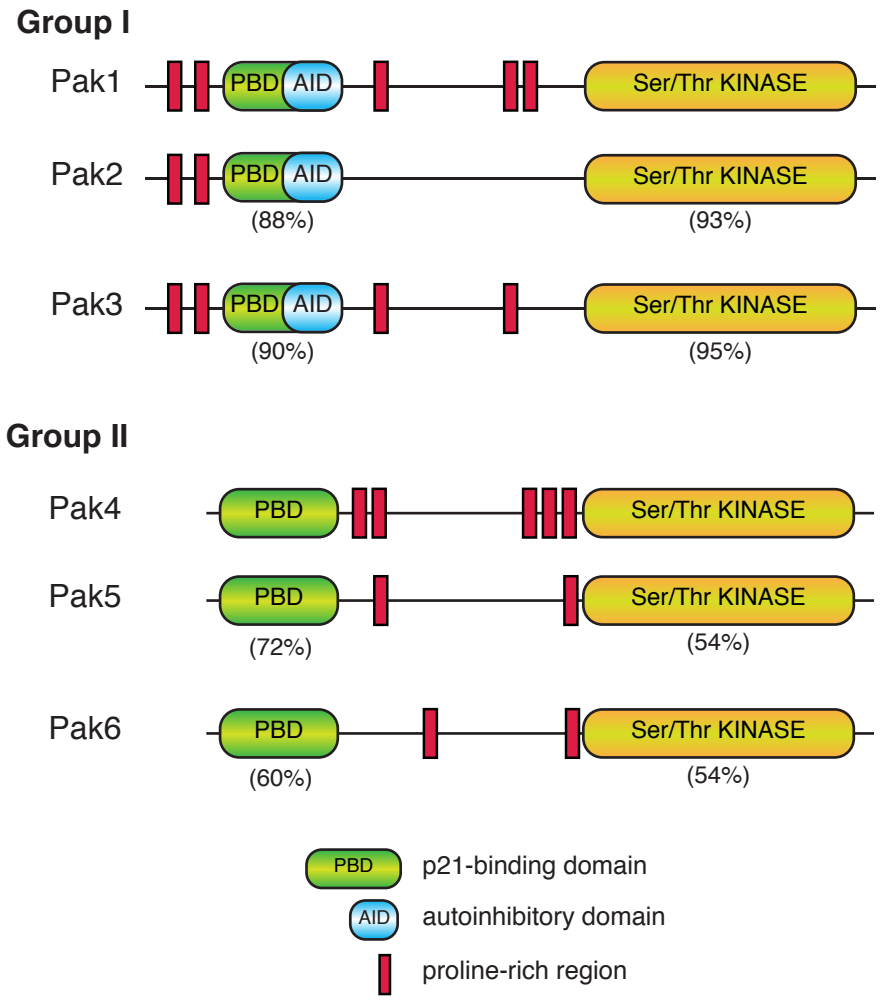


Figure 1.7: Pak family of Ser/Thr kinases. The Pak family of Ser/Thr kinases is composed of 6 family members that can be divided into two additional groups based on biochemical and structural features. Group I and Group II Paks contain a PBD and kinase domain, however, only Group I Paks possess a PBD that overlaps with an AID. All six Paks have conserved proline-rich motifs, where in the case of group I Paks, interactions with the SH3-domain-containing proteins Nck, Grb2 and PIX have been demonstrated. However, similar binding partners have not been identified for Group II Paks. Within brackets, the percentage identities for PBDs and kinase domains are indicated, relative to Pak1 for Group I and relative to Pak4 for Group II.

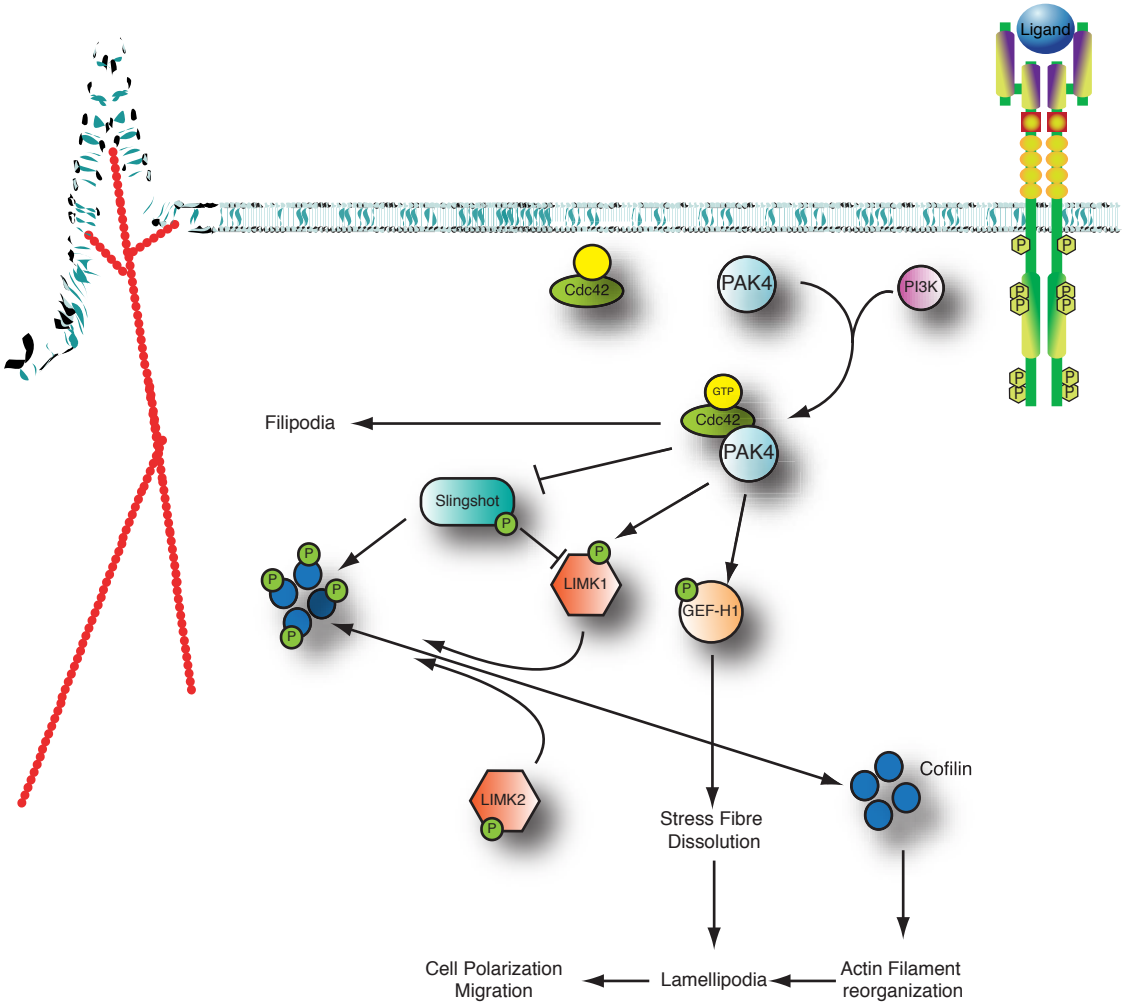


Figure 1.8: Pak4-mediated signalling pathways. Pak4 is a Ser/Thr kinase that is a downstream effector of Cdc42. Biological outcomes regulated by Pak4 include actin/cytoskeletal reorganization, filopodia formation, and migration.

Chapter 2

2 Protein Tyrosine Phosphatase 1B Deficiency Protects Against Fas-Induced Hepatic Failure

*These authors contributed equally to this work.

2.1 PREFACE

Activation of the Met receptor tyrosine kinase (RTK), induced by the binding of its ligand, hepatocytes growth factor (HGF), induces receptor dimerization, transphosphorylation of the twin tyrosine residues 1234 and 1235 in the kinase domain and activation of cellular pathways via the recruitment of multiple signalling proteins. Numerous studies have addressed mechanisms that activate the Met RTK and modulate the recruitment and activation of downstream signalling pathways, little is known regarding the attenuation of this signal. Attenuation of an RTK signal involves dephosphorylation of tyrosine residues by protein tyrosine phosphatases (PTPs), internalization and sequestration of the RTK into multivesicular bodies, RTK

ubiquitination and degradation in the lysosomes. Met receptor activation induces its ubiquitination, internalization and lysosomal degradation via the recruitment of the E3 ubiquitin ligase Cbl. However, the impact of PTPs in the downregulation of Met receptor signaling remains poorly understood. Protein tyrosine phosphatase 1B (PTP1B), a non-receptor tyrosine phosphatase, has been implicated in the modulation of multiple cytokine and growth factor-activated signaling pathways, as demonstrated by its ability to dephosphorylate growth factor receptors, including the insulin, IGF-1, EGF, PDGF, and erythropoietin receptors. PTP1B attenuates insulin receptor signaling via dephosphorylation of twin tyrosines present in its catalytic domain. The Met receptor possesses twin tyrosine residues within its kinase domain, supporting a potential role for PTP1B in the attenuation of Met receptor tyrosine kinase signalling.

The HGF/Met signaling axis plays an important role in liver regeneration, repair and tissue remodeling. HGF was originally identified as a potent mitogenic factor for hepatocytes and conditional Met-liver knock-out mice are sensitive to liver damage induced by heptotoxins and partial hepatechtomy. To examine the impact of PTP1B on Met-dependent activation, in a physiological context, we examined the consequence of hepatic liver damage in a PTP1B-null mice background. Our rational for this study was that WT mice would be sensitive to liver damage and KO mice, due to an increase in Met signalling, would be resistant to liver damage. This chapter addresses the importance of PTP1B as an important regulator of RTK signaling and demonstrates that Met receptor kinase activation is critical in the protection against Fas-induced hepatic failure in PTP1B-null mice.

2.2 ABSTRACT

Genetic disruption of protein tyrosine phosphatase 1B (PTP1B) in mice leads to increased insulin sensitivity and resistance to weight gain. Although PTP1B has been implicated as a regulator of multiple signals, its function in other physiological responses *in vivo* is poorly understood. Here we demonstrate that PTP1B-null mice are resistant to Fas-induced liver damage and lethality, as evident by reduced hepatic apoptosis in PTP1B-null versus wild-type mice and reduced levels of circulating liver enzymes. Activation of pro-apoptotic caspases-8, -9, -3 and -6 was attenuated in livers from PTP1B-null mice following Fas receptor stimulation, although components of DISC were intact. Activation of anti-apoptotic regulators, such as the HGF/Met receptor tyrosine kinase, as well as Raf, ERK1/2, $FLIP_L$, and the NF-κB pathway, was elevated in response to Fas activation in livers from PTP1B-null mice. Using PTP1B-deficient primary hepatocytes, we show that resistance to Fas-mediated apoptosis is cell autonomous and that signals involving the Met, ERK1/2 and NF-κB pathways are required for cytoprotection. This study identifies a previously unknown physiological role for PTP1B in Fas-mediated liver damage, and points to PTP1B as a potential therapeutic target against hepatotoxic agents.

2.3 ABBREVIATIONS

ALF	acute liver failure
ALT	alanine aminotransferase
AST	aspartate transaminase
DISC	Death-inducing signaling complex
Erk	Extracellular regulated Kinase
FasR	Fas receptor
HGF	Hepatocyte Growth Factor
KO	knockout
PTP1B	protein tyrosine phosphatase 1B
TCPTP	T cell protein tyrosine phosphatase
TGF	transforming growth factor
TUNEL	Terminal deoxynucleotide transferase-mediated dUDP nick-end labeling
WT	wild type

2.4 INTRODUCTION

The non-receptor tyrosine phosphatase PTP1B is the most widely studied prototype for the PTP superfamily and was first purified from human placenta (Tonks, Diltz et al. 1988). PTP1B is a ubiquitously expressed 50 kDa protein, possessing a N-terminal catalytic domain, followed by tandem proline-rich motifs (Liu, Hill et al. 1996; Tonks 2003). At its C-terminal, PTP1B contains a small hydrophobic ER-targeting sequence (Frangioni, Beahm et al. 1992). PTP1B has been implicated in the modulation of multiple cytokine and growth factor-activated signaling pathways, as demonstrated by its ability to dephosphorylate growth factor receptors, including the insulin, IGF-1 (Kenner, Anyanwu et al. 1996), EGF (Flint, Tiganis et al. 1997), PDGF (Liu and Chernoff 1997), erythropoetin (Cohen, Oren-Young et al. 2004) receptors, as well as non-receptor tyrosine kinases, Src (Cheng, Bal et al. 2001), p210Bcr-Abl (LaMontagne, Flint et al. 1998), Jak2 and Tyk2 (Myers, Andersen et al. 2001).

The role of PTP1B in regulating the activity of the insulin receptor has been extensively studied. PTP1B-null mice show hyperphosphorylation of the insulin receptor in liver and muscle tissue upon stimulation with insulin (Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000). Moreover, these mice are resistant to weight gain caused by a high fat diet through the ability of PTP1B to regulate the leptin receptor, via dephosphorylation and subsequent termination of signaling from the downstream kinase Jak2 (Cheng, Uetani et al. 2002; Zabolotny, Bence-Hanulec et al. 2002). PTP1B has also been shown to interact with a number of SH3-domain-containing proteins through its proline-rich motifs, including p130Cas (Liu, Hill et al. 1996), p62Dok (Dube, Cheng et

al. 2004), β-catenin (Balsamo, Arregui et al. 1998), Grb2 and Crk (Liu, Hill et al. 1996), that are thought to target this phosphatase to distinct cellular protein complexes.

Although PTP1B has been implicated as a regulator of diabetes and obesity, its function in other physiological responses regulated *in vivo* by tyrosine kinase signaling is poorly understood. For example, multiple cytokine and growth factor signals, such as TNF-α, EGF, HGF, IGF-1 and insulin, as well as IL-6, play important roles as anti-apoptotic and growth- promoting signals in response to surgical or chemical liver damage (Michalopoulos and DeFrances 1997; Borowiak, Garratt et al. 2004; Huh, Factor et al. 2004; Taub 2004). Several of these are targets for PTP1B (Flint, Tiganis et al. 1997; Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000; Buckley, Cheng et al. 2002). Deregulation of the apoptotic program is pathophysiologically involved in liver disease (Rust and Gores 2000). Acute hepatic failure induced by hepatic toxins such as Fas ligand (FasL), or the agonistic Fas antibody (Jo-2) is characterized by uncontrolled apoptosis of hepatocytes mediated by the death receptor Fas (FasR) (Ogasawara, Watanabe-Fukunaga et al. 1993). Moreover, the Fas receptor is present on the plasma membrane of normal hepatocytes, and is over-expressed in some chronic hepatic diseases such as that induced by hepatitis B (Mochizuki, Hayashi et al. 1996) or hepatitis C virus (Ogasawara, Watanabe-Fukunaga et al. 1993; Hiramatsu, Hayashi et al. 1994). Binding of FasL to its cognate receptor causes hepatic apoptosis via caspase activation. This occurs through FasR-mediated assembly of the Death-Inducing Signaling Complex (DISC) (Barnhart, Alappat et al. 2003). The DISC complex consists of the Fas receptor, caspase-8, caspase-10 and FADD. Auto-proteolytic cleavage of caspase-8 is an early detectable event in Fas-induced apoptosis, leading to caspase-3 activation either in a mitochondria-independent

manner or via a mitochondria-dependent pathway which proceeds via Bid and caspase-9 (Dabeva and Shafritz 1993; Scaffidi, Fulda et al. 1998).

To determine if PTP1B plays a role in regulating signals involved in liver damage and survival, we determined the sensitivity of PTP1B-null and wild-type (WT) mouse hepatocytes to Fas activation, using two complementary experimental systems; whole liver *in vivo* and *in vitro* primary culture. Protection from cell death and fulminant hepatic failure was evident by reduced blood levels of liver enzymes as well as reduced apoptosis in liver sections of PTP1B-null versus wild-type mice after injection with agonistic Fas antibody (Jo-2). This identifies a previously unknown function for PTP1B in modulating hepatic apoptosis in response to Fas-induced liver damage.

2.5 EXPERIMENTAL PROCEDURES

Animals and antibodies

WT and PTP1B-null mice are a hybrid of 129S/v and BALB/c backgrounds (Elchebly, Payette et al. 1999). Female mice (4-6 weeks) were used for experiments. Antibodies were obtained as follows: monoclonal anti-Jo-2, anti-c-Raf, anti-phosphotyrosine (PY20) and polyclonal anti-EGFR (BD Biosciences, San Diego, California); polyclonal anti-FLIP (Axxora LLC, San Diego, California), polyclonal anti-PTP1B antibody (Upstate Biotechnology, Lake Placid, NY); mouse monoclonal anti-Met, polyclonal anti-Fas, anti-IκBα, anti-insulin receptor and anti-IGF1 receptor (Santa Cruz Biotechnology, Santa Cruz, California); polyclonal anti-caspase-9 and anti-Caspase-8 (Stressgen, Victoria, BC, Canada); polyclonal anti-caspase-3, anti-caspase-6, anti-ERK1/2 antibody and anti-phospho-ERK1/2 $pThr^{202}/pY^{204}$ antibody (Cell Signaling Technology, Beverly, MA); rabbit polyclonal anti-c-Raf $pYpY^{340/341}$ antibody and polyclonal pIR/IGF1R$pYpYpY^{1158/1162/1163}$ (Biosource, Camarillo, California).

Injection of Jo-2 and histology

WT and PTP1B-null mice were injected intraperitoneally with 0.3 μg/g body weight of Jo-2 in 200 μl PBS. After 6 hours, mice were sacrificed and liver tissue recovered by dissection. Livers were fixed by immersion in 10% buffered formalin and embedded in paraffin. 4 μm sections were cut and stained with haematoxylin and eosin. All mouse manipulations were carried out in accordance with McGill University animal care guidelines.

Serum ALT and AST activity

Wild-type and PTP1B-null mice were injected intraperitoneally as above. After 4 or 6 hours, mice were anesthetized and exsanguinated by cardiac puncture and serum collected. Liver damage was quantitated by measuring serum ALT and serum AST levels using ALT IFCC and AST IFCC modified reagent without pyridoxal phosphate (Roche Diagnostics, Canada). Data were acquired using a Hitachi 911 analyzer.

Immunoblotting

Livers were homogenized using a Mixer Mill MM301 (Glen Mills Inc., Clifton, NJ) in 1% Triton Lysis Buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, pH 8.0, 1.5 mM $MgCl_2$, 10% Glycerol, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF, 1 mM Na_3VO_4). Homogenates were centrifuged at 13000 rpm to remove debris. Protein extracts were resolved on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Hybond, Amersham) and probed with antibodies as described, followed by appropriate horseradish-peroxidase-conjugated secondary antibodies. All immunoblots were visualized by enhanced chemiluminescence (Amersham).

Hepatocyte preparation, culture and flow cytometry

Hepatocytes were isolated according to a modified version of the two-step collagenase method originally developed for rats (Seglen 1976; Deschenes, Valet et al. 1980). Mouse livers were perfused with a 25 mM Ca^{2+}-free HEPES buffer, pH 7.5, containing insulin (0.5 µg/ml) and EGTA (0.5 mM), followed by DMEM/F12 modified medium containing collagenase (0.2 Wünsch U/ml) and Ca^{2+} (5 mmol/liter). 0.5 x 10^6 cells/cm^2 viable hepatocytes were plated on attachment media (DMEM/F12 with selenious acid (5 µg/l),

insulin (5 mg/l), transferrin (5 mg/l), and gentamicin (50 μg/ml) on fibronectin-coated dishes. Post-attachment, dexamethasone (10^{-7} M) and EGF (20 ng/ml) were added to the culture medium. For cell surface Fas receptor staining, hepatocyte cell suspensions were incubated with mouse Fc block (CD16/CD32) (BD Biosciences) and Fas-specific anti-phycoerythrin antibody (BD PharMingen, San Diego, California). CD62L hamster IgG2λ (PharMingen, San Diego, California) conjugated to phycoerythrin was used as an isotype control. Data were acquired using a FACS Scan flow cytometer (Becton Dickinson, San Jose, California) and analyzed with CellQuest software (Becton Dickinson, San Jose, California).

Thymocyte preparation and viability assay

Thymuses were removed aseptically from WT and PTP1B-null mice and single cells suspensions were prepared by gently teasing through a 70 μM nylon mesh. Thymocytes ($0.15x10^6$ cells/well) were cultured in 96-well flat-bottom plates in complete RPMI medium (with glutamine, β-mercaptoethanol and Gentamycin) supplemented with 10% FBS and treated with Jo-2 (0.0625 – 1 μg/ml) at 37°C and 5% CO_2 in a final volume of 100 μl for 6h. Cell viability was measured using the colorimetric WST-1 assay (Roche) as per the manufacturer's instructions.

Assessment of apoptosis

TUNEL assays- Livers were fixed in 10% buffered formalin, paraffin embedded, sectioned (4 μm), stained to visualize apoptosis using the Apoptag Fluorescein *In Situ* Apoptosis Detection Kit (Serologicals Corporation, Norcross, GA). Tissue sections were

stained with anti-digoxigenin fluorescein conjugate antibody and counterstained with DAPI (0.5 mg/ml).

Annexin-V analysis

Hepatocytes were plated at a density of $0.5x10^6$ cells/cm^2 on fibronectin-coated dishes and pre-incubated with HGF (20 ng/ml) for 6 hours and/or UO126 (20 μM) (BD Transduction Laboratories, Mississauga, ON, Canada), NF-κB inhibitor peptide (SN50) (50 μg/ml) and inactive control peptide (SN50M) (50 μg/ml) (Calbiochem, La Jolla, California), Genistein (50 μM) (Sigma-Aldrich) or PHA-665752 (0.1 μM) (Pfizer Pharmaceuticals) for 1 hr prior to treatment with Jo-2 antibody (0.5 μg/ml), FasL (10 ng/ml) (Sigma-Aldrich), or TNF-α (10 ng/ml) (as indicated in figure legend) for 4 hr. TNF-α treatment was carried out in the absence or presence of Actinomycin D (10 μg/ml). Annexin-V staining (Roche) was performed following the manufacturer's instructions. Annexin-V positive cells were visualized by fluorescence microscopy. Apoptosis observed in untreated hepatocytes was taken as baseline and subtracted from the treatment group.

2.6 RESULTS

2.6.1 PTP1B-null mice are protected against Fas-induced liver failure

Multiple cytokine and growth factor signals that play important roles as anti-apoptotic and growth- promoting signals in response to surgical or chemical liver damage (Michalopoulos and DeFrances 1997; Borowiak, Garratt et al. 2004; Huh, Factor et al. 2004; Taub 2004) are known targets for PTP1B (Flint, Tiganis et al. 1997; Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000). We therefore investigated the role of PTP1B in protection against liver damage. To induce acute liver failure, PTP1B-null and WT mice were intraperitoneally injected with a lethal dose of the FasR-specific antibody Jo-2 (0.3 μg/g of body weight). The majority of PTP1B-null mice showed no overt signs of clinical compromise, loss of appetite, or decreased activity after injection with Jo-2, and were resistant to the lethal effect of the Fas-specific antibody (75%, Table I). In contrast, 84% of their WT counterparts presented with signs of clinical compromise, including tachypnea, shallow breathing and prostration indicative of liver failure, and were sacrificed (Table 1). Livers from these WT mice turned dark red in color following injection of Jo-2, which is indicative of liver hemorrhage, whereas livers from PTP1B-null mice did not show this phenotype (Figure 2.1A). Histological analysis of livers from the Jo-2 injected WT mice that showed distress revealed parenchymal necrosis, hemorrhage and hepatocyte apoptosis, whereas livers from the majority of PTP1B-null mice showed no significant histologic pathological features (Figure 2.1B). As a measure of apoptosis, DNA fragmentation, a hallmark of apoptotic cells, was quantified by the incorporation of anti-digoxigenin-fluorescein in liver sections (TUNEL staining). Liver sections from Jo-2 injected WT mice showed a significant number of positive TUNEL

staining cells. In contrast, few positive-staining apoptotic cells were observed in sections from either saline-injected controls, or PTP1B-null mice injected with Jo-2 (Figure 2.1C and 2.1D). However, livers from the PTP1B-null mice that showed distress in response to Jo-2, (25%, Table 1), showed parenchymal necrosis, and hepatocyte apoptosis to similar levels as WT animals (Supplemental Figure 2.1A and 2.1B). Blood samples were collected from WT and PTP1B-null mice at different time points (4h and 6h) following Jo-2 injection, and assayed for levels of two circulating liver enzymes, alanine aminotransferase (ALT) and aspartate transaminase (AST), elevated levels of which are indicative of liver damage. Levels of ALT and AST were significantly elevated in WT mice 6 hours post Jo-2 treatment (Figure 2.1E). In contrast, ALT and AST levels were near baseline in PTP1B-null mice in response to Jo-2 6 hours post-injection (Figure 2.1E), and remained at these levels even when assessed at 24 hours (data not shown). Taken together, our data demonstrate that PTP1B-null mice are resistant to Fas- mediated liver damage.

2.6.2 Absence of caspase cleavage in livers from Fas-treated PTP1B-null mice

The current model of Fas engagement suggests that the Fas receptor, upon binding of FasL, recruits FADD, followed by caspase-8. Formation of this DISC complex activates caspase-8, thus initiating the caspase cascade leading to the activation of downstream caspase-9, caspase-7, caspase-6 and caspase-3 (Barnhart, Alappat et al. 2003). In order to determine the step in the apoptotic signaling cascade that is blocked due to loss of PTP1B, we studied the presence of activated caspases in liver lysates from WT and PTP1B-null mice post-Jo-2 treatment. Consistent with our histological data (Figure 2.1B-2.1D), cleavage of caspases -8, -9, -3 and -6, normally activated following

FasR oligomerization (Strasser, O'Connor et al. 2000), were detected in liver extracts from WT mice 6 hr post Jo-2 treatment, but was significantly abrogated in PTP1B-null mice (Figure 2A), even though FasR levels on the hepatocyte cell surface are similar in WT and PTP1B-null mice (Figure 2.2A and 2.2B). Since activation of caspase-8 is one of the first events following activation of the Fas receptor (Strasser, O'Connor et al. 2000), our data demonstrate that one of the earliest events in Fas-induced apoptosis is abrogated in PTP1B-null mice.

2.6.3 Induction of signals in livers from Fas-treated PTP1B-null mice

In liver development and T cells, resistance to apoptosis induced by death receptors is tightly regulated by genes activated by the transcription factor NF-κB (Beg, Sha et al. 1995; Liu, Hsu et al. 1996; Van Antwerp, Martin et al. 1996; Wajant, Pfizenmaier et al. 2003). An investigation of proteins involved in the NF-κB pathway in liver extracts revealed that levels of IκBα, the inhibitor of NF-κB signaling, were decreased in both Jo-2 treated PTP1B as well as WT mice (Figure 2.3). However, the decrease in IκBα levels was significantly greater in PTP1B-null mice when compared to WT (Figure 2.3), indicating greater activation of this pathway in PTP1B-nulll mice. Consistent with this, levels of $FLIP_L$, a downstream transcriptional target of NF-κB (Kreuz, Siegmund et al. 2001; Micheau, Lens et al. 2001) which can act as an inhibitor of caspase-8 activation by interacting with FADD and caspase-8 in the DISC complex (Schneider and Tschopp 2000), were elevated in PTP1B-null mice following Jo-2 treatment when compared with WT mice (Figure 2.3). In addition, MAPK enzymes (ERK1/2), which have been shown to promote survival in response to Fas (Aoudjit and Vuori 2001; Gilbert, Loranger et al. 2004), were activated in PTP1B-null mice in

response to Jo-2, but not in their WT counterparts, as assayed using anti-phospho-ERK antibodies (Figure 2.3). An upstream activator of ERK1/2, Raf kinase, was also activated, as determined using a phosphospecific antiserum, in Jo-2 treated PTP1B-null mice (Figure 2.3).

2.6.4 Hepatocytes from PTP1B-null mice are protected against FasL and TNF-α -induced damage, but thymocytes are not resistant to Fas-induced apoptosis

To establish if protection from apoptosis is cell autonomous, the sensitivity of primary hepatocytes isolated from PTP1B-null and WT mice to both Jo-2 and the physiological Fas ligand (FasL) was assessed quantitatively by counting apoptotic cells after staining for Annexin V, an early indicator of apoptosis. In support of our *in vivo* data, primary hepatocytes prepared from PTP1B-null mice were more resistant to Jo-2 or FasL induced apoptosis, when compared to hepatocytes prepared from WT mice. The number of apoptotic hepatocytes from WT mice was 25% and 30% in response to Jo-2 and FasL respectively, whereas PTP1B-null hepatocytes did not show an increase in apoptosis in response to these treatments (Figure 2.4A and 2.4B). In agreement with results obtained by Musallam et al. (Musallam, Ethier et al. 2001), hepatocytes from WT BALB/c mice mice are sensitive to Jo-2 in the absence of Actinomycin D. To test if the resistance of hepatocytes from PTP1B-null mice was specific to Fas-induced apoptosis, hepatocytes from these mice were treated with TNF-α in the presence and absence of Actinomycin D. As previously shown (Leist, Gantner et al. 1994), hepatocytes from WT animals are sensitive to TNF-α induced apoptosis in the presence of Actinomycin D (Figure 2.4A). In contrast, hepatocytes isolated from PTP1B-null mice are resistant to

apoptosis in response to TNF-α (with Actinomycin D) compared to WT mice (28% apoptotic cells) (Figure 2.4A and 2.4B).

To examine if the resistance to apoptosis observed in PTP1B-null mice was tissue- or cell type- specific, we examined the sensitivity of thymocytes isolated from PTP1B-null and WT mice to Jo-2 induced apoptosis. In contrast to PTP1B-null hepatocytes, PTP1B-null thymocytes are not protected from Fas-induced apoptosis *in vitro*, whereas thymocytes derived from WT or PTP1B-null mice undergo apoptosis to a similar extent (Figure 2.4C). In support of this, thymuses from both PTP1B-null and WT mice were involuted following Jo-2 treatment, indicating occurrence of apoptosis (data not shown). Our data show that the resistance to Fas-mediated apoptosis observed in PTP1B-null mice is cell type-specific.

2.6.5 Protection against Fas-induced apoptosis requires activation of both the ERK and NF-κB signaling pathways

We have shown that ERK1/2 and NF-κB signaling pathways are activated in PTP1B-null mice in response to Fas engagement (Figure 2.3). To examine whether ERK1/2 or NF-κB activity is required for the observed resistance of PTP1B-null mice to Jo-2 induced liver apoptosis *in vitro*, hepatocytes were treated with a pharmacological MEK-1/2 inhibitor (UO126) (Favata, Horiuchi et al. 1998), or a peptide inhibitor of NF-κB (SN50) (Lin, Yao et al. 1995) prior to Jo-2 exposure. Pretreatment with UO126 or SN50, but not vehicle or control peptide (SN50M), increased apoptosis of hepatocytes prepared from PTP1B-null mice in response to Jo-2 to a level similar to that of WT hepatocytes (Figure 2.5A and 2.5C). Moreover, pretreatment of primary hepatocytes derived from WT mice with activators of the MEK-ERK1/2 pathway, such as the

hepatocyte growth factor (HGF), protected WT hepatocytes from Jo-2 induced cell death (Figure 2.5B). Notably, the protection mediated by growth factor pre-treatment was abrogated in the presence of UO126 (Figure 2.5A and 2.5B). Together, these data indicate a critical role for MEK-ERK signals, as well as NF-κB-dependent signals, in the protection of PTP1B-null hepatocytes from Jo-2 induced apoptosis.

2.6.6 Receptor tyrosine kinase phosphorylation increases in response to Fas-induced apoptosis in PTP1B-null mice

Modulation of receptor tyrosine kinases (RTKs) including the insulin, EGF, PDGF, IGF-1 and erythropoetin receptors by PTP1B is thought to proceed via the ability of PTP1B to dephosphorylate these substrates (Kenner, Anyanwu et al. 1996; Flint, Tiganis et al. 1997; Liu and Chernoff 1997; Cohen, Oren-Young et al. 2004). To examine if the resistance of PTP1B-null mice to Fas-induced apoptosis is dependent on tyrosine kinase activity, primary hepatocytes from PTP1B-null and WT mice were pretreated with Genestein, a general protein tyrosine kinase inhibitor (Akiyama and Ogawara 1991), prior to Jo-2-induced apoptosis (Figure 2.6A). Following Genestein pretreatment, hepatocytes from PTP1B-null mice undergo apoptosis to a similar level as hepatocytes from WT mice, demonstrating that a tyrosine kinase-dependent signal is required for protection. In addition to the insulin receptor, murine livers express the EGF receptor (Michalopoulos and DeFrances 1997), JAK2 (Gu, Dube et al. 2003), TYK2 (Ruff, Chen et al. 1997) and IGF-1 receptor (Scharf, Dombrowski et al. 2001), known substrates for PTP1B *in vivo* or *in vitro* (Myers, Andersen et al. 2001).

To establish if any of the known PTP1B substrates shows an increased level of phosphorylation, or if an unknown substrate for PTP1B is highly phosphorylated in

PTP1B-null mice in response to hepatic injury, lysates prepared from livers of Jo-2 treated PTP1B-null or WT mice were subjected to immunoprecipitation with antibodies raised against phosphotyrosine to isolate tyrosine phosphorylated proteins (Figure 2.6B). Consistent with previous studies (Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000; Buckley, Cheng et al. 2002) the known PTP1B substrates, IR and IGF1R, show elevated phosphorylation in Jo-2 treated PTP1B-null mice livers when compared to WT (Figure 2.6B). However, immunoblotting with an antiserum against phosphotyrosine revealed that the prominent phosphoprotein(s) retained in lysates from Jo-2 treated PTP1B-null, but not in WT mice, was ca. 150 kDa in size (Figure 2.7A). Using an antiserum raised against the HGF/Met receptor tyrosine kinase, a known hepatic phosphoprotein, we concluded the 150 kDa protein corresponds to Met, a potent physiological protector against Fas-induced liver damage (Huh, Factor et al. 2004; Schulze-Bergkamen, Brenner et al. 2004) (Figure 2.7B). The importance of the Met receptor in protection of hepatocytes is underlined by abrogation of the ability of PTP1B-null hepatocytes to resist Fas-induced apoptosis after pretreatment with the Met-specific inhibitor PHA-665752 (Christensen, Schreck et al. 2003) (Figure 2.7C).

2.7 DISCUSSION

The role of PTP1B in the regulation of insulin signaling, and as a regulator of diabetes and obesity has been studied extensively (Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000; Asante-Appiah and Kennedy 2003). However, its function in other physiological responses regulated *in vivo* by tyrosine kinase signaling is poorly understood. Many signaling pathways regulated by tyrosine phosphorylation have been shown to play a role during liver regeneration and in the protection of hepatocytes from apoptosis (Michalopoulos and DeFrances 1997; Kosai, Matsumoto et al. 1998; Huh, Factor et al. 2004; Taub 2004). To address this, we investigated the role of PTP1B in response to liver damage induced by Fas. Our data indicate that the absence of PTPIB markedly protects against Fas-induced massive liver apoptosis and fulminant hepatic failure *in vivo* (Table 2.1 and Figure 2.1). Interestingly, the 75% of PTP1B-null mice that showed no signs of distress in response to Jo-2 (Table 2.1) showed no overt signs of liver damage as indicated by no elevation in circulating AST and ALT liver enzyme levels (Figure 2.1E), or liver apoptotic index, as assessed through quantitation of TUNEL positive nuclei (Figure 2.1C and 2.1D). Together these results identify PTP1B as an important modulator of the Fas-mediated apoptotic response. These data are also consistent with a recent report that injection of adenovirus expressing PTP1B into PTP1B–null mice consistently caused higher elevation of circulating transaminase liver enzymes than adenovirus alone, indicating that elevated levels of PTP1B may potentiate adenovirus-induced hepatic damage (Haj, Zabolotny et al. 2005).

Binding of the FasL or cross-linking of Fas with agonistic antibodies, such as Jo-2, promotes the recruitment and subsequent activation by proteolytic cleavage of

procaspase-8, (Nagata 1999). In hepatocytes the formation of the Death-Inducing Signaling Complex (DISC), and activation of caspase-8, is followed by a cascade of events leading to activation of caspase-9 and -3 and apoptosis (Barnhart, Alappat et al. 2003). In livers from PTP1B-null mice, protection from apoptosis occurred at the level of the initiator caspase-8; cleavage of caspase-8, as well as downstream caspases-3 and -9, was abrogated in PTP1B-null, but not WT animals (Figure 2.2A). No difference was seen in the protein levels of different components of the DISC complex in saline-injected WT vs. PTP1B-null mice. The level of Fas receptor present on the cell surface (Figure 2.2A and 2.2B), as well as FADD and caspase-8 (Figure. 2.2A), were similar between WT and PTP1B-null animals (Figure 2.2B), indicating that components of DISC in this pathway are intact. Primary hepatocytes prepared from PTP1B-null but not WT mice were protected against apoptosis when treated with either Jo-2 or the Fas ligand (Figure 2.4), indicating that resistance to apoptosis is cell autonomous.

In livers from PTP1B-null mice, protection against Fas-mediated apoptosis correlated with an elevation and/or activation of numerous anti-apoptotic signaling proteins that regulate the caspase and apoptotic cascade. These include $FLIP_L$, ERK1/2 and NF-κB (Figure 2.3) (Beg, Sha et al. 1995; Liu, Hsu et al. 1996; Van Antwerp, Martin et al. 1996; Tran, Holmstrom et al. 2001; Wajant, Pfizenmaier et al. 2003; Sharp, Lawrence et al. 2005). NF-κB activation, as measured by IκBα degradation, was observed in both WT and PTP1B-null mice, but activation was greater in PTP1B-null mice in response to Jo-2 (Figure 2.3). This data is in agreement with previous experiments (Costelli, Aoki et al. 2003) where mice lacking TNFα receptors were resistant to Fas-mediated liver damage 5-8h post-Jo-2 injection, and showed greater NF-

κB activation in comparison to WT. $FLIP_L$ has been characterized as a specific inhibitor of death receptor mediated apoptosis, possibly through its ability to compete with caspase-8 for recruitment to the DISC (Schneider and Tschopp 2000), or alternatively $FLIP_L$ can also promote the activation of anti-apoptotic NF-κB and ERK signaling pathways (Kataoka, Budd et al. 2000; Kataoka and Tschopp 2004). Consistent with this, in intact livers of PTP1B-null mice, elevated $FLIP_L$ levels correlate with ERK1/2 activation, IκB degradation and protection from Fas-mediated apoptosis (Figure 2.3). In support of this, inhibition of ERK1/2 activation by a MEK1/2 inhibitor (UO126) or inhibition of NF-κB by a peptide inhibitor (SN50), sensitizes primary hepatocytes derived from PTP1B-null mice from Fas-mediated apoptosis (Figure 2.5A and 2.5C) suggesting a critical role for both ERK1/2 and NF-κB in this cytoprotection. Similarly, a decrease in $FLIP_L$ levels correlated with decreased ERK1/2 activation and increased sensitivity to Fas-mediated apoptosis in keratin 8-null hepatocytes (Gilbert, Loranger et al. 2004). Although the exact mechanism is yet unknown, ERK1/2-mediated protection against Fas can occur via inhibition of caspase-8 activation (Holmstrom, Schmitz et al. 2000; Tran, Holmstrom et al. 2001; Tran, Meinander et al. 2004).

The resistance of PTPIB-null hepatocytes to Fas-mediated apoptosis requires tyrosine kinase activity, as PTP1B-null hepatocytes are rendered susceptible to Fas-mediated apoptosis by pretreatment with Genestein, a general inhibitor of tyrosine kinases (Figure 2.6A). Although multiple tyrosine kinases are expressed in liver, (Michalopoulos and DeFrances 1997; Ruff, Chen et al. 1997; Scharf, Dombrowski et al. 2001; Gu, Dube et al. 2003) and some known substrates of PTP1B (IR and IGF-1R) show elevated levels of phosphotyrosine following Fas activation (Figure 2.6B), several lines of evidence

support a role for the HGF/Met receptor in the hepatoprotection of PTP1B-null mice (Kosai, Matsumoto et al. 1998; Huh, Factor et al. 2004). The Met receptor was the predominant phosphoprotein elevated in Fas-treated PTP1B-null mice when compared to control mice (Figure 2.7A and 2.7B). In addition, the Met receptor is a substrate for PTP1B *in vitro* (Sangwan et al., unpublished) and HGF is a potent hepatoprotective agent against Fas-mediated liver damage both *in vivo* (Kosai, Matsumoto et al. 1998; Suzuki, Hayashida et al. 2000) and in primary hepatocytes *in vitro* (Schulze-Bergkamen, Brenner et al. 2004). Hepatocytes from liver-specific Met knockout mice are hypersensitive to Fas induced apoptosis (Huh, Factor et al. 2004) and Met is essential for efficient liver regeneration (Borowiak, Garratt et al. 2004; Huh, Factor et al. 2004). Noteably, ERK1/2 activation, which is important for efficient liver regeneration, requires the activation of the Met receptor *in vivo* (Borowiak, Garratt et al. 2004), whereas in primary hepatocytes *in vitro*, Akt signals appeared predominant over ERK1/2 (Schulze-Bergkamen, Brenner et al. 2004). We failed to observe an elevation in phospho-Akt in livers from Jo-2 treated PTP1B-null mice. This may reflect a robust negative regulatory pathway for Akt activation in intact livers. Nevertheless, pretreatment of primary hepatocytes from WT mice with HGF protects these cells from Fas-mediated apoptosis (Figure 2.5B) and this protection is diminished by inhibiting ERK1/2 with UO126 (Figure 2.5B), supporting a requirement for Met for ERK1/2 activation during liver regeneration (Borowiak, Garratt et al. 2004).

Hence, the anti-apoptotic effect observed in primary hepatocytes derived from PTPIB-null mice is dependent on the activation of physiological modulators of liver regeneration, such as the Met receptor and anti-apoptotic pathways involving NF-κB and ERK1/2 (Figure 2.3 and Figure 2.5). Although the Fas receptor is highly expressed in

thymocytes and hepatocytes (Timmer, de Vries et al. 2002) and PTP1B is expressed in both cell types, resistance to Fas-induced apoptosis is limited to hepatocytes (Figure 2.4). Thymocytes from PTP1B-null mice did not differ in their response to Jo-2 when compared to thymocytes from WT mice (Figure 2.4C). The observed tissue selectivity may reflect differences in the expression of targets for PTP1B, such as the Met receptor, which is highly expressed in hepatocytes, but is only weakly expressed in thymocytes from adult mice (Tamura, Sugawara et al. 1998). Similarly, the sensitivity of 25% of PTP1B-null mice to Jo-2 induced liver damage may reflect differences in the levels of expression of targets for PTP1B, resulting in differences in the threshold of anti-apoptotic pathways activated, such as Erk 1 and 2 (data not shown).

Fibroblasts from PTP1B-null mice are also resistant to ER-mediated stress (Gu, Nguyen et al. 2004). In this process, decreased levels of phosphorylated JNK and p38 MAPK correlated with protection (Gu, Nguyen et al. 2004). We failed to observe any difference in the levels of JNK or p38 in livers of PTP1B-null mice compared to WT mice (data not shown). This may reflect the time at which livers were harvested for analysis, i.e. 6 hours post Jo-2 injection, by which time changes in JNK or p38 MAPK activity may not be detected. Alternatively, this may reflect differences between PTP1B substrates in primary hepatocytes and immortalized fibroblasts.

Fas-mediated apoptosis is an efficient process through which damaged hepatocytes are excluded from the liver (Kanzler and Galle 2000). The present results regarding the response of primary hepatocytes to Fas and TNF-α show that PTP1B-null hepatocytes are resistant to both apoptosis-inducing signals. Since the TNF-α receptor can induce apoptosis in the presence of Actinomycin D via activation of caspase-8 in a

manner similar to Fas (Ozoren and El-Deiry 2003), this suggests a common role for PTP1B as a positive modulator of both of these signals in primary hepatocytes. A similar inhibition of caspase induction and death receptor-induced liver damage was recently demonstrated for the compound suramin (Eichhorst, Krueger et al. 2004). Notably, suramin is an inhibitor of PTP1B (Zhang, Keng et al. 1998) at concentrations shown to provide protection to death receptor-mediated liver damage (Eichhorst, Krueger et al. 2004). Together these data suggest a potential role for PTPIB in suramin-regulated resistance to death receptor-induced apoptosis.

Interestingly, a recent report has identified a role for a PTP1B-related phosphatase, T cell protein tyrosine phosphatase (TCPTP), in the negative regulation of ERK1/2 activity downstream from the TNF receptor (TNFR1), through dephosphorylation and inactivation of the Src kinase (van Vliet, Bukczynska et al. 2005). In contrast, PTP1B has been proposed to activate Src in fibroblasts through dephosphorylation of a negative regulatory site on Src (Y527) (Cheng, Bal et al. 2001). Consistent with this, we observed elevated Src Y527 phosphorylation in livers from Fas-treated PTP1B-null mice when compared to WT mice (data not shown). Thus our data do not implicate Src in the mechanism of ERK1/2 activation in Fas-treated PTP1B-null mice.

PTP1B-null mice live and reproduce normally (Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000). When these animals are subjected to the stress of a high fat diet, they show protection against diabetes and obesity through increased phosphorylation of signaling components associated with the insulin, leptin and growth hormone receptors (Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000; Cheng, Uetani et al. 2002; Zabolotny, Bence-Hanulec et al. 2002; Gu, Dube et al. 2003). In response to Fas

activation, a known stress inducer, we have shown, in PTP1B-null mice but not WT mice, elevated tyrosine phosphorylation and downstream signaling of receptor tyrosine kinases involved in liver injury, namely the Met RTK. Notably, only subtle differences in these signals were observed in untreated animals, consistent with previous studies (Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000; Cheng, Uetani et al. 2002; Zabolotny, Bence-Hanulec et al. 2002). Hence, PTP1B acts as a potential rheostat to integrate cellular responses to extracellular stress. Our observation that the majority of PTP1B-null mice are protected against Fas-mediated liver damage suggests that pharmacological manipulation of PTP1B activity may constitute a viable therapeutic modality for treatment against hepatotoxins and liver damage.

2.8 ACKNOWLEDGEMENTS

We are grateful to Dr. George Vande Woude for HGF, Dr. J. Desbarats for helpful discussion, reagents and FACS analysis; members of the Park laboratory for helpful discussions; Dr. N. Marceau and Dr. A. Loranger (U. Laval, Quebec) for training in hepatocyte isolation; Anie Monast for help with mouse experiments and hepatocyte preparation. This research was supported by a fellowship to V.S. from Cancer Research Society, Inc. and Terry Fox Foundation (National Cancer Institute of Canada). G.P. is a recipient of the Canadian Institutes of Health Research (CIHR) Cancer Consortium award and A.C. and N.D. are recipients of a CIHR studentship and doctoral research award respectively. This work was supported by the Cancer Research Society, Inc., and CIHR Operating Grant (MOP-62887), to M.L.T and National Cancer Institute of Canada Grant (011392) and CIHR Operating Grant (MOP-11545) to M.P. M.L.T. is a Scientist and M.P. is a Senior Scientist of the CIHR. We would like to thank Pfizer Phamaceuticals for the gift of the Met inhibitor, PHA-665752.

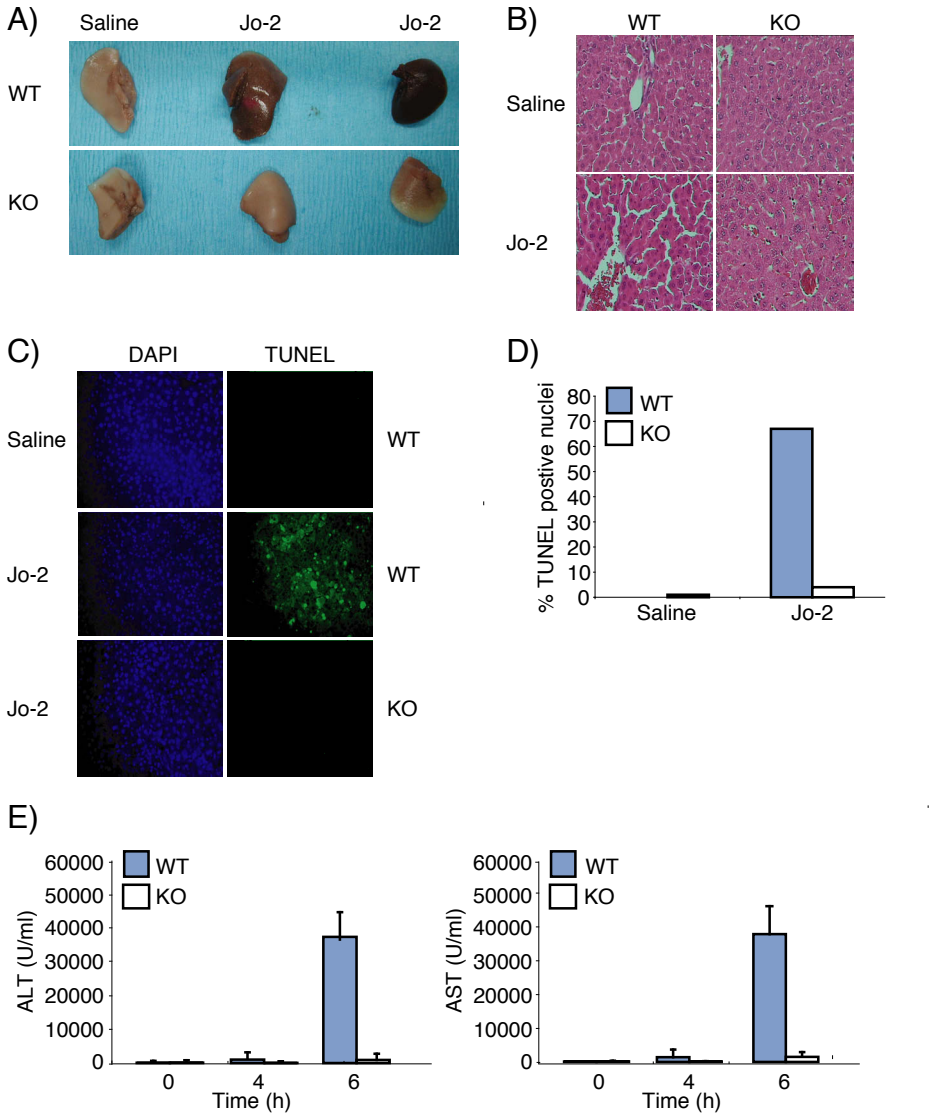


Figure 2.1: PTP1B deficiency protects against Fas-induced liver apoptosis. Mice were intraperitoneally injected with 0.3 µg/g body weight of the Fas receptor-specific antibody, Jo-2 and sacrificed upon signs of distress. **A-E)** Analysis of liver tissue from WT and PTP1B-null mice, removed 6h post-intraperitoneal injection, of either saline or Jo-2 (0.3 µg/g body weight). **A)** Whole livers were fixed in 10% formalin and photographed. **B)** Paraffin sections of liver tissue stained with haemotoxylin and eosin. **C)** TUNEL staining of sections from liver tissue. Tissue sections were stained with anti-digoxigenin fluorescein conjugate antibody and counterstained with DAPI (0.5 mg/ml). **D)** Percentage of TUNEL positive nuclei as measured by TUNEL staining. **E)** Levels of the liver enzymes ALT and AST in WT compared to Jo-2 injected PTP1B-null mice, indicating increased liver damage in WT mice. Error bars in (E) represent s.e.m. *KO*, knockout.

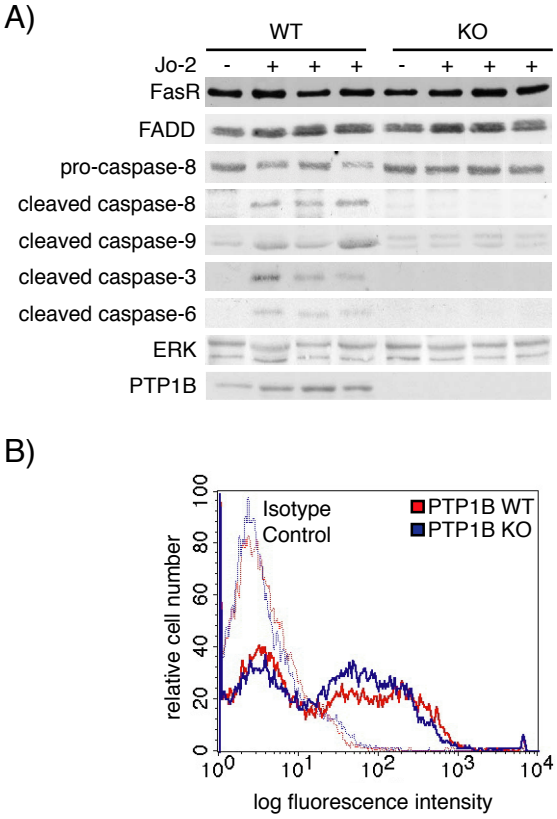


Figure 2.2: Activation of pro-apoptotic pathways in livers from WT and PTP1B-null mice following Jo-2 injections. A) Immunoblot analysis of total liver lysates (20 μg protein) prepared from liver tissue from WT and PTP1B-null mice following injection, of either saline or Jo-2 (0.3 μg/g body weight) harvested 6h post-injection using antisera for: FasR, FADD, pro-caspase-8, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3, and cleaved caspase-6. Each lane represents an independently injected mouse. **B)** FACS analysis comparing levels of Fas receptor at the cell surface of hepatocytes from WT and PTP1B-null mice. *KO*, knockout.

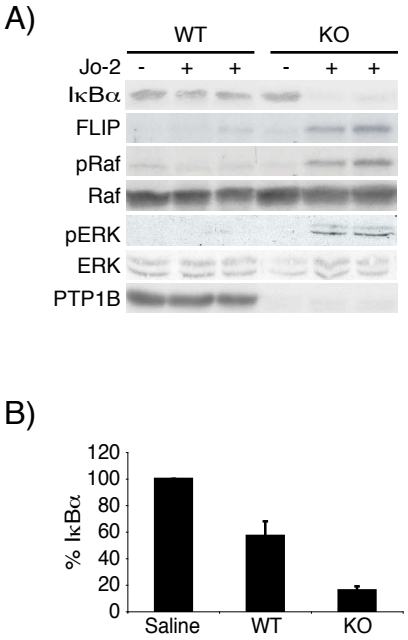


Figure 2.3: Activation of anti-apoptotic pathways in livers from WT and PTP1B-null mice following Jo-2 injections. A) Immunoblot analysis of total liver lysates (20 μ g protein) prepared from liver tissue from WT and PTP1B-null mice following injection, of either saline or Jo-2 (0.3 μg/g body weight) harvested 6h post-injection using antisera for IκBα, FLIPL, c-Raf pYpY$^{340/341}$, c-Raf, phospho-ERK1/2 pThr^{202}/pY^{204}, ERK1/2 and PTP1B. **B)** Densitometric analysis of IκBα shown in A were measured and compared to the saline injected control mice. *KO*, knockout.

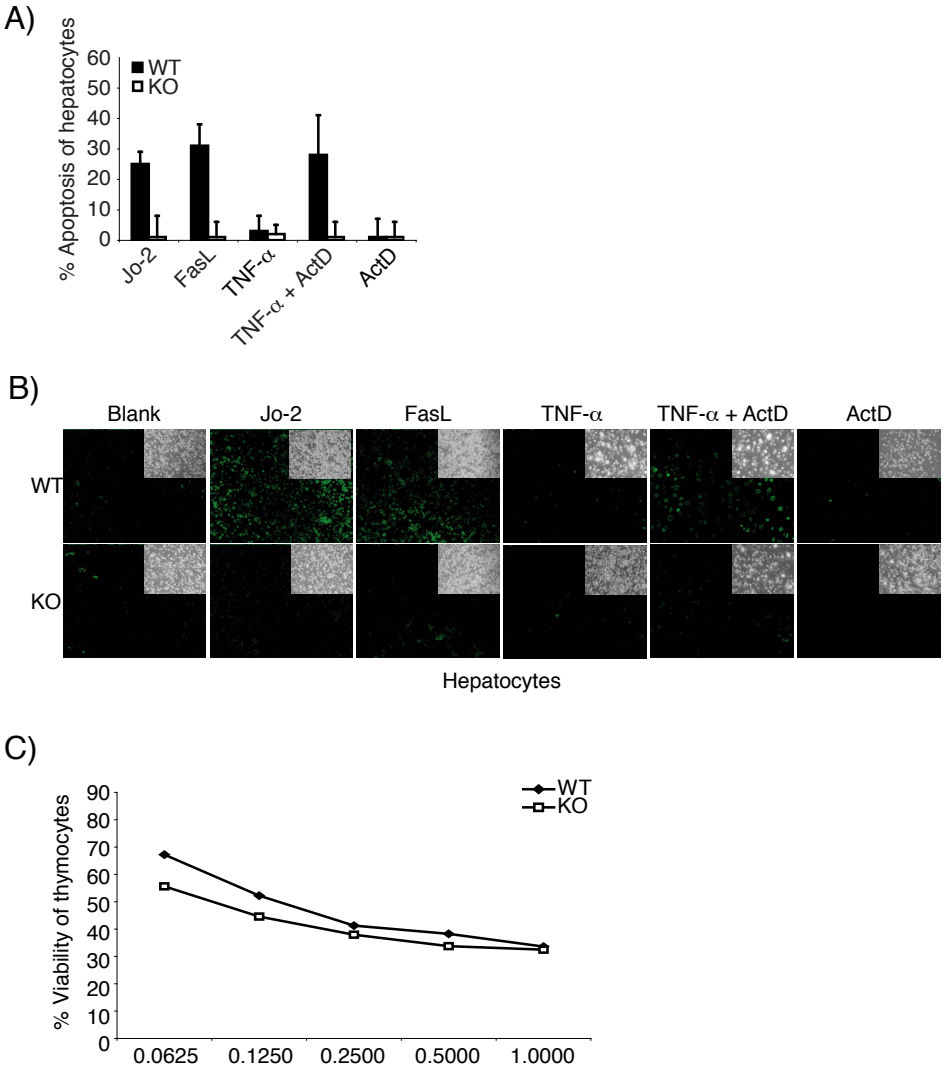


Figure 2.4: PTP1B-null hepatocytes, but not thymocytes, are protected against Jo-2 induced apoptosis. A-B) $0.5x10^6$ cells/cm^2 primary hepatocytes from WT and PTP1B-null mice were subjected to indicated treatments 24 h post-isolation. Apoptosis was assessed by staining with AnnexinV, followed by fluorescence microscopy. Apoptotic cells vs. total number of cells in 3 separate fields were counted. Each graph represents a minimum of 3 independent experiments. **A)** One day post-isolation, hepatocytes were subjected to Jo-2 antibody (0.5 μg/ml), FasL (10 ng/ml), TNF-α (10 ng/ml) or TNF-α (10 ng/ml) + Actinomycin D (10 μg/ml). **B)** Representative fluorescence images of Annexin V staining of hepatocytes following stimulation. **C)** Primary thymocytes ($0.15x10^6$ cells/well) isolated from WT and PTP1B-null mice plated in 96-well plates and exposed to indicated concentrations of Jo-2. Cell viability was measured using the colorimetric WST-1 assay (Roche). Percent viability is measured as a percentage of the untreated control. *KO*, knockout.

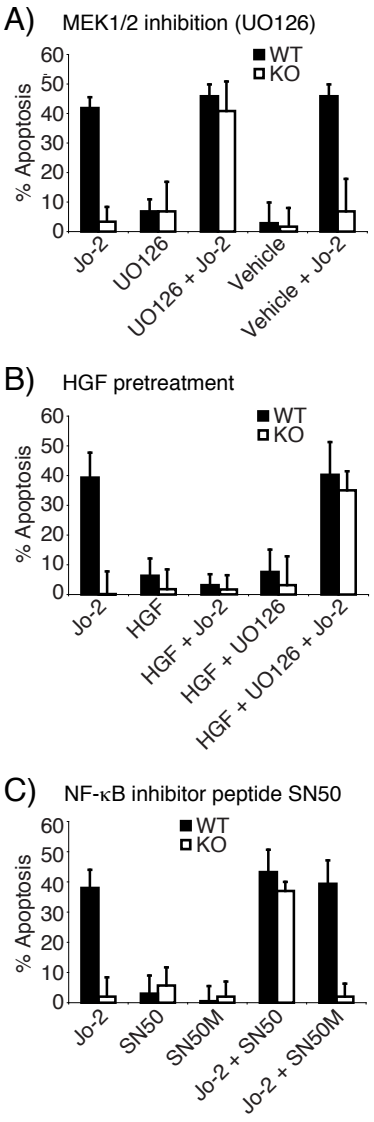


Figure 2.5: ERK1/2 and NF-κB activation is required to protect PTP1B-null hepatocytes against Jo-2 induced apoptosis. A-C) $0.5x10^6$ cells/cm^2 primary hepatocytes from WT and PTP1B-null mice were subjected to indicated treatments 24 h post-isolation. Apoptosis was assessed by staining with AnnexinV, followed by fluorescence microscopy. Apoptotic cells vs. total number of cells in 3 separate fields were counted. Each graph represents a minimum of 3 independent experiments. Primary hepatocytes from WT and PTP1B-null mice were stimulated with Jo-2 following pretreatment with : **A)** MEK inhibitor, UO126 (20 μM), **B)** Hepatocyte growth factor (HGF) (20 ng/ml) for ERK 1/2 activation, **C)** NF-κB inhibitor peptide SN50 (50 μg/ml) or control peptide SN50M (50 μg/ml). Error bars in A-C represent s.e.m.. *KO*, knockout.

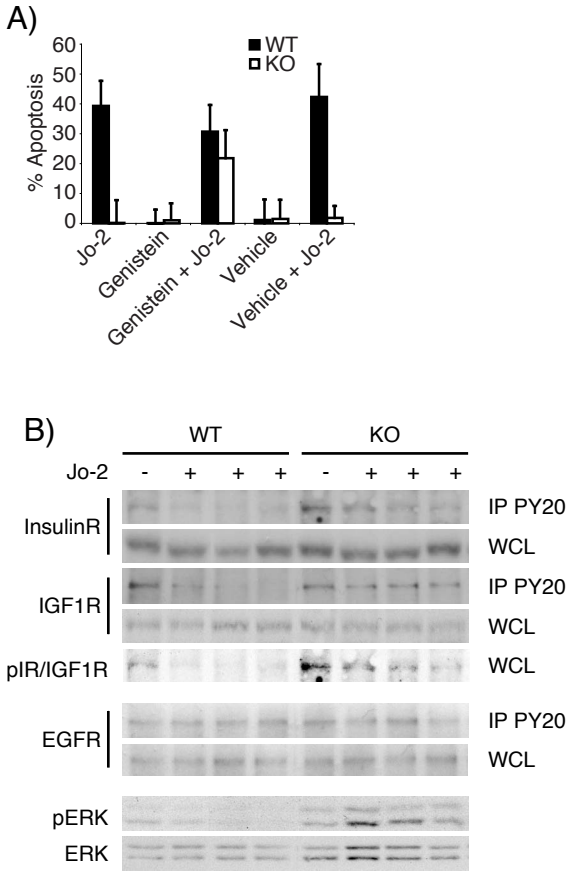


Figure 2.6: Tyrosine phosphorylation is required for protection against Jo-2 induced apoptosis. A) 24 h post-isolation, $0.5x10^6$ cells/cm^2 primary hepatocytes from WT and PTP1B-null mice were treated with the tyrosine kinase inhibitor, Genistein (50 μM) prior to Jo-2 treatment. Apoptosis was assessed as in Figure 4 and 5. Graph represents a minimum of 3 independent experiments. **B)** Phosphotyrosine complexes in livers from WT and PTP1B-null mice following Jo-2 treatment. Total liver lysate (3 mg) were immuprecipitated with anti-phosphotyrosine sera (PY20), separated by SDS PAGE and subjected to immunoblot analysis with sera against: Insulin receptor, IGF1 receptor and EGF receptor. Immunoblot analysis of whole cell lysate (WCL) was performed using antibodies against phospho-insulin receptor/IGF1R, Insulin receptor, EGFR, pERK and ERK. *KO*, knockout; *IP*, Immunoprecipitation.

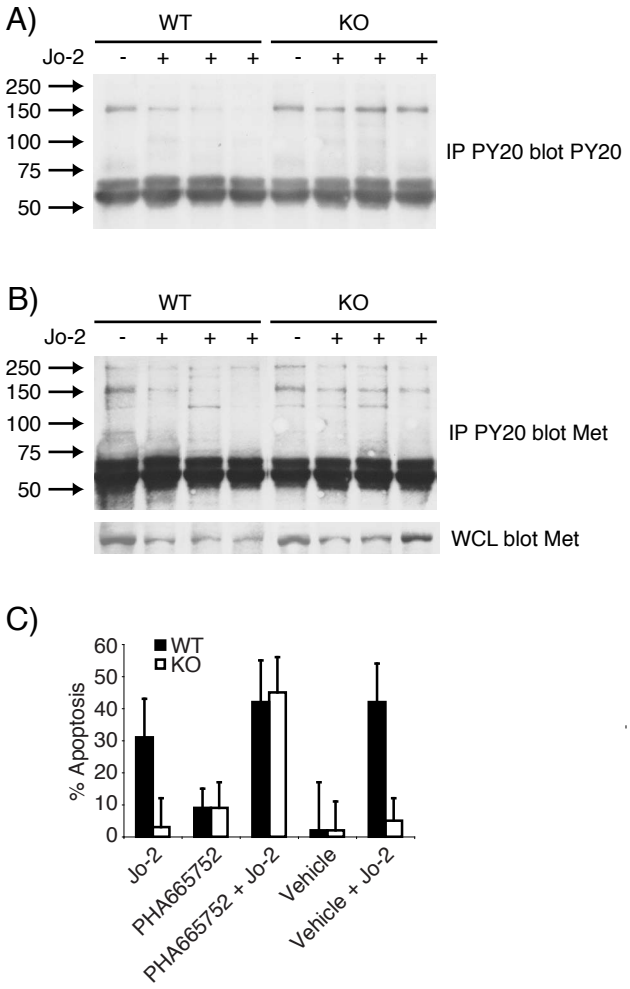
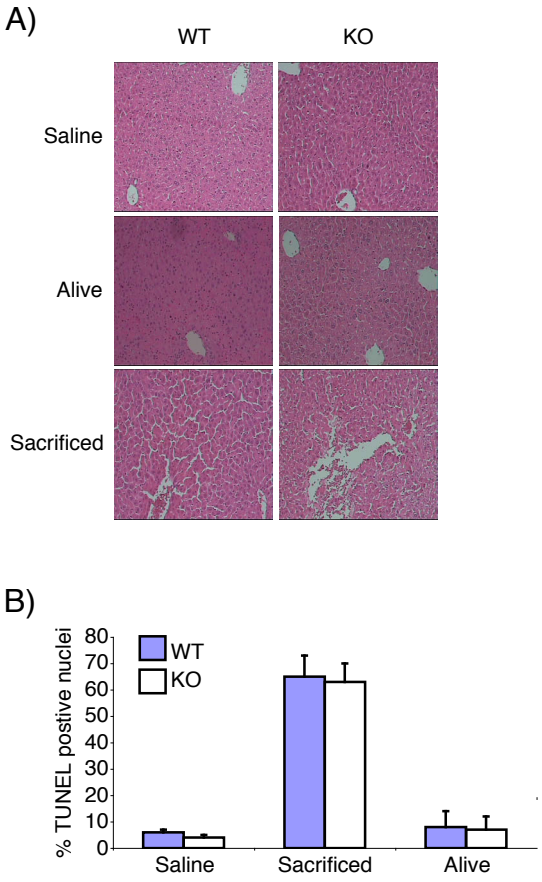


Figure 2.7: Met is the major phosphoprotein required for protection against Jo-2 induced liver apoptosis. A-B) Total liver lysate (3 mg) were immunoprecipitated with anti-phosphotyrosine sera (PY20), separated by SDS PAGE and subjected to immunoblot analysis with sera against: **A)** phosphotyrosine **B)** HGF/Met receptor. Immunoblot analysis of whole cell lysate (WCL) was performed using an antibody against HGF/Met. **C)** 24 h post-isolation, $0.5x10^6$ cells/cm^2 primary hepatocytes from WT and PTP1B-null mice were treated with the Met receptor tyrosine kinase inhibitor, PHA-665752 prior to Jo-2 treatment. Apoptosis was assessed as in Figure 2.4 and 2.5. Each graph represents a minimum of 3 independent experiments. *KO*, knockout; *IP*, Immunoprecipitation.

	Fas-specific antibody injection	
	Alive	Sacrificed[a]
PTP1B-WT	7/43 (16%)	36/43 (84%)
PTP1B-KO	24/32 (75%)	8/32 (25%)

[a] The mice were sacrificed within 6h upon showing signs of distress

Table 2.1: PTP1B deficiency prevents lethal effect of Fas-specific antibodies. All mice were intraperitoneally injected with 0.3μg/g body weight of the fas-specific antibody, Jo-2.



Supplemental Figure 2.1: Mice were intraperitoneally injected with 0.3 μg/g body weight of the Fas receptor-specific antibody, Jo-2 and sacrificed upon signs of distress. **A-B)** Analysis of liver tissue from WT and PTP1B-null mice, removed 6h post-intraperitoneal injection, of either saline or Jo-2 (0.3 μg/g body weight). **A)** Paraffin sections of liver tissue stained with haemotoxylin and eosin. **B)** Percentage of TUNEL positive nuclei as measured by TUNEL staining. *KO*, knockout.

Chapter 3

3 Pak4, a novel Gab1 binding partner, modulates cell migration and invasion by the Met receptor

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3.1 PREFACE

Activation and phosphorylation of Met initiates the recruitment of multiple signalling proteins. Proper regulation of this process is critical in maintaining a delicate balance between the positive and negative signals needed to maintain tissue homeostasis. In the previous chapter, I addressed the importance of dephosphorylation in regulating the activation of signals needed in the protection against Fas-induced hepatic failure. I identified that the Met receptor was major phosphorylated protein in PTP1B-null mice following Jo-2 induced liver damage and inhibition of the Met receptor activation was sufficient to ablate the protection seen in PTP1B-null hepatocytes. Therefore. dephosphorylation of Met is important in regulating the physiological activity of the receptor. The Met receptor is deregulated in many cancers resulting in aberrant activation of multiple signaling pathways. Therefore a greater understanding of signaling pathways

activated downstream from an activated Met receptor is necessary in furthering our understanding of Met receptor mediated biological responses.

One unique feature of Met receptor is presence of a multi-substrate binding site at the C-terminus that is responsible for recruiting the majority of signaling proteins mediating Met biological function. The principal protein recruited downstream of an activated Met receptor is the scaffold protein Gab1. Gab1 is a critical modulator of cell dispersal, invasion and epithelial morphogenesis downstream from the Met receptor. Since Gab1 acts as a scaffold integrating signals downstream from multiple RTKs that are deregulated in cancer, I propose that Gab1 is a key modulator of the invasive response downstream of the Met receptor. The functions of known proteins recruited and activated downstream from Gab have been shown to be involved in cellular migration, invasion, adhesion, apoptosis and proliferation. However, a thorough analysis of proteins associated to Gab1 following RTK activation has not been performed that would further support a role of Gab1 in the actin-cytoskeletal reorganization necessary in promoting morphogenesis, migration and invasion. To further our understanding of Gab1-dependent signals recruited downstream of the Met receptor, I undertook a proteomic approach to isolate novel-Gab1 associated binding partners (Appendix I). One protein identified from my analysis was the serine/threonine p21-activated kinase (Pak) 4. In this chapter, I characterize the novel association between Gab1 and Pak4 following Met receptor activation and demonstrate a physiological requirement for Gab1-Pak4 association. The data presented in this chapter identify Pak4 as a key integrator of epithelial cell dispersal and invasive morphogenic growth downstream from the Met receptor.

3.2 ABSTRACT

Hepatocyte growth factor (HGF), the ligand for the Met receptor tyrosine kinase, induces epithelial cell dispersal, invasion and morphogenesis, events that require remodeling of the actin cytoskeleton. The scaffold protein Gab1 is essential for these biological responses downstream from Met. We have identified p21-activated kinase 4 (Pak4) as a novel Gab1-interacting protein. We show that in response to HGF, Gab1 and Pak4 associate and co-localize at the cell periphery within lamellipodia. The association between Pak4 and Gab1 is dependent on Gab1 phosphorylation but independent of Pak4 kinase activity. The interaction is mediated through a region in Gab1, which displays no homology to known Gab1 interaction motifs and through the GEF-interacting domain of Pak4. In response to HGF, Gab1 and Pak4 synergize to enhance epithelial cell dispersal, migration and invasion whereas knockdown of Pak4 attenuates these responses. A Gab1 mutant unable to recruit Pak4 fails to promote epithelial cell dispersal and an invasive morphogenic program in response to HGF, demonstrating a physiological requirement for Gab1-Pak4 association. These data demonstrate a novel association between Gab1 and Pak4 and identify Pak4 as a key integrator of cell migration and invasive growth downstream from the Met receptor.

3.3 ABREVIATIONS

ASM	association motif
BSA	bovine serum albumin
Cdc42	Cell Division Cycle 42
Crk	CT10 Regulator of Kinase
CSF	Colony Stimulation Factor
DMEM	Dubelcos Modified Eagles Medium
EGFR	epidermal growth factor receptor
ErbB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
Erk	Extracellular regulated kinase
Gab1	Grb2-associated binding protein 1
Gab2	Grb2-associated binding protein 2
Gab3	Grb2-association binding protein 3
GEF-H1	guanine nucleotide exchange-H1
GFP	green fluorescent protein
GID	GEF-interacting domain
HA	hemagglutinin tag
HEK	human embryonic kidney
HGF	Hepatocyte growth factor
LIMK	LIM domain kinase
MDCK	Madin-Darby Canine Kidney
Myr	myristoylation

Pak4	p21-activated kinase 4
PBS	phosphate buffered saline
PFA	paraformaldehyde
PH	pleckstrin homology
PI3K	phosphatidylinositol-3-kinase
PIP3	phosphoinositide 3,4,5 tri-phosphate
PLCγ	phospholipase C gamma
Rac	ras-related C3 botulinum toxin substrate 1
Rho	ras homolog gene family
RTK	receptor tyrosine kinase
SH2	Src-homology 2
siRNA	short-interfering ribonucleic acid
TAP-tag	Tandem Affinity Purification-tag
WGA	wheat germ agglutinin
WT	wild type

3.4 INTRODUCTION

The dispersal of sheets of epithelia is a complex biological process that requires the coordinated function of numerous cellular proteins. One of the initial steps of cell motility involves the reorganization of the actin cytoskeleton and the initiation of a dynamic actin meshwork at the leading edge of the cell in ruffles or lamellipodia (Small and Resch 2005; Faix and Rottner 2006). The actin cytoskeleton is tightly controlled via multiple signaling enzymes which when deregulated can promote defects in the processes in cell invasion and motility and in oncogenic transformation.

The activation of the Met receptor tyrosine kinase (RTK), induced by the binding of its ligand, HGF, modulates epithelial cell proliferation, survival, scatter of epithelial colonies and invasion (Peschard and Park 2007). Epithelial cells stimulated with HGF undergo a dramatic remodeling of their actin cytoskeleton, which is required for branching morphogenesis, cell migration and invasion (Ridley, Comoglio et al. 1995; Royal, Lamarche-Vane et al. 2000). These biological processes downstream from the Met RTK are dependent on the scaffold protein Gab1, which is the major substrate/phosphoprotein recruited to Met (Weidner, Di Cesare et al. 1996; Nguyen, Holgado-Madruga et al. 1997; Maroun, Holgado-Madruga et al. 1999; Maroun, Moscatello et al. 1999; Lock, Maroun et al. 2002).

Gab1 is a member of a family of adaptor proteins that act as a scaffold downstream from a broad range of growth factor, cytokine, and antigen receptors, linking them to downstream intracellular signaling pathways through the assembly of multi-protein complexes (Holgado-Madruga, Emlet et al. 1996; Liu and Rohrschneider 2002; Gu and Neel 2003). Three mammalian Gab genes have been identified, Gab1, Gab2 and

Gab3 (Liu and Rohrschneider 2002; Gu and Neel 2003). Gab1, but not Gab2 or Gab3, is a critical modulator of cell dispersal, invasion and epithelial morphogenesis downstream from the Met receptor (Lock, Maroun et al. 2002). Activation of the Met RTK by HGF promotes recruitment and tyrosine phosphorylation of Gab1, which in turn provides docking sites for the recruitment of multiple SH2 domain containing signaling molecules, including the p85 subunit of PI(3)kinase (PI3K), PLCγ, the adapter protein Crk and the tyrosine phosphatase Shp2 (Maroun, Holgado-Madruga et al. 1999; Gual, Giordano et al. 2000; Maroun, Naujokas et al. 2000; Sakkab, Lewitzky et al. 2000; Lamorte, Royal et al. 2002). Structure function studies have shown that the association of Gab1 with Shp2, Crk and the PH domain of Gab1 are critical for Met–induced branching morphogenesis (Maroun, Holgado-Madruga et al. 1999; Maroun, Naujokas et al. 2000; Schaeper, Gehring et al. 2000; Lamorte, Rodrigues et al. 2002). Hence, Gab1 subcellular localization, as well as its ability to form signaling complexes, is critical for Met-dependent biological responses.

The HGF-Met signaling axis activates Rho GTPases that play key roles in regulating the organization of the actin cytoskeleton in mammalian cells (Ridley, Comoglio et al. 1995; Royal, Lamarche-Vane et al. 2000; Wells, Ahmed et al. 2005). Three members of this family, Cdc42, Rac, and Rho, induce the production of filopodia, lamellipodia, and stress fibers respectively. This occurs in a variety of cell types including epithelial cells and fibroblasts (Hall 2005). These proteins are modulated downstream from HGF and are required for dispersal of epithelial cells in response to HGF (Ridley, Comoglio et al. 1995; Royal, Lamarche-Vane et al. 2000). Activation of Rac by HGF is mediated in part through the recruitment of Crk to Gab1. Overexpression of Crk enhances lamellipodia formation and cell spreading in response to HGF and

consequently enhances cell motility and promotes epithelial dispersal (Lamorte, Royal et al. 2002; Lamorte, Rodrigues et al. 2003).

Members of the p21-associated kinase (Pak) family are major effectors of the RhoGTPases Cdc42 and Rac (Jaffer and Chernoff 2002) and function to induce reorganization of the actin cytoskeleton (Abo, Qu et al. 1998; Adam, Vadlamudi et al. 1998; Edwards, Sanders et al. 1999). Pak proteins are Ser/Thr kinases that promote the reorganization of the actin cytoskeleton in response to upstream signals (Sells, Knaus et al. 1997). The Pak family consists of six members, which are subdivided into two groups: Pak 1-3 (group I) and Pak 4-6 (group II) (Jaffer and Chernoff 2002). This distinction is based on sequence similarities, and also on the presence of an autoinhibitory region in group I, which is not present in group II Pak proteins. Pak1 and Pak4 are the best-studied members of each group and are widely expressed in a variety of tissue types (Jaffer and Chernoff 2002). Both Pak1 and Pak4 have been shown to be involved in adhesion to the cell-substratum and to link RhoGTPase signaling to the actin cytoskeleton (Abo, Qu et al. 1998; Edwards, Sanders et al. 1999; Dan, Kelly et al. 2001; Wells, Abo et al. 2002; Zhang, Li et al. 2002; Zhou and Kramer 2005). HGF activates Pak1 (Royal, Lamarche-Vane et al. 2000) and stimulates the translocation of Pak4 to the leading edge of the epithelial cells in a PI3K-dependent manner (Wells, Abo et al. 2002). However, the mechanism or molecular function for this HGF-dependent response is not understood.

In this study, we have identified Pak4 as a novel Gab1-interacting protein downstream from the Met receptor. We define a new Gab1-Pak4 interaction domain, and show that Pak4 is required for HGF-dependent epithelial cell dispersal, invasion and morphogenesis.

3.5 EXPERIMENTAL PROCEDURES

Antibodies

Polyclonal anti-Erk1/2, anti-phospho-Erk1/2 (pTpY202/204), anti-phospho-Pak4(Ser474)/Pak5(Ser602)/Pak6(Ser560), anti-phospho-Gab1(Tyr627) and monoclonal anti-myc (9E10) antibodies were purchased from Cell Signaling Technology (Mississauga, ON). Monoclonal anti-Pak4 and anti-PY20 antibodies were purchased from BD Biosciences (San Diego, CA). Polyclonal anti-Gab1 was purchased from Upstate Biotechnology Inc (Lake Placid, NY). Rabbit monoclonal anti-Pak1 antibody was purchased from Epitomics (Burlingame, CA). Polyclonal anti-GFP, wheat germ agglutinin (WGA) Alexa 555 and Alexa 488 conjugated phalloidin were purchased from Molecular Probes (Burlington, ON). Monoclonal anti-HA.11 antibody was purchased from Covance (Berkeley, CA). Polyclonal anti-actin, anti-cofilin and anti-phospho-cofilin (Ser3) antibodies were purchased from Sigma-Aldrich (St. Louis, MI). Polyclonal anti-V5 antibody was purchased from GeneTex (San Antonio, TX). Anti-Met antibody (148) was generated by immunizing rabbits with the carboxy-terminal 16 amino acids of the human Met sequence as previously described (Rodrigues, Naujokas et al. 1991).

DNA Constructs

Pak4 cDNA was purchased from Origene (Rockville, MD). Myc-tagged Pak4 deletion constructs were generated by PCR and cloned into the NheI and Not1 sites of pEBB (Supplemental Table 1). Gab1 deletion constructs containing an N-terminal HA-tag were generated by PCR amplification and cloning into BamHI and EcoRI sites of pcDNA1.1

(Supplemental Table 1). Pak4 and Gab1 amplifications were performed using High Fidelity Taq polymerase (ROCHE Diagnostics, Laval, QC) as per the manufacturer's instructions. To generate Gab1ΔPak4, SmaI/XmaI restriction sites were generated at amino acid position 116 and 234 of Gab1 pcDNA1.1 (Supplemental Table 1). Following restriction digest with XmaI (New England Biolabs, Pickering, ON) and agarose gel-purification (Qiagen, Mississauga, ON), the Gab1 pcDNA1.1 fragment was religated using T4 DNA ligase (New England Biolabs, Pickering, ON). The following constructs were described previously; pcDNA1.1 pcDNA1.1 HA-Gab1, pcDNA1.1 HA-Gab1ΔPH, GFP-Gab1, HA-Gab1ΔCrk, HA-Gab1ΔShp2, Gab1ΔPI3K and HA-Gab1ΔGrb2 (Maroun, Holgado-Madruga et al. 1999; Maroun, Moscatello et al. 1999; Lock, Royal et al. 2000; Lamorte, Rodrigues et al. 2002; Frigault, Naujokas et al. 2008).

Cell culture and DNA transfections

All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 μg/ml Gentamicin (Invitrogen, Carlsbad, CA). Madin-Darby Canine Kidney (MDCK) epithelial cells expressing GFP-Gab1 and/or mcherry-Pak4 were maintained in geneticin (400 μg/ml) (Invitrogen, Carlsbad, CA) and/or hygromycin (75 μg/ml) (Invitrogen, Carlsbad, CA). For transient expression of proteins, 1 x 10^6 HEK293 cells were seeded 24 hours (h) prior to performing transient transfections using Lipofectamine Plus (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Media was replaced 3h post-transfection, and cells lysed 24-48h post-transfection in 1% NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, pH 8.0, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM

phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4). The homogenates were centrifuged at 13,000 rpm for 15 minutes (min) to remove debris.

Immunoprecipitation and Immunoblotting

HEK293 cell protein lysates (500 µg) were used for each immunoprecipitation. Antibodies were allowed to bind for 1h at 4°C with gentle rocking, and 10µl of Protein A or Protein G sepharose beads were then added to collect immune complexes. Beads were washed 3 times in lysis buffer, resuspended in 20µl of Laemmlli sample buffer and boiled for 5 min. For lambda phosphatase treatment, prior to immunoprecipitation, lysates were incubated with 2000 units of lambda phosphatase (New England Biolabs, Pickering, ON) for 30 min at 30°C. Samples were resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with 3% bovine serum albumin (BSA) and probed with appropriate antibodies as described, followed by horseradish peroxidase-conjugated secondary antibodies. All immunoblots were visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

Confocal Immunofluorescence microscopy

HeLa, MDCK or MDCK expressing cells CSF-Met N1358H (G17) (Fournier, Kamikura et al. 1996) together with Gab1ΔPH (Maroun, Holgado-Madruga et al. 1999) or the rescue myristoylation-Gab1ΔPH (Maroun, Naujokas et al. 2003) (2×10^4) were seeded on glass coverslips (Bellco Glass Inc. Vineland, NJ, USA) in 24-well plates (Nalgene NUNC, Rochester, NY, USA) and were transfected with the indicated DNA using Lipofectamine Plus (Invitrogen, Carlsbad, CA) 16h post-plating, following

manufacturer's instructions. Cells were serum starved for 2h prior to HGF or CSF-1 treatment. Coverslips were washed once with PBS and then fixed with 2% paraformaldehyde (PFA) (Fisher Scientific) in PBS for 20 min. Coverslips were then washed four times in PBS and residual PFA was removed with three 5-minute washes in 100 mM glycine in PBS. Cells were permeabilized with 0.3% Triton X-100/PBS and blocked for 30 min in blocking buffer (5% BSA, 0.2% Triton X-100, 0.05% Tween 20, PBS). Coverslips were incubated with primary and secondary antibodies diluted in blocking buffer for 1 h and 40 min, respectively, at room temperature. Coverslips were mounted with Immumount (Thermo-Shandon, Pittsburgh, PA). Confocal images were taken using a Zeiss 510 Meta laser scanning confocal microscope (Carl Zeiss, Canada Ltd, Toronto, ON) with 100x objective. Image analysis was carried out using the LSM 5 image browser (Empix Imaging, Mississauga, ON). Confocal live cell imaging was performed with a Spinning disk confocal microscope from Quorum Technologies. Data from the spinning disk microscopy was analyzed using Volocity 4.1 software (Improvision, Coventry, England).

Scatter Assay

MDCK cells or stable cell lines expressing CSF-Met N1358H (G17) alone or expressing HA-Gab1 (WT3) (Maroun, Holgado-Madruga et al. 1999) or HA-Gab1ΔPak4 were seeded (5 x 10^3/well) overnight in 24-well dishes (Nalgene NUNC, Rochester, NY) and 24h later, HGF, 0.34 ng/ml or 1.35 ng/ml, defined as 0.25 units and 1.00 units, respectively (Stoker, Gherardi et al. 1987) or CSF-1 (50ng) (Zhu, Naujokas et al. 1994) was added for 24h. Phase contrast images were captured with a Zeiss Axiovision 135 microscope with a 10x objective (Carl Zeiss Canada Ltd, Toronto, ON).

Collagen Assay

The ability of MDCK cells to form branching tubules was assayed as previously described with a few modifications (Zhu, Naujokas et al. 1994). Briefly, 5 x 10^3 cells were suspended in 500 µl of Vitrogen 100 collagen solution (now commercially available as Pur-Col by INAMED Biomaterials, Fremont, CA), following manufacturer's protocols and layered over 350 µl of the collagen solution in a 24-well plate. Cells were maintained in Leibowitz medium containing 5% FBS and allowed to form cysts for 6 days. Cysts were stimulated through the addition of HGF (20.25 ng/ml) or CSF-1 (50 ng/ml) to Leibowitz medium supplemented with 2% FBS. The medium was replaced every 5 days for the duration of the assay. Images were then acquired with a Zeiss Axiovision 135 microscope with a 10x objective (Carl Zeiss Canada Ltd, Toronto, ON).

Migration and Invasion Assays

MDCK cells (5 x 10^4) were counted and seeded directly onto 6.5-mm Corning Costar transwell chambers for migration, or transwells coated with 100 µg/cm^2 matrigel (BD Biosciences, San Jose, CA) for invasion assays. Complete media was added to both the top and bottom wells, and cells were incubated at 37°C overnight. For HGF stimulations, 34 ng/ml of HGF was added to the bottom wells. Following overnight incubation, cells on both sides of the transwells were fixed with 10% neutral buffered formalin for 20 min at room temperature. After washing with double-distilled water, cells were stained with 0.1% crystal violet in 20% methanol for 20 min at room temperature. Cells on the top layer were scraped off and membranes were left to dry overnight. Images were captured using a Retiga 1300 digital camera (QIMAGING, Burnaby, BC) and a Zeiss Axioskop

microscope (Carl Zeiss Canada Ltd., Toronto, ON). Image analysis and quantification was performed using the Scion Image-NIH equivalent program for Microsoft Windows (Scion Company, Frederick, MD).

Pak4 knockdown

siRNA-mediated knockdown of Pak4 in MDCK cells was accomplished using the Reverse-Transfection of Adherent Cells with siRNA in 6-Well Plates (Qiagen, Mississauga, ON) as per the manufacturer's protocol. Briefly, 1 x 10^5 cells were seeded per well in complete medium. 24 hours later, 150 nM of Pak4 siRNA duplex (Duplex 1: CCGGCTGGTGGCCGTCAAGAA or Duplex 4: CGAGAACGTGGTGGAGATGTA) was mixed with 20 µl HiPerfect transfection reagent (Qiagen, Mississauga, ON). AllStars Negative control siRNA (#1027281, Qiagen, Mississauga, ON) was used as the scrambled siRNA negative control. Cells were collected 96 hrs post-transfection and used to perform assays.

3.6 RESULTS

3.6.1 Pak4 is a novel Gab1 binding partner

Recruitment of Gab1 is critical for Met-dependent biological responses in epithelial cells. To date, multiple proteins have been shown to associate with Gab1 downstream from Met that play key roles in Met signaling. These interactions were identified based on predicted SH2 domain binding sites (Holgado-Madruga, Emlet et al. 1996). However, no unbiased screens for Gab1 associated proteins have been performed. To identify novel Gab1-associated protein complexes following Met receptor activation, we generated a Gab1 TAP-tag fusion protein and used this in a proteomic screen to identify Gab1-binding partners. This technique has been successful in isolating and identifying protein complexes in yeast and mammalian cells (Rigaut, Shevchenko et al. 1999; Knuesel, Wan et al. 2003). Stable lines of HEK293 cells expressing a Gab1 TAP-tag fusion protein were generated and Gab1 TAP-tag associated protein complexes were isolated following stimulation with HGF for 5 min. Protein complexes were analyzed by mass spectrometry (data not shown). As a proof of principle, we were able to isolate the known Gab1-binding partners Grb2, Shp2 and the p85 subunit of PI3K by mass spectrometry and by western blot analysis in a Gab1 TAP-tag, HGF-dependent protein complex (Supplemental Figure 3.1 and data not shown). We were interested to pursue novel Gab1-binding partners and identified Pak4, a Ser/Thr kinase.

To establish if Pak4 was a Met-dependent Gab1-binding protein, HEK293 cells were transiently transfected with HA-tagged Gab1 and myc-tagged Pak4, in the absence, or presence of the Met RTK. Transient overexpression of Met leads to its activation in the absence of ligand (Rodrigues, Naujokas et al. 1991). Although weak binding of Gab1

and Pak4, as established by co-immunoprecipitation, is observed in the absence of Met, this association was significantly increased in the presence of active Met (Figure 3.1A). Moreover, phosphorylation of serine 474 of Pak4, which is thought to play a positive role in regulating the activity and function of Pak4 (Qu, Cammarano et al. 2001), was significantly elevated in the presence of Met (Figure 3.1A). To examine if the association of Pak4 was unique to Gab1 alone or capable of binding to all Gab family members downstream from the Met receptor, transient transfections of Pak4 with Gab1, Gab2 and Gab3 were performed. Interestingly, Pak4 was found to associate solely with Gab1 and not with Gab2 or Gab3, suggesting a specific interaction between Pak4 with Gab1 (Supplemental Figure 3.2).

To evaluate the possibility that group I Pak family members could also bind to Gab1, we tested the ability of Pak1 to associate with Gab1. We have previously shown that Pak1 is activated, phosphorylated and translocated to membrane ruffles at the edge of lamellipodia 15 min post-HGF stimulation (Royal, Lamarche-Vane et al. 2000). However, transient transfection of myc-Pak1 together with HA-Gab1 and Met showed no association between Gab1 and Pak1, whereas under these conditions, Pak4 co-immunoprecipitated with Gab1, delineating a specific Gab1-Pak4 complex (Figure 3.1B). We next determined whether the association between Pak4 and Gab1 could occur with endogenous proteins. Following stimulation of HeLa cells with HGF, co-immunoprecipitation of endogenous Pak4 with Gab1 was detected as early as 5 min post HGF-stimulation and maintained up to 30 min post-stimulation (Figure 3.1C). To examine the requirements for the Gab1-Pak4 association, we established if Gab1 phosphorylation and Pak4 activation was required. A requirement for Gab1 phosphorylation was examined by pre-treating protein lysates prepared from HEK293

cells transiently transfected with Met, Gab1 and Pak4 with lambda phosphatase, which acts to nonspecifically remove tyrosine, serine and threonine phosphate residues (Zhuo, Clemens et al. 1993). Pretreatment with lambda phosphatase abrogates co-immunoprecipitation of Gab1 with Pak4 supporting a requirement for Gab1 and/or Pak4 phosphorylation for this interaction (Figure 3.1D). To establish if Pak4 catalytic activity was necessary, constitutively active Pak4 (Pak4S445NS447E) (Qu, Cammarano et al. 2001) and kinase dead Pak4 (Pak4K350M) (Abo, Qu et al. 1998) were transiently transfected with Met and Gab1 in HEK293 (Figure 3.1E). The Pak4K350M protein associated with Gab1 to similar levels as the constitutively active Pak4 demonstrating that Pak4 activation was not necessary for the association between Gab1 and Pak4 (Figure 3.1E).

HGF stimulation for two hours leads to the translocation of Pak4 to the cell periphery in MDCK cells (Wells, Abo et al. 2002). To establish if Pak4 rapidly translocated to the cell cortex in response to HGF, we generated MDCK cells that stably expressed GFP-Pak4. In response to HGF, Pak4 rapidly translocated to the cell periphery and localized within newly forming peripheral ruffles as early as 3 min post-HGF stimulation and subsequently to newly forming lamellipodia at 7 min (Figure 3.2A). To establish that Pak4 is recruited to lamellipodia, MDCK cells expressing GFP-Pak4 were stimulated with HGF and stained with wheat germ agglutinin (WGA) Alexa 555, a marker for the plasma membrane. Localization of GFP-Pak4 and WGA (555) as examined by confocal microscopy on fixed cells through multiple z-stacks (Figure 3.2B), revealed that Pak4 is not localized to the plasma membrane in the absence of HGF stimulation (no overlap with WGA staining) but is recruited following stimulation with HGF to the limits of the cell colony and is further enhanced in lamellipodia (Figure 3.2B

and data not shown). Moreover, following transient transfection of HA-Gab1 and myc-Pak4 in HeLa cells, confocal images of fixed cells further demonstrated that these two proteins co-localize at the cell periphery within lamellipodia at 5 min post-HGF stimulation (Figure 3.2C), supporting the ability of these two proteins to form a complex following HGF stimulation. To determine if recruitment of Pak4 to the cell cortex is dependent on Gab1, MDCK cells stably expressing HA-Gab1ΔPH were stimulated and localization of Pak4 examined. Membrane recruitment of Gab1 via its PH domain is required for the biological activity of Gab1 (Maroun, Holgado-Madruga et al. 1999) and this can be rescued through the addition of the myristoylation (Myr) signal from c-src (Myr-Gab1ΔPH) (Maroun, Naujokas et al. 2003). In MDCK cell expressing Gab1ΔPH, neither Gab1ΔPH nor Pak4 translocated to the cell cortex. However, in MDCK cells expressing the Myr-Gab1ΔPH protein, which localizes to the cell membrane in the absence of HGF stimulation, Pak4 was recruited to the membrane in response to HGF but not in the absence (Figure 3.2D). Therefore, Pak4 and Gab1 co-localize in a complex within lamellipodia, following Met receptor stimulation.

3.6.2 Association of Gab1 with Pak4 requires the GID domain of Pak4

To identify which region of Pak4 was required for association with Gab1, Pak4 deletion constructs were created and a structure function analysis was performed. Pak4 possesses an N-terminal p21-binding (CRIB) domain and a C-terminal kinase domain (Figure 3.3A). Deletion mutants generated to remove the CRIB domain still retained the ability to bind Gab1 (Figure 3.3B and data not shown). Deletion of the kinase domain and the newly identified GEF-interacting domain, GID (Callow, Zozulya et al. 2005), abrogated Gab1 binding. However, a Pak4 mutant that possessed a GID domain and

kinase domain retained binding to Gab1, whereas a similar Pak4 mutant lacking the GID domain failed to bind (Figure 3.3B). Taken together, our data localized the region in Pak4 required for binding to Gab1 to a previously described region in Pak4 which is responsible for associating with the guanine exchange factor GEF-H1 (Figure 3.3B) (Callow, Zozulya et al. 2005).

3.6.3 Association of Pak4 with Gab1 is independent of known Gab1 binding sites

In response to HGF, Gab1 associates with multiple proteins in a phosphotyrosine-dependent manner through specific interactions with the SH2 domains of Shp2, Crk, PLCγ, and the p85 subunit of PI3K. To determine the mechanism through which Pak4 is recruited to Gab1, Gab1 mutants deficient in their ability to recruit known binding proteins were examined for their ability to associate with Pak4 (Figure 3.4A). Mutant Gab1 proteins unable to recruit Grb2, PI3K, Crk/PLCγ or Shp2, still retain the ability to recruit Pak4 (Figure 3.4B), providing evidence that the association of Pak4 and Gab1 is mediated by an unidentified domain within Gab1. To delineate the region of Gab1 responsible for mediating the association with Pak4, Gab1 deletion constructs were generated (Figure 3.5A). Deletion constructs revealed that Pak4 recruitment to Gab1 is dependent on a region of Gab1 localized between the PH domain and the first Crk phosphotyrosine binding site (amino acids 116-234) (Figure 3.5A and 3.5B). This region in Gab1 possesses no known binding sites for other proteins (Figure 3.5A). A Gab1 mutant, Gab1 (1-234), that contains only the PH domain and amino acids 116-234, was still capable of associating with Pak4 (Figure 3.5B). Similarly, decreased association is

observed between Pak4 and a Gab1 mutant lacking the PH domain, which fails to localize to the plasma membrane (Figure 3.5B).

To test if amino acids (aa.) 116-234 are essential for Pak4 association with Gab1 we generated a Gab1 mutant that lacks this domain (Figure 3.5C). Following transient co-transfection of HEK293 cells, the Gab1Δ116-234 (Gab1ΔPak4) mutant failed to co-immunoprecipitate with Pak4 (Figure 3.5D), demonstrating that aa. 116-234 are essential for Pak4 recruitment. Importantly, the Gab1ΔPak4 mutant was robustly phosphorylated downstream from Met (Figure 3.5D), and recruited other known signaling proteins, including Shp2 and Crk, to similar levels as the wild type (WT) Gab1 protein (Figure 3.5E). To establish whether the Gab1 aa. 116-234, which are required for Pak4 recruitment, reflects a Pak4-Associating Motif (ASM) on Gab1, we examined if increasing overexpression of the Pak4-ASM (aa. 116-234) of Gab1 (HA-Pak4-ASM, Figure 3.5F and 3.5G) would compete for Pak4 association with Gab1. With increasing titration, we observe both a loss of association between Pak4 and full-length Gab1, as well as a decrease in phosphorylation on serine 474 of Pak4 (Figure 3.5F and 3.5G). Notably the Pak4-ASM of Gab1 co-immunoprecipitated with full-length Pak4, demonstrating that it was both sufficient and necessary for Pak4 interaction (Figure 3.5F and 3.5G).

3.6.4 Pak4 loss decreases cofilin phosphorylation

To examine the requirement of Pak4 in Met-dependent signaling, the consequence of loss of Pak4 on known downstream effectors of Pak4 in response to HGF was examined. We employed a siRNA strategy against Pak4 to knockdown endogenous protein levels. Efficient knockdown of Pak4 was achieved in MDCK cells (Figure 3.6A)

when compared to MOCK and scrambled siRNA (Figure 3.6A). Pak4 is a known regulator of LIMK and consequently cofilin activity (Dan, Kelly et al. 2001). Inactivation of cofilin results in part through phosphorylation on serine 3 by LIMK, preventing association of cofilin with actin. We therefore examined the level of phosphorylation of cofilin in MDCK cells treated with Pak4 siRNA or scrambled siRNA and stimulated with HGF. In response to HGF, no significant change in the level of cofilin phosphorylation was observed at early time points (Figure 3.6B and 3.6C). However, at later time points following HGF stimulation (45-60 min), the level of cofilin phosphorylation in cells transfected with scrambled when compared to Pak4 siRNA was 2-fold higher (Figure 3.6C). This change was consistent in 3 independent experiments. As a control, the phosphorylation status of Erk was examined to look at the efficiency of stimulation. No significant difference was observed in Erk activation in scrambled compared to Pak4 siRNA treated cells. This demonstrates a requirement for Pak4 in the regulation of enzymes involved in the remodeling of the actin cytoskeleton downstream from Met. Furthermore, we observed marked differences in the actin cytoskeleton of MDCK cells depleted of Pak4 upon HGF stimulation. Phalloidin staining of Pak4 siRNA treated MDCK cells reveals a decrease in lamellipodia formation and the actin cytoskeletal network on cells at the edge of the colony (Figure 3.6D). In contrast, in scramble treated MDCK cells, polymerized actin was visualized by phalloidin staining at the cell cortex (Figure 3.6D, arrows).

3.6.5 Pak4/Gab1 enhances the breakdown of cell-cell contact and epithelial cell scatter

To address the biological relevance for the association of Gab1 with Pak4, we created stable MDCK cells lines overexpressing Gab1 and/or Pak4 (Figure 3.7A). MDCK cells form tight colonies when grown in culture (Figure 3.7) (Gherardi, Gray et al. 1989; Weidner, Behrens et al. 1990). Stimulation of these colonies with HGF promotes the breakdown of cell-cell contacts, remodeling of the actin cytoskeleton and dispersal of cells 12-16 h post-stimulation resulting in an event that resembles an epithelial-mesenchymal transition (EMT) (Royal and Park 1995). To address the impact of Pak4 on the ability for cells to undergo cell scatter, multiple stable cell lines overexpressing Gab1 (HA-Gab1 (7D6), GFP-Gab1 (B1-3)), Pak4 (GFP-Pak4 CL9, CL11 and mCherry-Pak4 CL12) and both Gab1 and Pak4 (GFP-Gab1 and mCherry-Pak4 CL13, CL14, CL15 and CL16) were used to examine HGF-induced cell scatter. MDCK cells, or cells expressing either GFP alone (A2), GFP-Gab1 (B1-3), GFP-Pak4 (CL9) or both GFP-Gab1 and mcherry-Pak4 (CL14 and CL15) form tight colonies (Figure 3.7B and Supplemental Figure 3.3). Interestingly, cells overexpressing both GFP-Gab1 and mcherry-Pak4 (CL13, CL14, CL15, CL16) scattered in response to sub-optimal levels of HGF (0.34 ng/ml), whereas cells over-expressing Gab1 or Pak4 alone failed to scatter in response to low levels of HGF (Figure 3.7B and data not shown). As expected, all MDCK cells and those that expressed Gab1 or Pak4 alone were capable of scatter in response to higher levels of HGF (1.35 ng/ml, Supplemental Figure 3.3). Together, these data support that Gab1 and Pak4 synergize to mediate the scatter of MDCK cells downstream from the Met receptor.

3.6.6 Pak4 mediates Met-dependent cell migration and invasion

Scatter of epithelial colonies reflects the breakdown of cell-cell junctions as well as cellular migration. To establish if Pak4 and Gab1 synergize to promote cell migration and invasion, we tested the ability of stable cell lines expressing Pak4 and Gab1 to enhance migration and invasion of single cells using Boyden chamber transwell assays. Overexpression of Gab1 or Pak4 alone resulted in no increase in migration or invasion in the absence of HGF (Figure 3.8A and 3.8B). Similarly, overexpression of Gab1 and Pak4 had no significant effect on the ability of MDCK cells to migrate in the presence of HGF. However, when Pak4 and Gab1 were over-expressed together, we observed an increase in basal cell migration, but not in cell invasion, in the absence of HGF in 4 clones tested (Figure 3.8A and 3.8B). We observed a significant increase in HGF induced migration (5-10 fold) and invasion (3-6 fold) in cells expressing both proteins when compared to cells overexpressing either Pak4 or Gab1 alone. Hence, this provides further evidence of synergy between Gab1 and Pak4 that plays a role in mediating the migratory and invasive response downstream from the Met receptor.

3.6.7 Pak4 is required for scatter and invasion downstream of Met

The above data demonstrate that Pak4-Gab1 overexpression enhances epithelial cell migration and invasion. To assess a requirement for Pak4 on these processes, the consequence of Pak4 knockdown was examined. MDCK cells in which Pak4 protein levels were reduced by 2 different siRNA (Figure 3.6A) failed to scatter following HGF stimulation, as compared to control MDCK or cells transfected with scrambled siRNA (Figure 3.9 and data not shown). Moreover, following Pak4 knockdown, MDCK and HeLa cells showed decreased cell migration and invasion in single cell Boyden chamber

assays (Figure 3.9B, 3.9C and Supplemental Figure 3.4). An overall decrease of 46% in migration and 44% in invasion was observed in MDCK cells (Figure 3.9B and 3.9C, respectively) and 45% in migration and 62% in invasion was observed in HeLa cells (Supplemental Figure 3.4).

3.6.8 Gab1-Pak4 association is critical for HGF induced cell scatter, invasion and tubulogenesis

To test the requirement for recruitment of Pak4 to a Gab1 complex, we utilized MDCK cells expressing a chimeric CSF-Met receptor mutant where their ability to undergo a morphogenic program in response to CSF-1 is dependent on overexpression of Gab1 (Fournier, Kamikura et al. 1996; Maroun, Holgado-Madruga et al. 1999). This has allowed structure function analyses of the requirement of Gab1-associated proteins for cell scatter and invasive tubulogenesis (Maroun, Holgado-Madruga et al. 1999; Lamorte, Rodrigues et al. 2002; Lock, Maroun et al. 2002). This MDCK (G17) model was used to test the requirement for Pak4-Gab1 interaction by overexpressing the Gab1ΔPak4 mutant. Multiple clones of MDCK (G17) cells expressing Gab1ΔPak4 (CL3A4, CL3A7, CL3A8, and CL3A9) (Figure 3.9D) failed to scatter or undergo a tubulogenic response to CSF-1 (Figure 3.9D, 3.9E, 3.9F and Supplemental Figures 3.5 and 3.6). Since we demonstrated that increasing concentrations of the Pak4-ASM of Gab1 can compete for association between Pak4 and Gab1 (Figure 3.5F and 3.5G) to further test the requirement for Gab1-Pak4, migration/invasion assays were performed on HeLa cells in which the Pak4-ASM of Gab1 was overexpressed. Consistent with Pak4 knockdown causing a decrease in the migratory and invasive capacity of HeLa cells (Supplemental Figure 3.4), the overexpression of the Pak4-ASM of Gab1 (Figure 3.9G) resulted in a 2-fold decrease in

the migratory (Figure 3.9H) and invasive (Figure 3.9I) capacity of HeLa cells in response to HGF. Hence, these data are consistent with a requirement of the Pak4-Gab1 association for an HGF-dependent scatter, invasive and tubulogenic response.

3.7 DISCUSSION

Our results have provided insight into the molecular mechanism through which a Gab1-Pak4 complex modulates Met-dependent signals for cell migration and invasion. Pak4 was previously shown to translocate to membrane ruffles and lamellipodia following the stimulation of MDCK cells with HGF (Wells, Abo et al. 2002). We have now demonstrated that Pak4 is a downstream target of the Met receptor (Figure 3.1). We provided a mechanism through which Pak4 recruitment to Gab1 promotes Pak4 localization to the cell periphery and newly forming lamellipodia and is essential for HGF-dependent, Gab1-mediated, biological responses including epithelial cell scatter, invasion and tubulogenesis (Figures 3.7, 3.8, 3.9 and Supplemental Figures 3.4, 3.5 and 3.6). Pak4 and Gab1 interact, as demonstrated by co-immunoprecipitation of proteins from both transiently transfected cells as well as endogenous proteins (Figure 3.1). Notably, Pak4 associated with Gab1 in response to HGF stimulation and Met activation and not with other Gab family members, including Gab2 and Gab3 (Supplemental Figure 3.2). This is consistent with our data that a Gab1-Pak4 association is essential for HGF-induced cell migration, invasion and tubulogenesis which are biological responses dependent on Gab1 downstream from the Met receptor (Maroun, Holgado-Madruga et al. 1999; Gual, Giordano et al. 2000; Maroun, Naujokas et al. 2000; Lamorte, Rodrigues et al. 2002; Lock, Maroun et al. 2002) and not on Gab2 (Lock, Maroun et al. 2002) or Gab3 (our unpublished data).

We have shown that Pak4 recruitment to Gab1 is dependent on Met activity (Figure 3.1). From structure function analysis, we have determined that recruitment of Pak4 to Gab1 is dependent on a domain in Gab1 not previously identified as a protein

binding domain. From 2D modeling studies, the Pak4-ASM of Gab1 (aa. 116-234) displays no similarity to known protein binding motifs. When overexpressed, the Gab1-Pak4-ASM is sufficient to compete with Met-dependent recruitment of Pak4 to Gab1 (Figure 3.5) and this inhibited migration and invasion following HGF stimulation when increasing concentrations of Pak4-ASM were expressed in HeLa (Figure 3.9G-3.9I). Hence the Gab1-Pak4-ASM is both necessary and sufficient to interact with Pak4.

Interestingly, treatment of protein lysates with lambda phosphatase, which is active on serine, threonine and tyrosine residues, results in a decrease in the association of Gab1 and Pak4 (Figure 3.1D), supporting a potential role for tyrosine phosphorylation of Gab1 for this interaction. The Gab1-Pak4-ASM contains tyrosine, serine and threonine residues. Hence phosphorylation of the Gab1-Pak4-ASM may generate a phosphotyrosine or phospho-serine/threonine-dependent recruitment site for Pak4 or may modulate the structure of Gab1, enhancing the interaction with Pak4. The Pak4 domain required for binding to Gab1 is a domain previously identified to bind to GEF-H1 (GEF-H1 interacting domain, [GID]). Interaction of the Pak4 GID with GEF-H1 occurs in an apparently phosphorylation independent manner, demonstrating that phosphorylation is not essential for Pak4-GID binding (Callow, Zozulya et al. 2005). A comparison of the primary and secondary structures of the Gab1-Pak4-ASM and GEF-H1 does not reveal similarities in their Pak4 interacting motifs, indicating that the Pak4 GID may reflect a multi-protein interacting domain. Since the GID domain of Pak4 interacts with both proteins this would also indicate that Gab1 and GEFH1 may compete for Pak4 binding and or recruit Pak4 to distinct subcellular localizations.

The recruitment of Pak4 to Gab1 provides a mechanism to modulate subcellular localization of Pak4. The Gab1 scaffold protein is recruited to membrane ruffles and

lamellipodia in response to HGF and this is dependent on an intact Gab1 PH domain that binds to phosphoinositide 3,4,5 tri-phosphate (PIP3) phospholipids, in a PI3K-dependent manner (Maroun, Holgado-Madruga et al. 1999). In support of a role for Gab1 for Pak4 localization, Pak4 fails to be recruited to the cell cortex in cells that overexpress a Gab1 mutant lacking its PH domain, that is still competent for Pak4 interaction (Figure 3.5B), and this is rescued by targeting the Gab1ΔPH domain mutant to the plasma membrane through the addition of the c-src myristoylation signal (Figure 3.2D). Consistent with a requirement for a Pak4-Gab1 complex for Met-induced epithelial cell invasion and migration (Figures 3.7-3.9), a Gab1ΔPak4 mutant that is unable to associate with Pak4, fails to promote an invasive morphogenic program in response to HGF (Figure 3.9F and Supplemental Figure 3.6) even though recruitment of other signaling proteins such as Shp2 and Crk is unaltered (Figure 3.5E), identifying Pak4 recruitment to Gab1 as critical for Gab1-mediated biological responses. Hence, Gab1 may act as a scaffold protein to localize Pak4 to subcellular compartments promoting association of Pak4 upstream regulators, as well as downstream effectors. This is consistent with our previous data where upstream regulators of Pak4, activated Rac and Cdc42 (Manser, Leung et al. 1994), re-localize to membrane protrusions in MDCK cells in response to HGF (Ridley, Comoglio et al. 1995; Royal, Lamarche-Vane et al. 2000). Moreover, Pak4 activity is associated with enhanced activation of integrins and cell spreading (Qu, Cammarano et al. 2001; Callow, Clairvoyant et al. 2002; Liu, Xiao et al. 2008) and Gab1 may act to accumulate Pak4 in integrin-rich protrusions (Figure 3.2).

We show that a Gab1-Pak4 complex is critical for the breakdown of cell-cell contacts and for inducing cytoskeletal changes required for migration and invasion of

epithelial cells in response to HGF (Figures 3.7-3.9). The requirement for Pak4 for full activation of LIMK, which regulates filopodia and lamellipodia outgrowth (Dan, Kelly et al. 2001), provides a mechanism through which Pak4 could modulate HGF-dependent cell migration and invasion. Consistent with a role for Pak4 in HGF-induced migration of a prostate cancer cell line being dependent on cofilin phosphorylation (Ahmed, Shea et al. 2008), we observed maximum stimulation of cofilin phosphorylation by 30-45min post-HGF stimulation. A decrease in HGF-induced cofilin phosphorylation following Pak4 knockdown was seen (Figure 3.6B and 3.6C) and we observed a decrease of actin network at the leading edge of cells following knockdown of Pak4 (Figure 3.6D). Phosphorylation of cofilin is thought to suppress its activity due to its inability to bind F-actin (Condeelis 2001). However, recent data suggests that the phosphorylation status of cofilin is necessary for determining the direction of cellular protrusions (Mouneimne, DesMarais et al. 2006; Song, Chen et al. 2006). In support of this, we observed a decrease in actin remodeling in MDCK cells following knockdown of Pak4 (Figure 3.6D).

Pak1 also regulates LIMK phosphorylation and HGF can regulate Pak1 localization through an undetermined mechanism (Royal, Lamarche-Vane et al. 2000). Since Pak1 was unable to associate with Gab1 downstream from the Met receptor (Figure 3.1B), Pak4 is a specific Gab1 effector downstream of Met. Hence, even though Pak1 and Pak4 both localize to the leading edge of migrating cells in response to HGF, they are likely to be regulated independently, and may be present within different subcellular compartments or complexes to regulate actin dynamics downstream from Met. Interestingly, sequence alignment of group I and group II Paks, show that only the family of group II Paks possess a GEF-HI interaction domain (GID), providing support for the

specificity that we observe (Callow, Zozulya et al. 2005), whereas group 1 Pak proteins such as Pak1 interact with GEFs of the Cool/Pix family (Bagrodia and Cerione 1999).

In summary, our data provide direct evidence for a novel signaling complex downstream from HGF, involving a Gab1-Pak4 complex and identify a role for this complex in mediating the migratory and invasive morphogenic responses of sheets of epithelial cells downstream from the Met receptor. These findings have particular significance for human cancer. Many RTKs deregulated in human cancer (ErbB2, Met and EGFR), signal through Gab1. Oncomine analysis of Gab1 revealed overexpression in multiple human cancers. Group I and group II Pak family members are elevated in multiple human cancers. A screen of human cancer cell lines revealed Pak4 to be overexpressed in 78% of these cancer lines and implicated Pak4 in ras transformation and anchorage-independent growth (Callow, Clairvoyant et al. 2002; Liu, Xiao et al. 2008). Therefore, given the importance of Gab1 in the invasive growth of epithelial cells downstream from the Met receptor, plus the observation that deregulation of Met is associated with many human cancers (Birchmeier, Birchmeier et al. 2003) and that Gab1 and Pak4 expression is elevated in human cancer, this highlights the importance in identifying the molecular mechanisms through which these proteins act together to enhance tumorigenesis, invasion and the metastasis.

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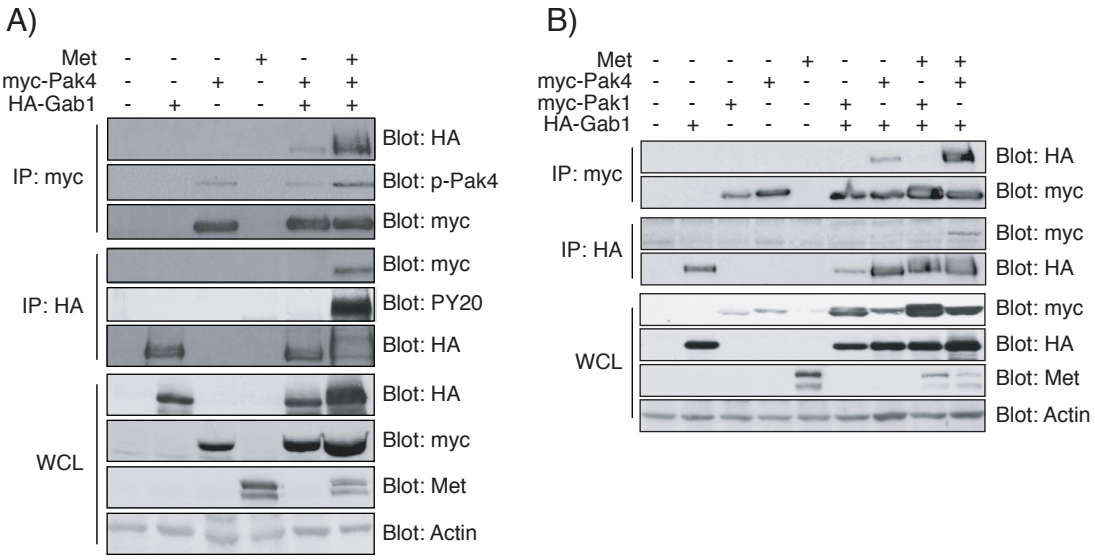


Figure 3.1: Pak4 associates with Gab1 following Met receptor activation. **A)** Following transient transfection of HA-Gab1, myc-Pak4 and Met in HEK293 cells, total cell lysate (500 μg) were immunoprecipitated with anti-HA and anti-myc (9E10) sera. Immunoprecipitations and whole cell lysates (30 μg) were separated by SDS-PAGE and immunoblot analysis was performed with indicated antibodies. **B)** Transient transfection of HA-Gab1, myc-Pak4, myc-Pak1 and Met in HEK293 cells. Proteins were extracted and total cell lysate (500 μg) were immunoprecipitated by anti-HA and anti-myc (9E10) sera, separated by SDS-PAGE and immunoblot analysis performed. Immunoblot blot analysis was performed on whole cell lysate (30 μg) and probed with antisera against HA, myc, Met and actin. **C)** HeLa cells were plated at a density of 1x106 cells/10cm dish and were stimulated for the indicated time points with 135ng/ml of HGF. Proteins from total cell lysate (500 μg) were immunoprecipitated with anti-Gab1 sera, separated by SDS-PAGE and immunobloted with antisera against Pak4. Proteins from whole cell lysate (30 μg) were probed with antisera against phospho-Pak4(S474), phospho-ERK1/2 (pTpY202/204) and total ERK1/2. **D)** HEK293 cells were transiently transfected with myc-Pak4, HA-Gab1 and Met. Prior to co-immunoprecipitation, cells were treated with 2000 units of lambda phosphatase for 30 min at 30oC. Immunoprecipitation and whole cell lysate (30 μg) lysates were separated by SDS-PAGE and immunoblotted with antisera againt HA, myc (9E10), Met, phospho-MetY1234/35, phospho-Gab1Y627 and actin. **E)** HEK293 cells were transiently trasnsfected with constitutively active HA-Pak4 (S445NS447E) and kinase dead HA-Pak4(K350) together with GFP-Gab1 and Met. Proteins were extracted and total cell lysate (500 μg) were immunoprecipitated by anti-HA and anti-GFP sera, separated by SDS-PAGE and immunoblot analysis performed. Immunoblot blot analysis was performed on whole cell lysate (30 μg) and probed with antisera against HA, GFP, phospho-Pak4(Ser474), Met and actin.

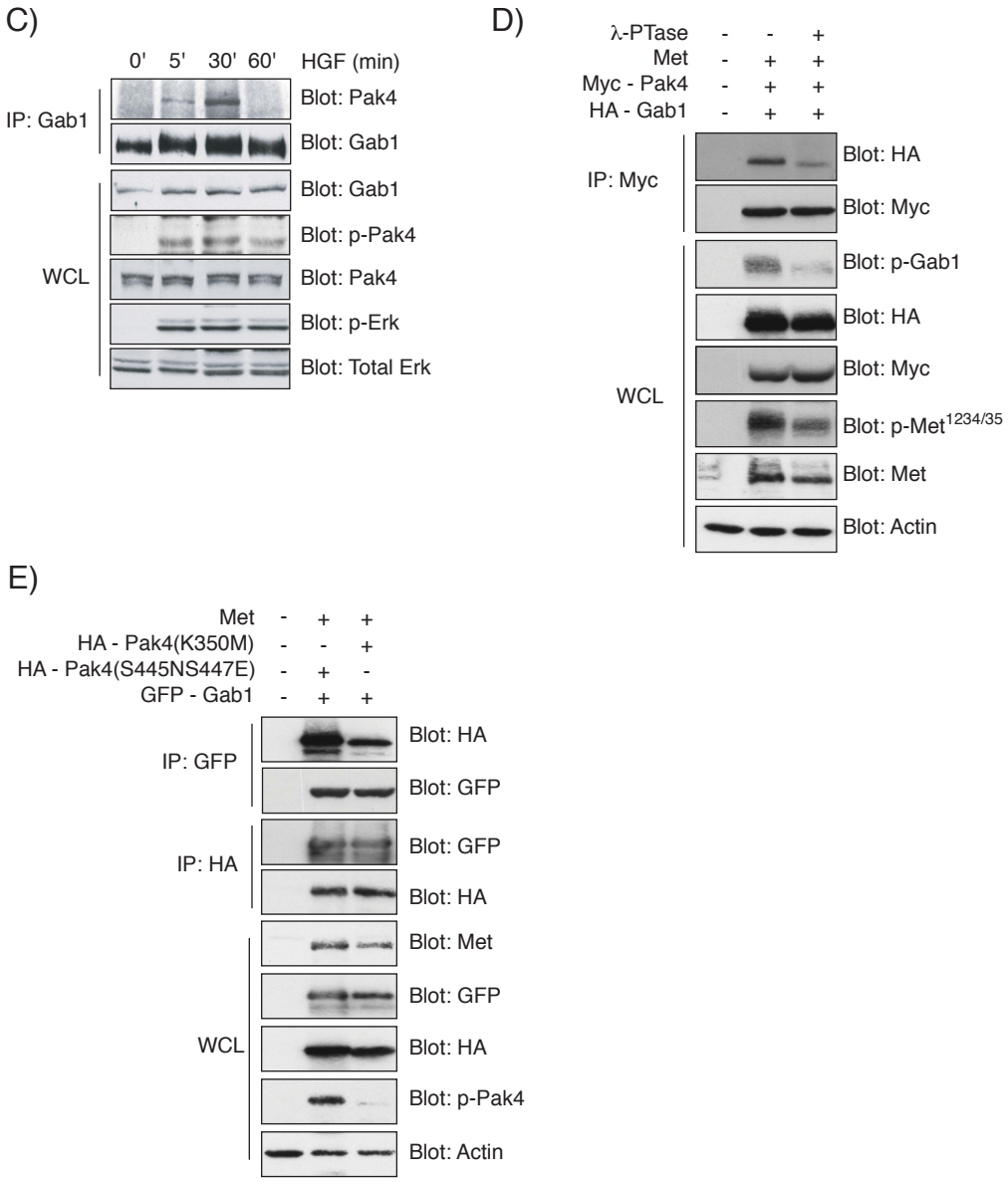


Figure 3.1: Pak4 associates with Gab1 following Met receptor activation.

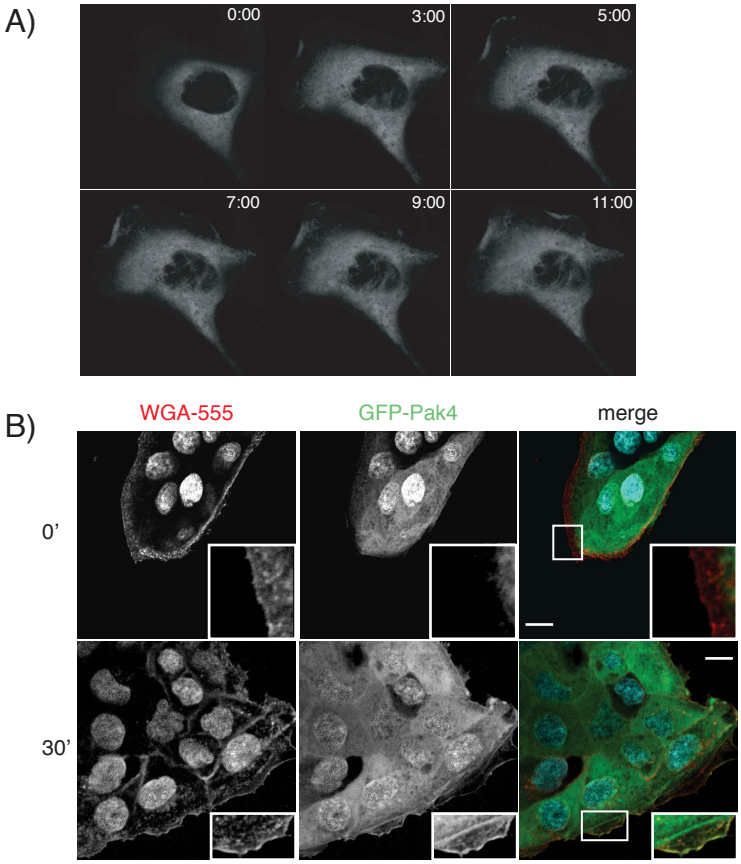


Figure 3.2: Pak4 co-localizes with Gab1 at the leading edge in lamellipodia in HGF stimulated cells. A) MDCK cells stably expressing GFP-Pak4 (2 x 10^4) were plated on glass bottom dishes, and the next day stimulated with HGF. Spinning disk time-lapse microscopy of living cells with single time frames taken at the indicated times post-HGF stimulation with a 63x objective. Images shown represent 0.2 µm thickness slice of z-stack. **B)** MDCK cells stably expressing GFP-Pak4 were plated on coverslips, and the next day stimulated with HGF. Following stimulation, cells were stained with plasma membrane specific protein wheat germ agglutinin (WGA) Alexa Fluor 555. Confocal images were taken using a Zeiss 510 Meta laser scanning confocal microscope. Confocal images were taken with a 100x objective. Image representative of 0.2 µm z-stack. Bar represents 10 µm. **C)** HeLa cells were plated on coverslips, and were transiently transfected with HA-Gab1 and myc-Pak4. Following overnight incubation, cells were serum starved for 2 h, and stimulated with 135 ng/ml HGF at 37°C for the indicated time points. Coverslips were fixed in 3% PFA, and stained with anti-HA (first panel) and anti-myc sera (second panel). Confocal images were taken with a 100× objective. Bar represents 10 µm. **D)** Stable MDCK cells expressing chimeric receptor CSF-MetΔGrb2 and overexpressing HA-Gab1ΔPH or myr-HA-Gab1ΔPH, were plated on coverslips and transiently transfected with mCherry-Pak4. Following overnight incubation, cells were serum starved for 2 h, and stimulated with 50ng of CSF-1 and then analyzed for the ability of Gab1 to be properly recruited to the plasma membrane. Coverslips were fixed in 3% PFA, and stained with anti-HA. Confocal images were taken with a 100x objective. Bar represents 10 µm.

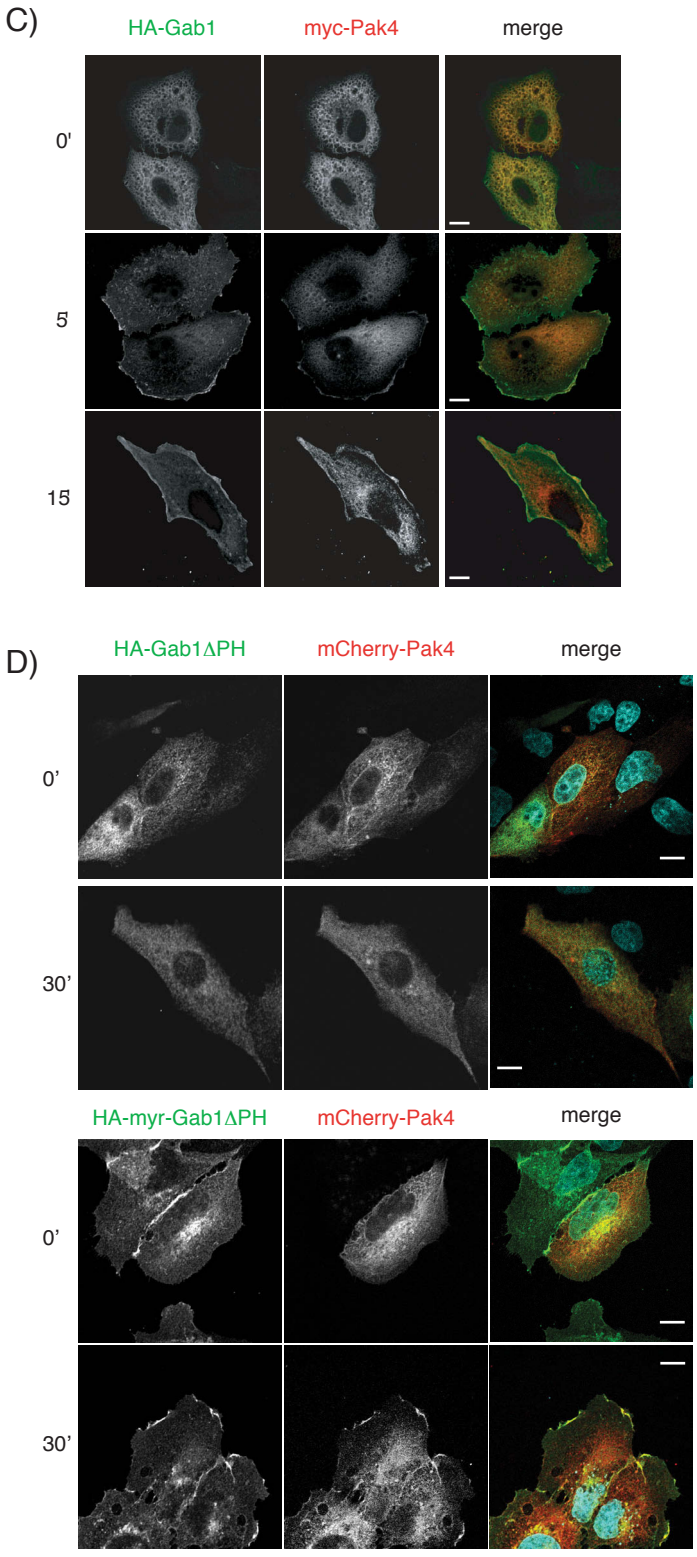


Figure 3.2: Pak4 co-localizes with Gab1 at the leading edge in lamellipodia in HGF stimulated cells.

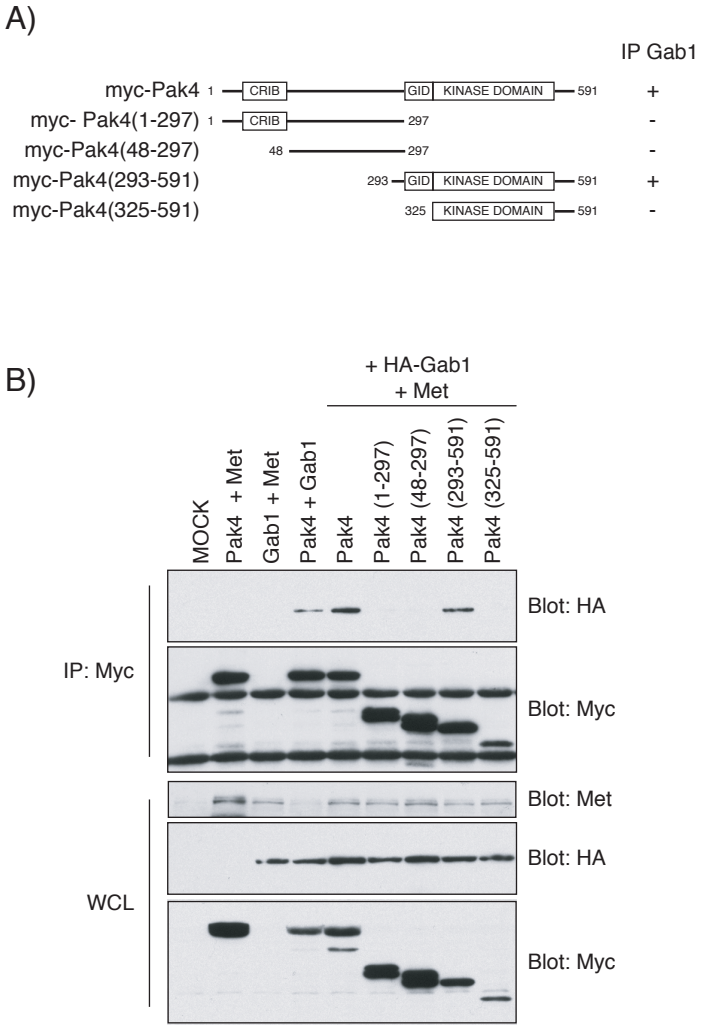


Figure 3.3: Association of Pak4 with Gab1 occurs through GID domain of Pak4. **A)** Schematic representation of Pak4 deletion constructs. Numbers in parentheses represent amino acids in Pak4 sequence. **B)** HEK293 cells were transiently transfected with myc-Pak4 deletion constructs together with HA-Gab1 and Met. Proteins from total cell lysate (500 μg) were immunoprecipitated with anti-myc antisera, separated by SDS-PAGE and immunoblotted with antisera for HA and myc (9E10). Proteins from whole cell lysates (30 μg) were immunoblotted with antisera against HA, Met, and myc (9E10).

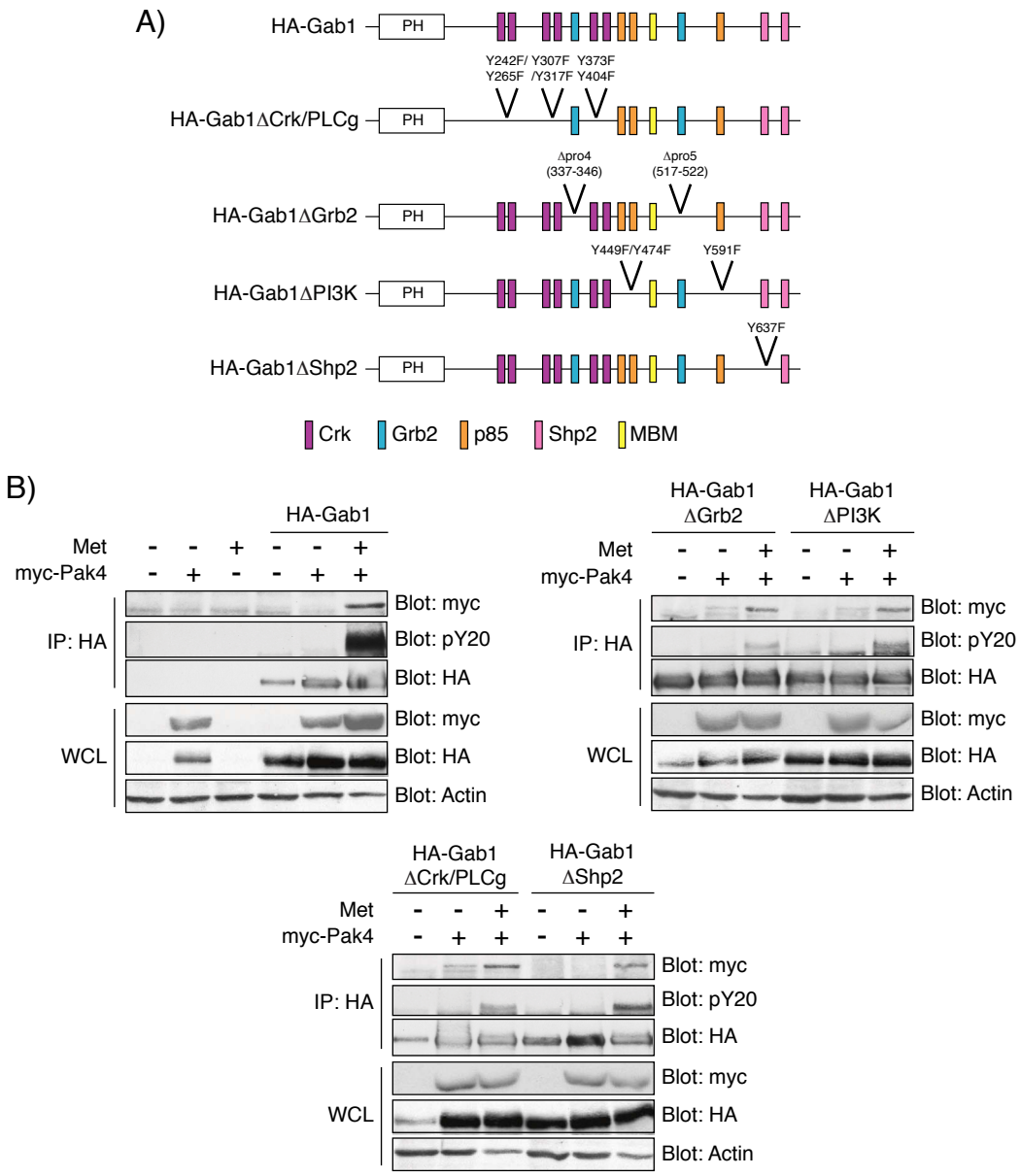


Figure 3.4: Association of Pak4 and Gab1 is not mediated through known Gab1 binding sites. A) Schematic representation of Gab1 mutant constructs. **B)** HEK293 cells were transiently transfected with HA-tagged Gab1 mutants lacking the bindings sites for Grb2, p85 subunit of PI3K, Crk/PLCγ, and Shp2 in combination with myc-Pak4 and/or Met. Gab1 protein lysates (500 µg) were immunoprecipitated with sera against anti-HA and resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was immunoblotted with antisera against myc (9E10), phosphotyrosine (PY20) and HA and actin.

Figure 3.5: Pak4 association to Gab1 is mediated through an unidentified domain of Gab1. A) Schematic representation of Gab1 deletions constructs. Numbers in parentheses represent amino acids in Gab1 sequence. **B)** HA-tagged Gab1 deletion constructs were transiently transfected in combination with myc-Pak4 and Met. Proteins from total cell lysates (500 μg) were immunoprecipitated with anti-myc (9E10) sera, separated by SDS-PAGE and immunoblotted with antisera against HA and myc (9E10). Proteins from whole cell lysates (30 μg) were immunoblotted with antisera against HA, myc (9E10) and actin. * Delineate the mature Gab1 construct. **C)** Schematic model of Gab1ΔPak4. **D)** Gab1 deletion construct lacking amino acid 116-234 (Gab1ΔPak4) were examined for its ability to associate to Pak4 downstream from Met. HA-Gab1 and HA-Gab1ΔPak4 were transiently transfected in combination with myc-Pak4 and Met. Proteins from total cell lysates (500 μg) were immunoprecipitated with anti-myc (9E10) sera, separated by SDS-PAGE and immunoblotted with antisera against HA and myc (9E10). Proteins from whole cell lysates (30 μg) were immunoblotted with antisera against HA, myc (9E10), phospho-Gab1Y627, Met and actin. **E)** Gab1ΔPak4 was examined for its ability to associate with known Gab1 binding partners. HA-Gab1 and HA-Gab1ΔPak4 were transiently transfected in combination with Met. Proteins from total cell lysates (500 μg) were immunoprecipitated with anti-HA sera, separated by SDS-PAGE and immunoblotted with antisera against phosphotyrosine (PY20), HA, Shp2 and Crk. Proteins from whole cell lysates (30 μg) were immunoblotted with antisera against Crk, Shp2 and actin. **F)** A competition assay was performed with increasing concentrations of HA-tagged Pak4-association motif (ASM) of Gab1 (HA-Pak4-ASM) to compete for binding of Pak4 with Gab1. HEK293 cells were transiently transfected with GFP-Gab1, myc-Pak4, Met and HA-Pak4-ASM. Proteins from total cell lysates (500 μg) were immunoprecipitated with anti-myc (9E10) sera, separated by SDS-PAGE and immunoblotted with antisera against HA and GFP. Proteins from whole cell lysates (30 μg) were immunoblotted with antisera against HA, myc (9E10), GFP and actin. **G)** Bar graphs representing the data shown in Figure 5F. The proportion of Gab1 associating to Pak4, Pak4-ASM binding to Pak4 and the proportion of phospho-Pak4 compared to Total Pak4 following increasing expression of Pak4-ASM of Gab1.

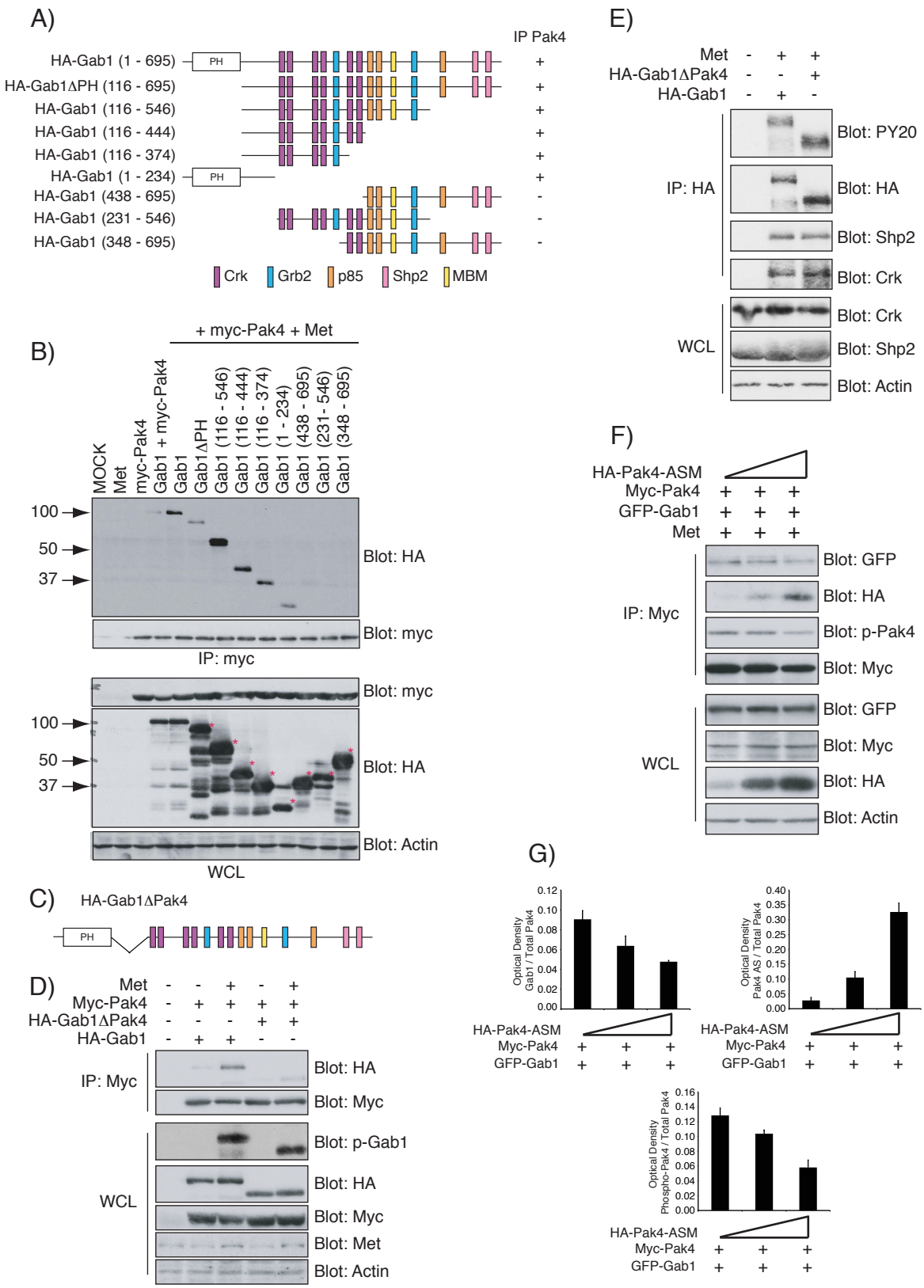


Figure 3.5: Pak4 association to Gab1 is mediated through an unidentified domain of Gab1.

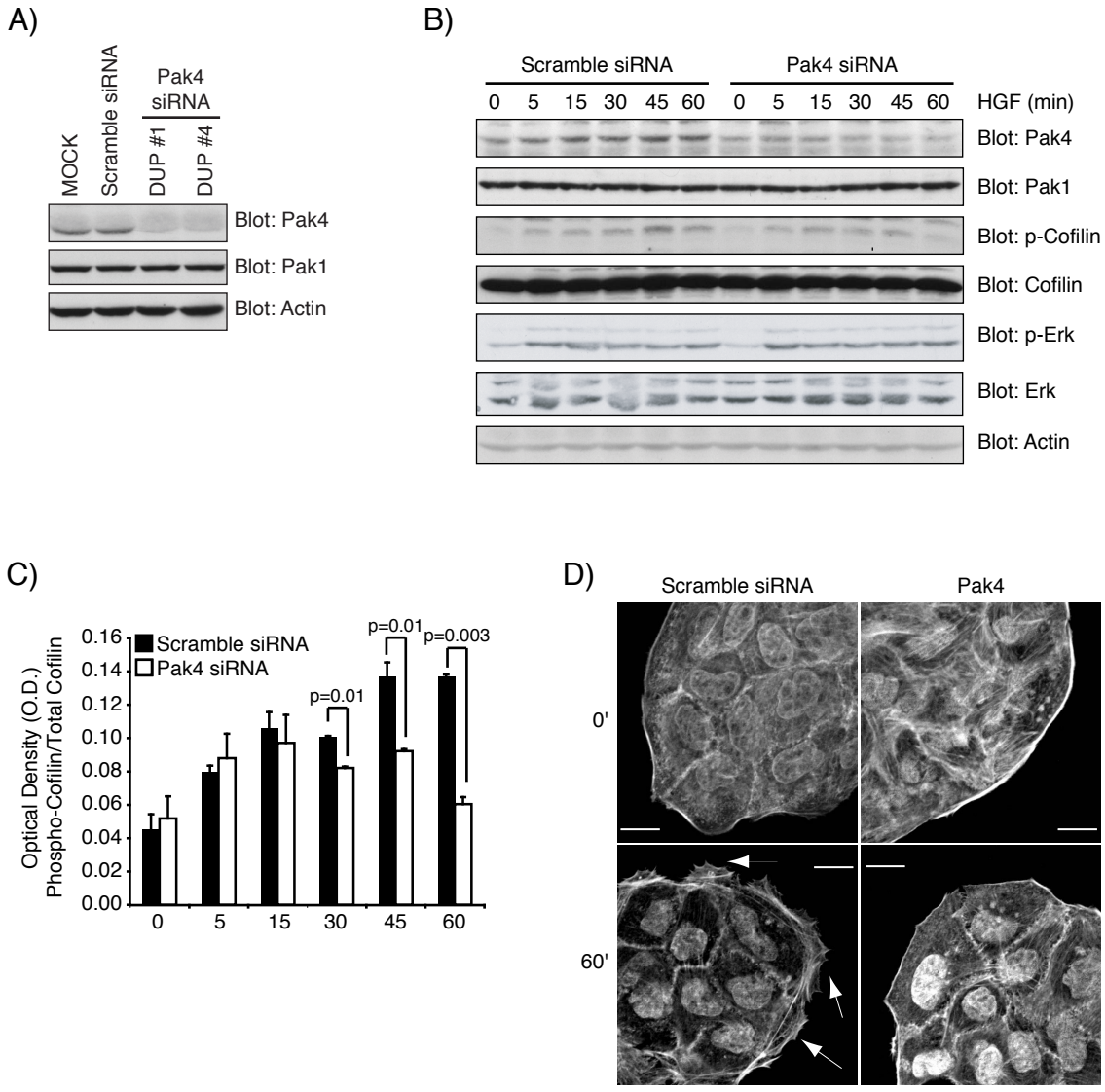


Figure 3.6: Pak4 knockdown decrease HGF-induced cofilin phosphorylation and actin dynamics. A) MDCK ($1x10^5$ cells) were plated in 6 well dish and the following day were transfected with 150 nM of Pak4 siRNA. Knockdown was examined 96 h later. Cells were lysed and proteins from whole cell lysates (20 μg) were immunoblotted with antisera against Pak4, Pak1 and actin. **B)** MDCK cells treated with scramble or Pak4 siRNA were stimulated with HGF for the indicated time points. Whole cell lysates (20 μg) were immunoblotted with antisera against Pak4, Pak1, phospho-cofilin, cofilin, phospho-Erk, total Erk and actin. **C)** Densitometric analysis of phospho-cofilin shown in 6B were measured by NIH Image and compared with the total cofilin levels. Bars represent standard error of the three separate experiments. **D)** MDCK cells stained with phalloidin. Confocal images were taken with a 100x objective. Bar represents 10 μm.

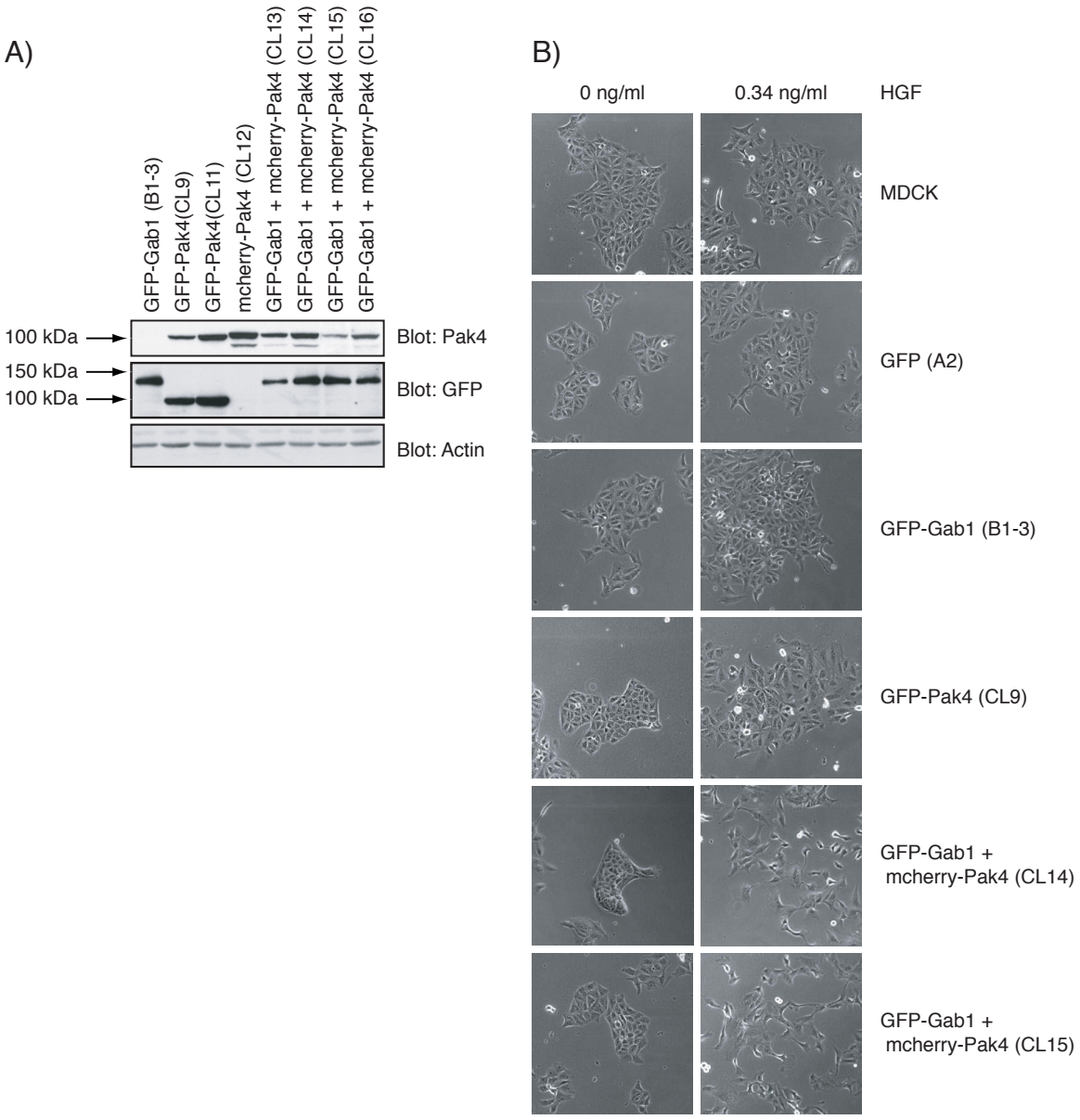


Figure 3.7: Overexpression of Gab1 and Pak4 induces scatter of MDCK cells. A) Proteins from whole cell lysate (30 µg) from MDCK cells and cells overexpressing GFP-Gab1 and/or mcherry-Pak4 were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-GFP and anti-Pak4. **B)** MDCK cells stably expressing GFP alone (A2), GFP-Gab1, GFP-Pak4 and GFP-Gab1 and mcherry-Pak4 were plated ($5x10^3$) in 24 well plates. The following day, 0.34 ng/ml HGF were added to each well for 24 h. Phase contrast images were taken to observe the extent of cell scatter.

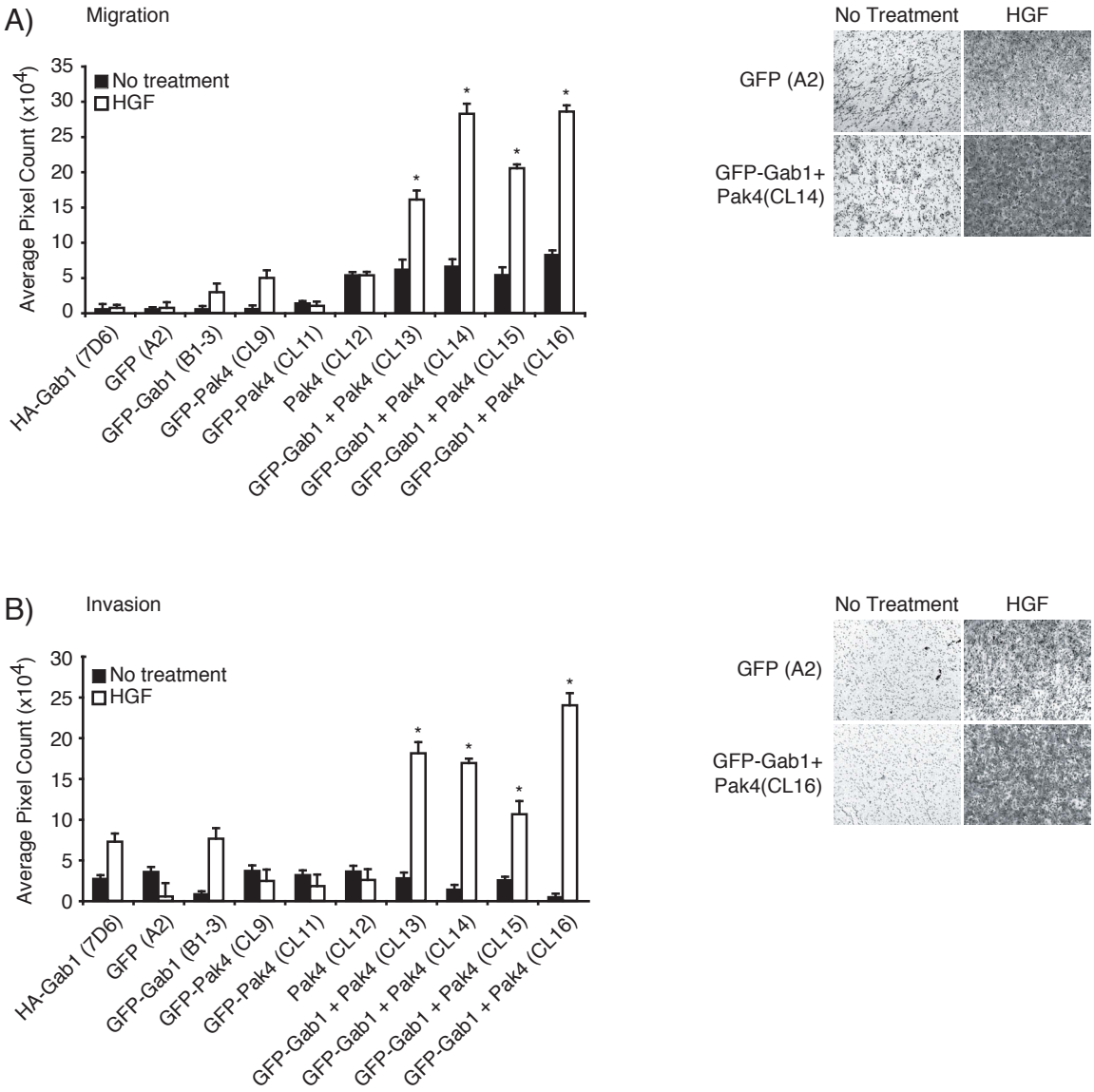


Figure 3.8: Overexpression of Gab1 and Pak4 induced migration and invasion of MDCK cells. MDCK cells overexpressing Gab1 and/or Pak4 were analyzed for their migration and invasion capacity in the presence of HGF (34 ng/ml). Cells were seeded (5 x 10^4 cells) onto **A)** modified Boyden chambers or **B)** onto matrigel-coated Boyden chamber and assayed for their migration and invasion capacity respectively. Using a Zeiss Axioskop microscope, bottom layers of the transwell were imaged in five separate fields for each condition using a 10x objective in phase contrast. Image analysis of these assays was carried out using Scion Image. Bars represent standard error of the three experiments.

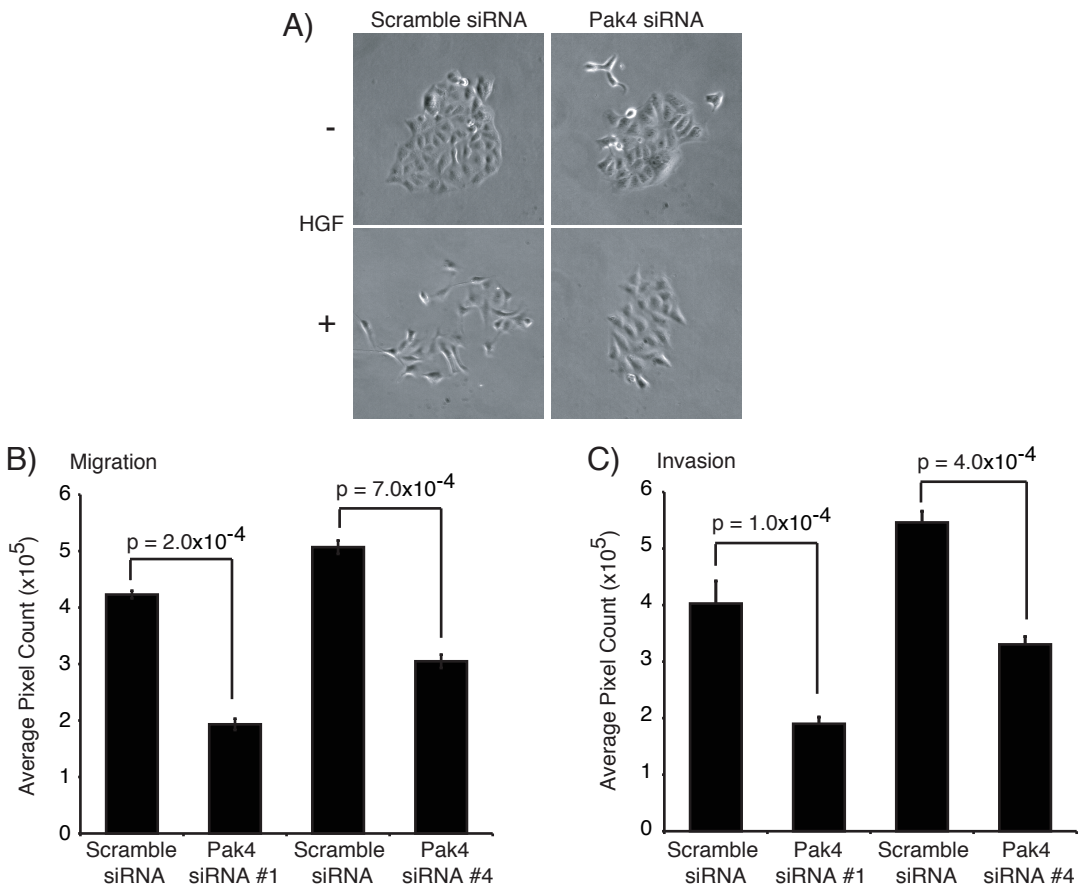


Figure 3.9: Pak4 knockdown affects HGF induced cell scatter, migration, invasion. **A)** MDCK cells (5x104) were treated with Pak4 siRNA for 96 h in a 24 well plate and a scatter assay performed. The following day, 1.35 ng/ml of HGF were added to each well and incubated for 24 h. Cells were then photographed with a Zeiss Axioskop microscope. **B)** A migration assay was performed using a modified Boyden chamber seeded with MDCK cells transfected with scramble or 2 different Pak4 siRNA **C)** Invasion assay was performed using a modified Boyden chamber coated with matrigel and MDCK cells transfected with scramble or 2 different Pak4 siRNA. **D)** Stable G17 cell lines expressing Gab1ΔPak4 were constructed. Blots represent relative levels of Gab1DPak4 expressed in the 4 different Gab1ΔPak4 used. **E)** Stable MDCK cells (5x104) expressing CSF-MetΔ Grb2 and Gab1ΔPak4 were plated in a 24-well plate and a scatter assay performed. The following day, 50 ng/ml of CSF-1 were added to each well and incubated for 24 hrs. Cells were then photographed with a Zeiss AxiosKop microscope. **F)** Stable MDCK cells lines were seeded in collagen and allowed to form cysts for 5 days, then were stimulated with CSF-1 (5 ng/ml). Representative images are shown. Pictures were acquired at a magnification of 10x. **G)** HeLa cells transiently transfected with 0, 2 μg, and 4 μg of HA-Pak4-ASM. **H)** A migration assay was performed using modified Boyden chambers seeded with HeLa cells transfected with 0, 2 μg, and 4 μg of HA-Pak4-ASM. **I)** Invasion assay was performed using a modified Boyden chamber coated with matrigel and MDCK cells transfected 0, 2 μg, and 4 μg of HA-Pak4-ASM. Results represent data performed in triplicate. Error bars represent standard error mean.

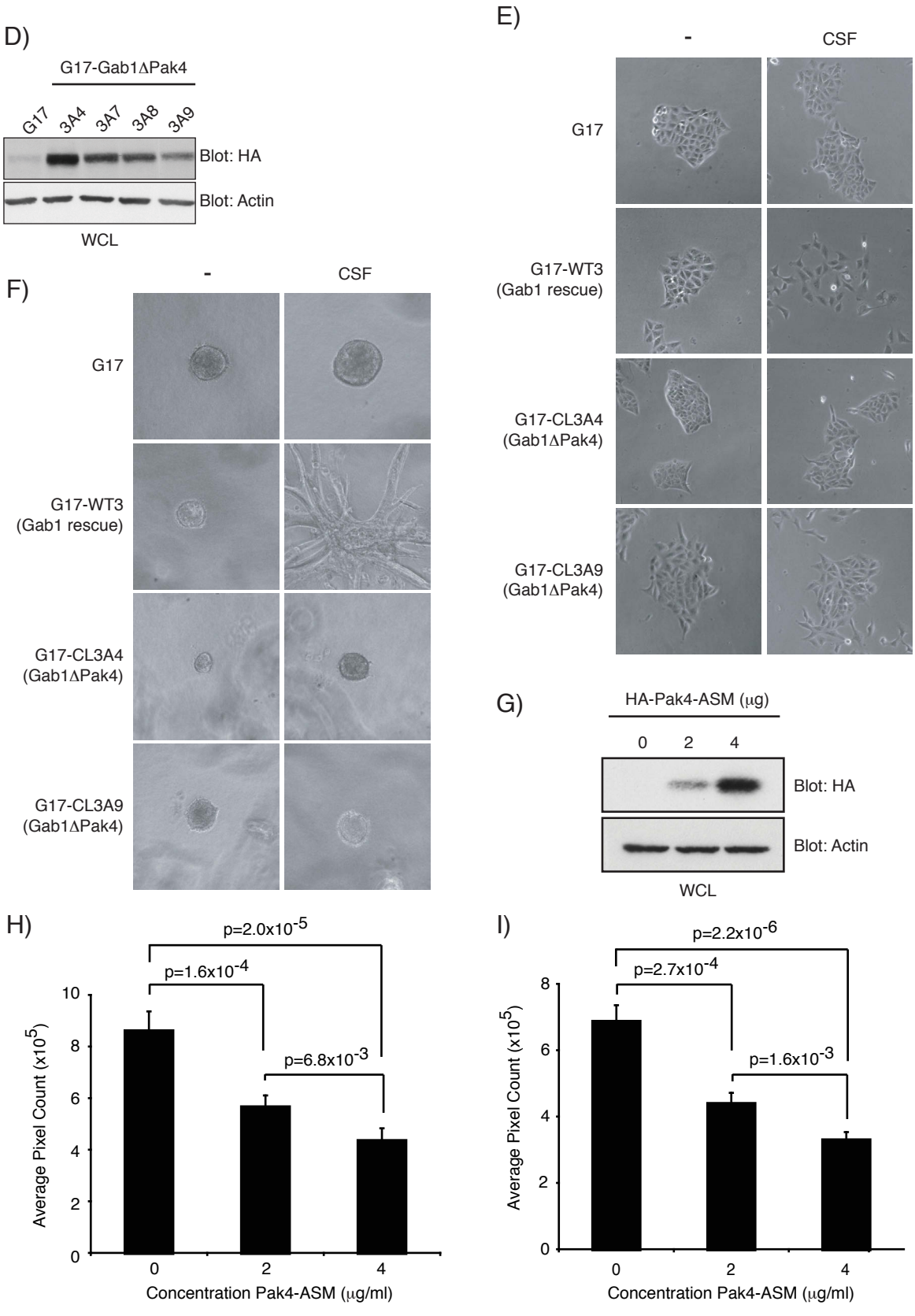
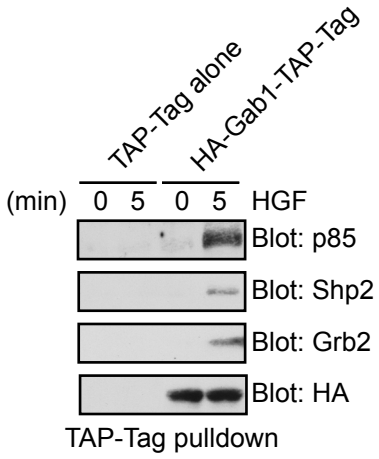
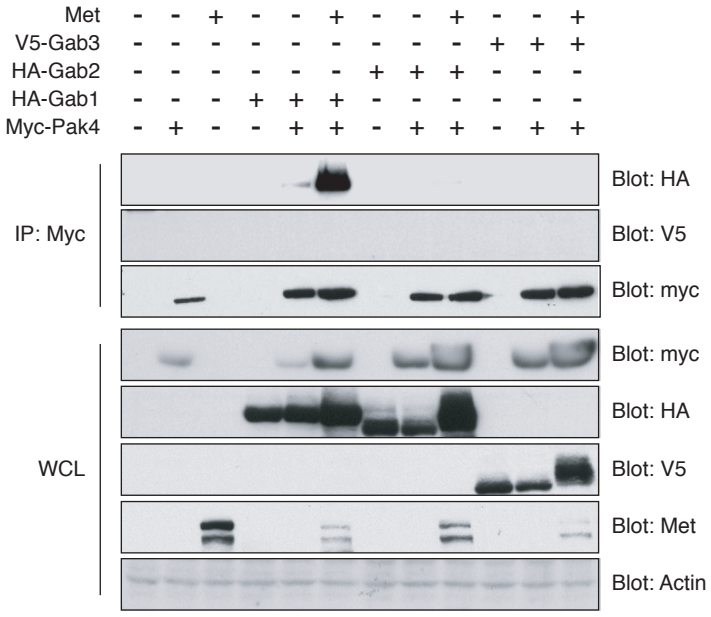


Figure 3.9: Pak4 knockdown affects HGF induced cell scatter, migration, invasion.

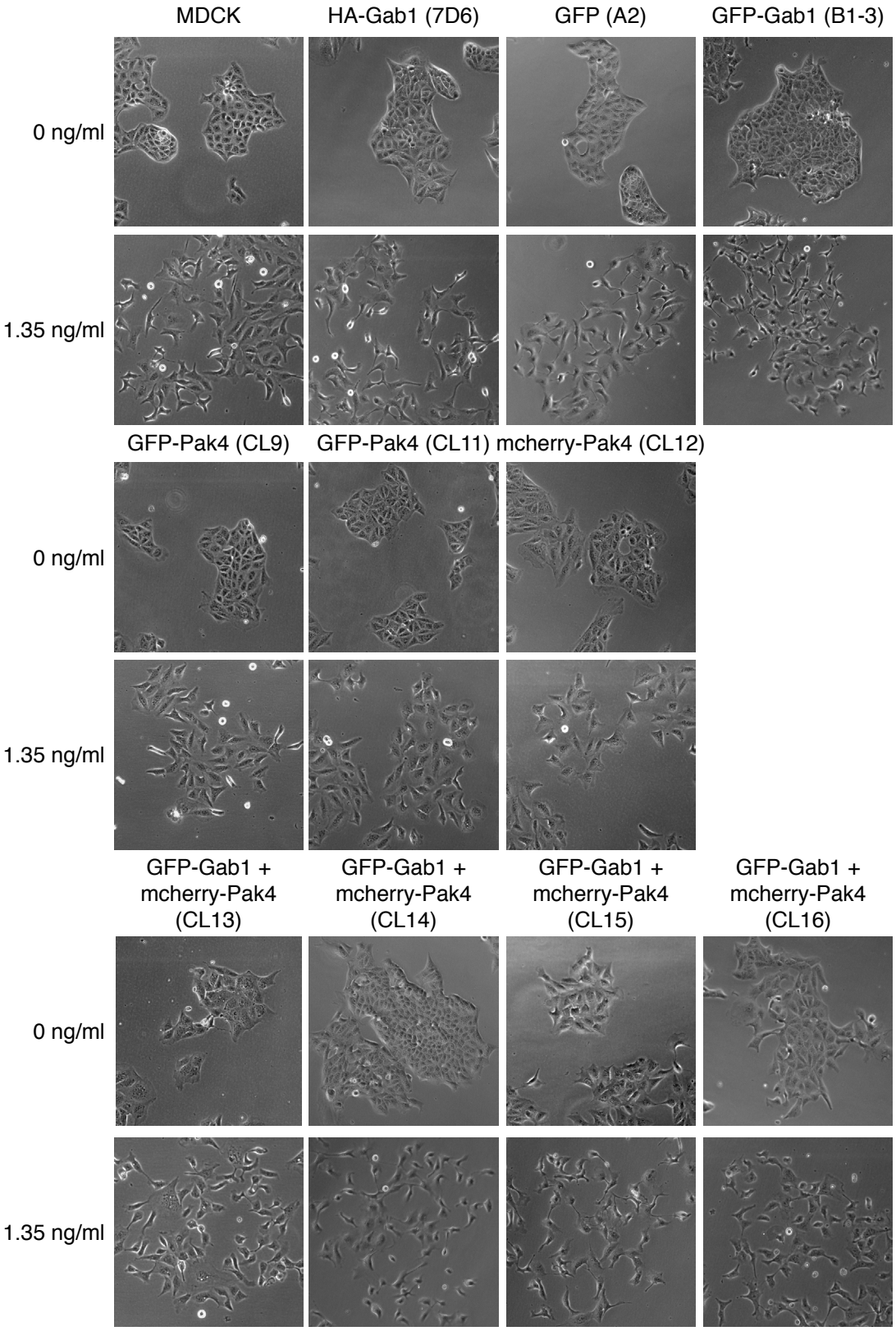


Supplemental Figure 3.1: Gab1-TAP tag protein associates with known Gab1-binding proteins. HEK293 stably expressing a TAP-tag vector alone or a Gab1-TAP-tag were stimulated for 5 min with HGF. TAP-tag proteins were coupled to Protein A, followed by TEV protease cleavage, and subsequent calmodulin binding. Pulldowns were separated by SDS-PAGE and immunoblot analysis performed with antisera against the p85 subunit of PI3K, Shp2, Grb2 and HA.

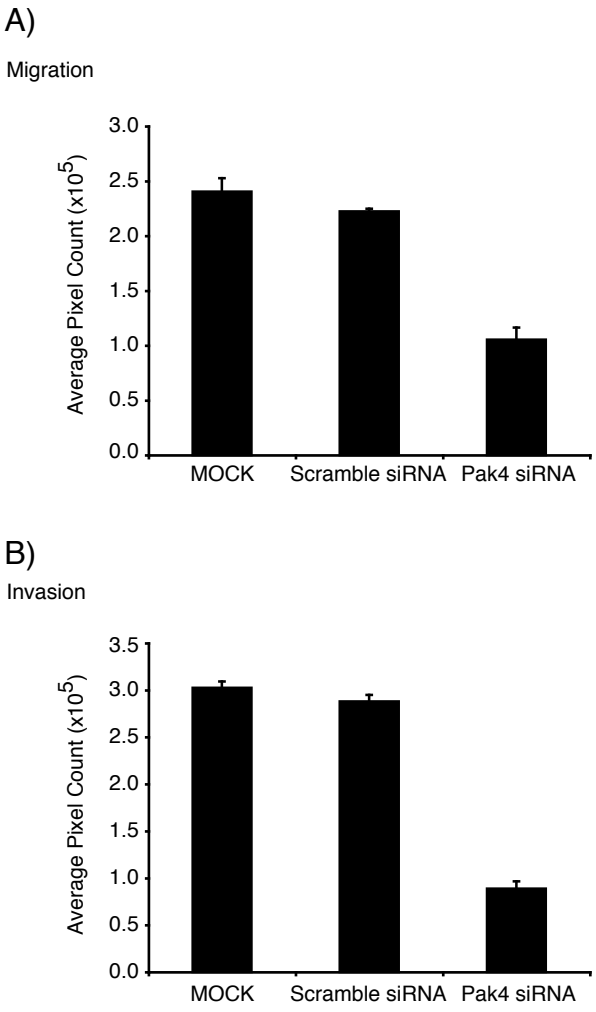


Supplemental Figure 3.2: Pak4 associates with Gab1 and not Gab2 or Gab3. HEK293 cells were transiently transfected with different combinations of HA-Gab1, HA-Gab2, V5-Gab3, myc-Pak4 and Met. Proteins from total cell lysate (500 μg) were immunoprecipitated with anti-myc antisera, separated by SDS-PAGE and immunoblotted with antisera for HA, V5, phosphotyrosine (PY100) and myc (9E10). Proteins from whole cell lysates (30 μg) were immunoblotted with antisera against HA, V5, myc (9E10) and actin

Supplemental Figure 3-3: MDCK cells expressing Gab1, Pak4 or both Gab1 and Pak4 undergo cell scatter in response to HGF. MDCK cells stably expressing Gab1, Pak4 or both Gab1 and Pak4 were plated ($5x10^3$) in 24 well plates. The following day, 1.35 ng/ml of HGF were added to each well and incubated for 24 h.

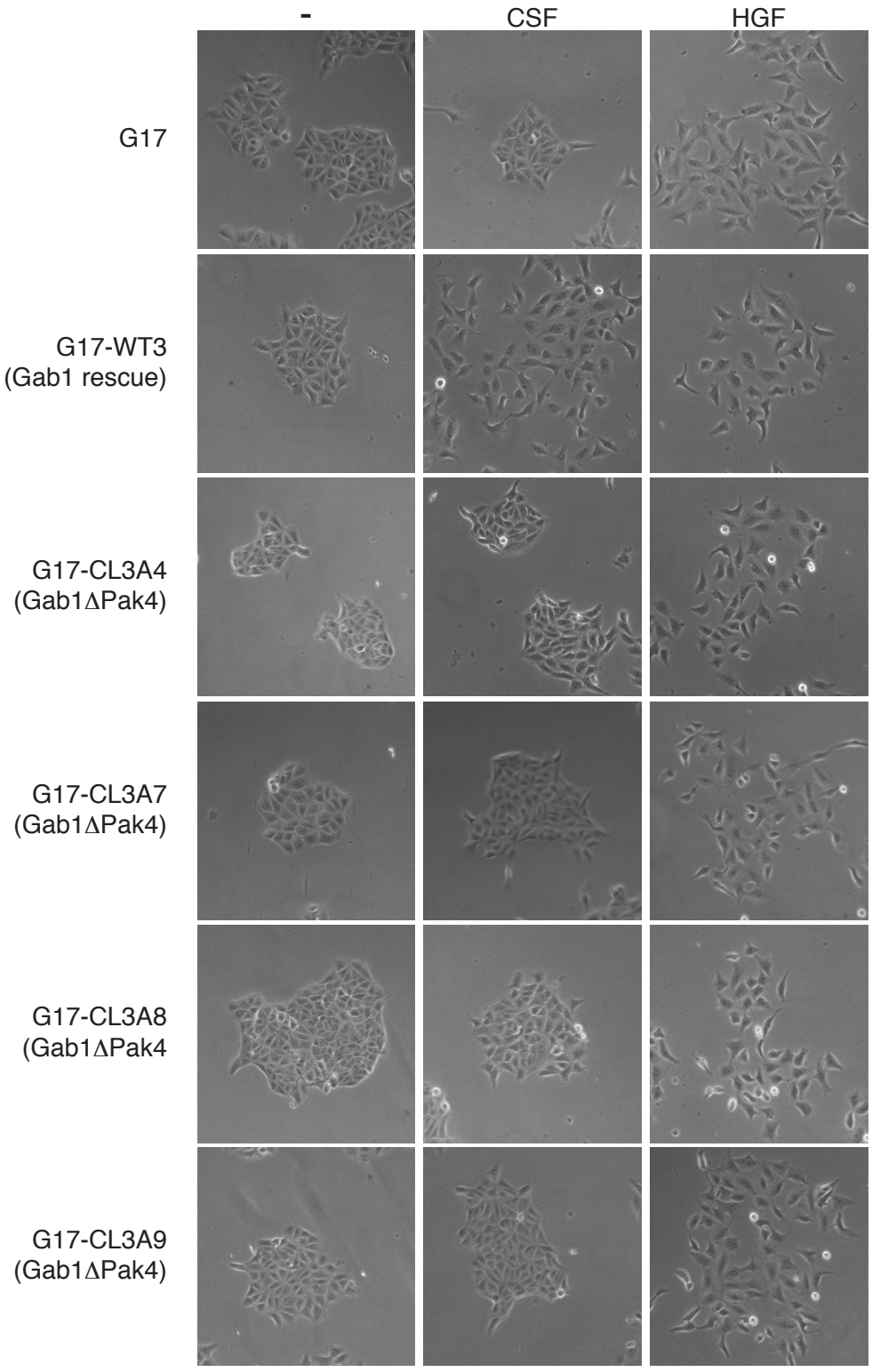


Supplemental Figure 3.3: MDCK cells expressing Gab1, Pak4 or both Gab1 and Pak4 undergo cell scatter in response to HGF.



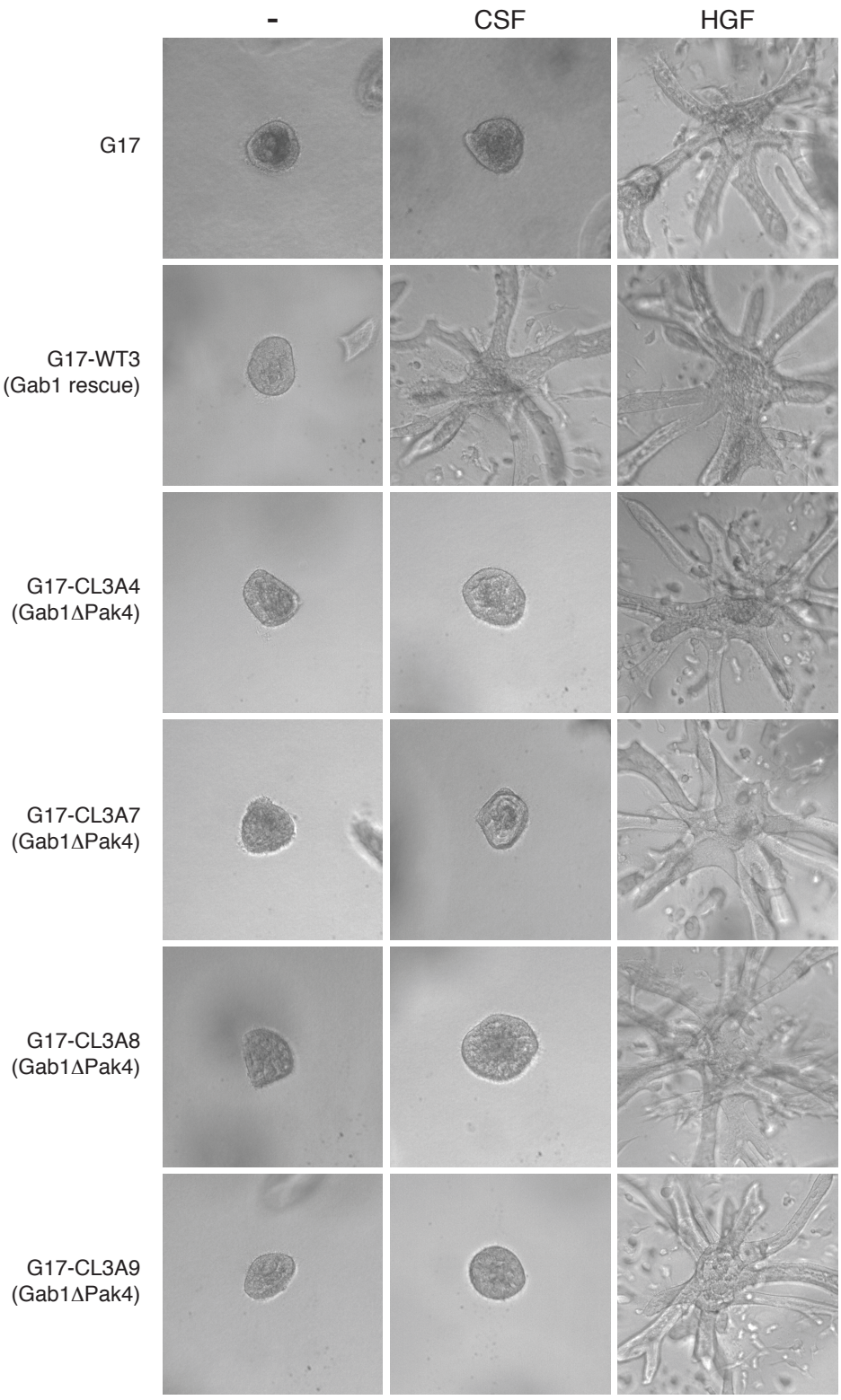
Supplemental Figure 3.4: Pak4 knockdown decreases the ability of HeLa cells to undergo migration and invasion. HeLa cells were treated with Pak4 siRNA for 72 h and migration and invasion assays were performed. **A)** A migration assay was performed using modified Boyden chambers seeded with HeLa cells untreated (MOCK) or transfected with scramble siRNA or Pak4 siRNA. **B)** Invasion assay was performed using a modified Boyden chambers coated with matrigel and HeLa cells untreated (MOCK), transfected with scramble siRNA or Pak4 siRNA. Bars represent standard error mean of the three experiments.

Supplemental Figure 3-5: Gab1ΔPak4 mutants fail to induce cell scatter following Met receptor activation. Stable MDCK cells ($5x10^4$) expressing CSF-MetΔGrb2 and Gab1ΔPak4 were plated in a 24-well plate and a scatter assay performed. The following day, 50 ng/ml of CSF-1 were added to each well and incubated for 24 h. Cells were then photographed with a Zeiss Axioskop microscope.



Supplemental Figure 3.5: Gab1ΔPak4 mutants fail to induce cell scatter following Met receptor activation

Supplemental Figure 3-6: Gab1ΔPak4 mutants fail to induce a morphogenic response downstream of the Met receptor. Stable MDCK cells ($5x10^4$) expressing CSF-MetΔGrb2 and Gab1ΔPak4 were seeded in collagen and allowed to form cysts for 5 days, then were stimulated with CSF-1 (5 ng/ml). Representative images are shown. Pictures were taken at a magnification of 10x.



Supplemental Figure 3.6: Gab1ΔPak4 mutants fail to induce a morphogenic response downstream of the Met receptor.

Supplemental Table 3-1: Primers used to generate multiple Gab1 and Pak4 mutants.

Construct	Primers
myc-Pak4 (1-297)	Fwd: 5' TCTGGCTAGCGGCATGTTTGGGAAGAGGAAGAAGCG Rev: 5' CCATGCGGCCGCTCACTGTGGCTCCCGCTGTGGTGAGC
myc-Pak4 (48-297)	Fwd: 5' TCTGGCTAGCGGCATGTTCACGGGGCTGCCCCGCCAGTGGC Rev: 5' CCATGCGGCCGCTCACTGTGGCTCCCGCTGTGGTGAGC
myc-Pak4 (293-591)	Fwd: 5' TCTGGCTAGCGGCATGCAGCGAGTATCCCATGAGCAGTTCCGG Rev: 5' CCATGCGGCCGCTCACTGTGGCTCCCGCTGTGGTGAGC
myc-Pak4 (325-591)	Fwd: 5' TCTGGCTAGCGGCATGCTGCCACCCCGACTGAAGAACCTGCACAAGG Rev: 5' CCATGCGGCCGCTCACTGTGGCTCCCGCTGTGGTGAGC
GFP-Pak4	Fwd: 5' TCTGGCTAGCGGCATGTTTGGGAAGAGGAAGAAGCG Rev: 5' CCATGCGGCCGCTCACTGTGGCTCCCGCTGTGGTGAGC
mCherry-Pak4	Fwd: 5' AAATTTGAATTCGGCTTTGGGAAGAGGAAGAAGCGGG Rev: 5' CCATGCGGATCCTCATCTGGTGCGGTTCTGGCGC
HA-Gab1 (116-546)	Fwd: 5' GCATGGGATCCCCGACATCTGTGGATTCAATCCCACAGAAG Rev: 5' GCATGAATTCTCACAGCTCTTCCCATTCTGACAGAGG
HA-Gab1 (116-444)	Fwd: 5' GCATGGGATCCCCGACATCTGTGGATTCAATCCCACAGAAG Rev: 5' GCATGAATTCTCACAGCTCTTCACCCGAGACACCTCCCG
HA-Gab1 (116-374)	Fwd: 5' GCATGGGATCCCCGACATCTGTGGATTCAATCCCACAGAAG Rev: 5' GCATGAATTCTCAACAGTAACTGCTGTCAGTGTCCGAGG
HA-Gab1 (1-234)	Fwd: 5' GCATGGGATCCCCATGAGCGGCGGCGAAGTGGTTTGCTCG Rev: 5' GCATGAATTCTCAGCCATTCATTCCGTGTTTGCTCTGGG
HA-Gab1 (438-695)	Fwd: 5' GCATGGGATCCCCGTCTCGGGTGAAGAGCTGGATGAG Rev: 5' GCATGAATTCTCACTTTACATTCTTGGTGGGTGTCTCGG
HA-Gab1 (231-546)	Fwd: 5' GCATGGGATCCCCAATGGCTTTTTCCAGCAACAAATGATG Rev: 5' GCATGAATTCTCACAGCTCTTCCCATTCTGACAGAGG
HA-Gab1 (348-695)	Fwd: 5' GCATGGGATCCCCCATCCAACTCATGACCGGTCTCCTGTGG Rev: 5' GCATGAATTCTCACTTTACATTCTTGGTGGGTGTCTCGG
HA-Gab1 (116-234)	Fwd: 5' GCATGGGATCCCCATGGACATCTGTGGATTCAATCCC Rev: 5' GCATGAATTCTCAGCCATTCATTCCGTGTTTGCTCTGGG
HA-Gab1ΔPak4	Fwd: 5' GCATCCCGGGTTTTTCCAGCAACAAATGATGTATGACTGCCCACCG Rev: 5' GCATCCCGGGATTGAATCCACAGATGTCACAGATACAACGGACCC

Chapter 4

4 GEF-H1, a Gab1 binding protein and a mediator of cellular migration, downstream of the Met receptor.

Grigorios N. Paliouras, Patrick De Koninck, Dongmei Zuo, Monica Naujokas and Morag Park. 2009. GEFH1, a Gab1 binding protein and a mediator of cellular migration, downstream of the Met receptor. *Manuscript in preparation.*

4.1 PREFACE

Following HGF binding to the Met receptor, the scaffold protein Gab1 is recruited to the activated receptor and phosphorylated. This generates binding sites for multiple downstream signaling proteins, such as the tyrosine phosphatase Shp2, PLCγ, the p85 subunit of PI3K and the adaptor protein Crk. In the previous chapter, I identified the Ser/Thr kinase, Pak4, as a novel Gab1-associated binding protein following Met receptor activation. The association between Pak4 and Gab1 was necessary for promoting epithelial cell dispersal and an invasive morphogenic response downstream from the Met receptor. The recruitment of Pak4 to the cell cortex within lamellipodia was dependent on Gab1 subcellular localization. Pak4 was further identified an integral component of Met-dependent migration and invasion. These data support a role for Pak4 as a mediator of actin dynamics involved during cellular migration. However, little is known regarding the signaling pathways activated downstream of Pak4. One protein found to interact with Pak4 is the microtubule bound guanine exchange factor, GEF-H1. Interestingly, phosphorylation and activation of GEF-H1 by Pak4 promotes the release of GEF-H1 from

microtubules and the formation of lamellipodia. This proposes a role for a Pak4-GEF-H1 signaling complex as a regulator of actin dynamics. In chapter IV, I wanted to explore the possibility of Gab1, Pak4 and GEF-H1 to form a complex downstream of the Met receptor and examine the consequence of GEF-H1 activation following HGF stimulation. Having established that Pak4 is critical for Met-dependent biology downstream of Gab1, the role of GEF-H1 downstream of Met is unknown and was examined in the following chapter.

4.2 ABSTRACT

Cell migration is a complex, highly regulated process that involves the coordinated activity of numerous proteins. Hepatocyte growth factor (HGF), the ligand for the Met receptor tyrosine kinase, induces epithelial cell migration, invasion and morphogenesis, events that require remodeling of the actin cytoskeleton. Downstream from Met, the adaptor protein Gab1 is essential for these biological processes. Here, we identify that HGF promotes the formation of a multi-protein complex involving Gab1 with the RhoA guanine nucleotide exchange factor, GEF-H1, and the p21-activated kinase 4 (Pak4). HGF stimulation promoted the subcellular redistribution of GEF-H1 from microtubules to the cytoplasm within membrane ruffles. GEF-H1 expression enhances cell migration and invasion downstream of the Met receptor and rates of wound closure correlate with GEF-H1 expression levels. GEF-H1-deficient cells form adhesions positive for paxillin, but negative for focal adhesion kinase (Fak). These data support a role for GEF-H1 in focal adhesion maturation and turnover and demonstrate an expanding role for Gab1-associated signals as important regulators of cellular migration and invasion downstream from the Met receptor.

4.3 ABBREVIATIONS

BSA	bovine serum albumin
Cdc42	Cell Division Cycle 42
Crk	v-crk sarcoma virus CT10 oncogene homolog
DMEM	Dubelcos Modified Eagles Medium
DOS	daughter of sevenless
Erk	extracellular signal-regulated kinase
Fak	focal adhesion kinase
Gab1	growth factor receptor bound protein 2-associated protein 1
GEF-H1	guanine exhange factor-H1
GFP	green fluorescent protein
GID	GEF-interacting domain
Grb2	growth factor receptor-bound protein 2
HA	hemagglutinin tag
HEK	human embryonic kidney
HGF	hepatocytes growth factor
Lbc	lymphoid blast crisis
Lfc	first cousin of Lbc
Limk	LIM domain kinase
MDCK	Madin-Darby Canine Kidney
Pak4	p21-activated kinase 4
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase

Rac	ras-related C3 botulinum toxin substrate 1
Rho	ras homolog gene family
Shp2	protein tyrosine phosphatase, non-receptor type 11
siRNA	short-interfering ribonucleic acid
SOC-1	suppressor of clear-1

4.4 INTRODUCTION

Cellular migration requires the coordinated activities of multiple proteins to alter the actin cytoskeleton to generate the protrusive forces at the leading edge and retraction at the cell rear. At the leading edge, microtubules regulate cortical polarity by delivering the necessary cues to activate the Rho GTPases such as Cdc42, Rac1 and RhoA, whose activation is correlated with the formation of filopodia, lamellipodia and stress fiber/focal adhesion formation, respectively. The spatial and temporal activation of these proteins regulates actin dynamics and is critical for the formation of filopodia and lamellipodia, formation and maturation of focal adhesions and cell retraction (Wittmann and Waterman-Storer 2001).

The HGF-Met signaling axis plays a significant role in promoting cellular migration. *In vivo*, genetic evidence from HGF- and Met- knockout mice emphasize a critical role for both proteins in the migration of myogenic precursor cells from the dermomyotome to the limb buds, which is lost upon genetic ablation of each gene (Bladt, Riethmacher et al. 1995; Schmidt, Bladt et al. 1995; Uehara, Minowa et al. 1995). The ability for the Met receptor to induce migration is evident when examining the consequence of HGF treatment of epithelial cells grown in culture. Upon activation of the Met receptor, tight and adherens junctions are disassembled and epithelial cells acquire a fibroblast-like shape before becoming motile and "scattering" (Stoker, Gherardi et al. 1987; Montesano, Matsumoto et al. 1991). This morphogenic event is dependent on the scaffold protein Gab1 (Maroun, Holgado-Madruga et al. 1999).

Gab1 is a member of the Gab family of adaptors/scaffold proteins which includes the mammalian Gab2 and Gab3, the *Drosophila melanogaster* DOS (daughter of

sevenless) and the *Caenorhabditis elegans* SOC-1 (suppressor of clear-1) (Herbst, Carroll et al. 1996; Holgado-Madruga, Emlet et al. 1996; Raabe, Riesgo-Escovar et al. 1996; Gu, Pratt et al. 1998; Schutzman, Borland et al. 2001; Wolf, Jenkins et al. 2002). The importance of Gab1 downstream of Met is highlighted by the Gab1 knock-out mouse which phenocopies the HGF and Met knock-out models, displaying many of the same phenotypical abnormalities, including a defect in the migration of myogenic precursor cells (Sachs, Brohmann et al. 2000). The binding of Gab1 to activated Met, leads to phosphorylation of Gab1 on tyrosine residues and the recruitment of multiple signaling proteins that are central modulators of the Met-induced morphogenic responses (Maroun, Holgado-Madruga et al. 1999; Maroun, Moscatello et al. 1999; Gual, Giordano et al. 2000; Maroun, Naujokas et al. 2000; Schaeper, Gehring et al. 2000; Lamorte, Rodrigues et al. 2002; Lock, Maroun et al. 2002). Gab1 provides binding sites for the tyrosine phosphatase, Shp2, the p85 subunit of PI3K, phospholipase Cγ, the adaptor protein Grb2, the Crk adaptor protein and the serine/threonine kinase Pak4 (Holgado-Madruga, Emlet et al. 1996; Garcia-Guzman, Dolfi et al. 1999; Maroun, Holgado-Madruga et al. 1999; Gual, Giordano et al. 2000; Maroun, Naujokas et al. 2000; Schaeper, Gehring et al. 2000; Paliouras, Naujokas et al. 2009). The association between Pak4 and Gab1 identified Pak4 as a key integrator of Met-dependent biology, as uncoupling their interaction resulted in a significant defect in cellular migration and invasive growth downstream from the Met receptor (Paliouras, Naujokas et al. 2009).

Pak kinases have been shown to be involved in many cellular signaling pathways including cell survival, cell transformation and actin cytoskeletal reorganization (Jaffer and Chernoff 2002; Arias-Romero and Chernoff 2008). The Pak family of Ser/Thr kinases consists of six members, which are subdivided into two groups: Pak 1-3 (group I)

and Pak 4-6 (group II) (Jaffer and Chernoff 2002). Only a limited number of pathways are found to be activated downstream of Pak4. This includes the Limk-cofillin pathway which is a critical regulator of actin dynamics and important in regulating migration following activation of the Met RTK (Ahmed, Shea et al. 2008; Paliouras, Naujokas et al. 2009). Another protein found to associate with Pak4 is the RhoA guanine nucleotide exchange factor GEF-H1 (Callow, Zozulya et al. 2005). Phosphorylation of GEF-H1 by Pak4 promotes the release of GEF-H1 from microtubules and into the cytoplasm, leading to the formation of actin-rich lamellipodia in NIH3T3 cells (Callow, Zozulya et al. 2005). The association of GEF-H1 with Pak4 occurs through the GEF interaction domain (GID) in Pak4 (Callow, Zozulya et al. 2005). Interestingly, the same region of Pak4 has been identified to mediate the association with the adaptor protein Gab1 (Paliouras, Naujokas et al. 2009), suggesting that Gab1 and GEF-H1 may compete for binding to Pak4 or they may form a multi-protein complex.

GEF-H1 was originally identified as a microtubule-associated RhoA GEF (Ren, Li et al. 1998; Glaven, Whitehead et al. 1999; Krendel, Zenke et al. 2002). Few Rho GEFS have been found to localize to microtubules; they include p190Rho-GEF, Lfc (first cousin of Lbc [lymphoid blast crisis-1] and the murine homolog of GEF-H1) and GEF-H1. The subcellular localization of GEF-H1 to microtubules and its activity is regulated by the amino-terminal C1 domain and the carboxy-terminal region of GEF-H1 (Krendel, Zenke et al. 2002). GEF-H1 binding to microtubules inhibits its activity, however, microtubule depolymerization leads to the activation of GEF-H1 and RhoA-dependent reorganization of the actin cytoskeleton (Krendel, Zenke et al. 2002). Multiple protein kinases regulate GEF-H1 activity via phosphorylation of specific residues. Phosphorylation of Ser885 of GEF-H1 by Pak1 induces the recruitment of 14-3-3 to

GEF-H1 and translocation of 14-3-3 to microtubules, suppressing the activity of GEF-H1 (Zenke, Krendel et al. 2004). The mitotic kinases Aurora A/B and Cdk1/Cyclin B phosphorylate GEF-H1 on Ser885 and Ser959 respectively, thereby inhibiting GEF-H1 catalytic activity and implicating GEF-H1 activation of RhoA in cytokinesis (Birkenfeld, Nalbant et al. 2007). Two proteins have been found to positively regulate GEF-H1 activity. Recently, Erk1/2 phosphorylation of GEF-H1 on Thr678 enhanced the activity of GEF-H1, however, the mechanism is still unknown (Fujishiro, Tanimura et al. 2008). Similarly, the activation of GEF-H1 by Pak4 positively regulates GEF-H1 activity, however the mechanism regulating their association is unknown.

The impact of growth factor stimulation on GEF-H1 activity has not been examined to date. In this study, we have identified a GEF-H1-Gab1-Pak4 protein complex downstream from the Met receptor. We show that GEF-H1 expression increases the migratory and invasive capabilities of MDCK cells and show that GEF-H1 is important in epithelial wound closure and the formation of mature focal adhesions downstream from the Met receptor.

4.5 EXPERIMENTAL PROCEDURES

Antibodies

Monoclonal anti-Pak4 and anti-Fak antibodies were purchased from BD Biosciences (San Diego, CA). Monoclonal anti-myc (9E10) antibodies were purchased from Cell Signaling Technology (Mississauga, ON). Polyclonal anti-GFP, monoclonal anti-paxillin and Alexa 488 conjugated phalloidin were purchased from Molecular Probes (Burlington, ON). Monoclonal anti-HA.11 antibody was purchased from Covance (Berkeley, CA). Polyclonal anti-actin was purchased from Sigma-Aldrich (St. Louis, MI). Polyclonal anti-GEF-H1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Met antibody (148) was generated by immunizing rabbits with the carboxy-terminal 16 amino acids of the human Met sequence as previously described (Rodrigues, Naujokas et al. 1991).

Cell culture and DNA transfections

All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 μg/ml Gentamicin (Invitrogen, Carlsbad, CA). Madin-Darby Canine Kidney (MDCK) epithelial cells expressing GFP-GEF-H1 were maintained in geneticin (400 μg/ml) (Invitrogen, Carlsbad, CA) and/or hygromycin (75 μg/ml) (Invitrogen, Carlsbad, CA). For transient expression of proteins, 1 x 10^6 HEK293 cells were seeded 24 hours (h) prior to performing transient transfections using Lipofectamine Plus (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Media was replaced 3h post-transfection, and cells were lysed 24-48h post-

transfection in 1% NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, pH 8.0, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4). The homogenates were centrifuged at 13,000 rpm for 15 minutes (min) to remove debris.

Immunoprecipitation and western blotting

HEK293 cell protein lysates (500 µg) were used for each immunoprecipitation. Antibodies were allowed to bind for 1h at 4°C with gentle rocking, and 10µl of Protein A or Protein G sepharose beads were then added to collect immune complexes. Beads were washed 3 times in lysis buffer, resuspended in 20µl of Laemmlli sample buffer and boiled for 5 min. Samples were resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with 3% bovine serum albumin (BSA) and probed with appropriate antibodies as described, followed by horseradish peroxidase-conjugated secondary antibodies. All immunoblots were visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

Confocal Microscopy & Live cell imaging

HeLa or MDCK (2×10^4) were seeded on glass coverslips (Bellco Glass Inc. Vineland, NJ, USA) in 24-well plates (Nalgene NUNC, Rochester, NY, USA) and, if required, were transfected with the indicated DNA using Lipofectamine Plus (Invitrogen, Carlsbad, CA) 16h post-plating, following manufacturer's instructions. Cells were serum starved for 2h prior to HGF treatment. Coverslips were washed once with PBS and then fixed with 2% paraformaldehyde (PFA) (Fisher Scientific) in PBS for 20 min. Coverslips were then washed four times in PBS and residual PFA was removed with three 5-minute washes in

100 mM glycine in PBS. Cells were permeabilized with 0.3% Triton X-100/PBS and blocked for 30 min in blocking buffer (5% BSA, 0.2% Triton X-100, 0.05% Tween 20, PBS). Coverslips were incubated with primary and secondary antibodies diluted in blocking buffer for 1 h and 40 min, respectively, at room temperature. Coverslips were mounted with Immumount (Thermo-Shandon, Pittsburgh, PA). Confocal images were taken using a Zeiss 510 Meta laser scanning confocal microscope (Carl Zeiss, Canada Ltd, Toronto, ON) with 100x objective. Image analysis was carried out using the LSM 5 image browser (Empix Imaging, Mississauga, ON). Confocal live cell imaging was performed with a Spinning disk confocal microscope from Quorum Technologies. Data from the spinning disk microscopy was analyzed using Volocity 4.1 software (Improvision, Coventry, England).

Migration/Invasion Assay

MDCK cells (5×10^4) were counted and seeded directly onto 6.5-mm Corning Costar transwell chambers for migration, or transwells coated with 100 $\mu g/cm^2$ matrigel (BD Biosciences, San Jose, CA) for invasion assays. Complete media was added to both the top and bottom wells, and cells were incubated at 37°C overnight. For HGF stimulations, 34 ng/ml of HGF was added to the bottom wells. Following overnight incubation, cells on both sides of the transwells were fixed with 10% neutral buffered formalin for 20 min at room temperature. After washing with double-distilled water, cells were stained with 0.1% crystal violet in 20% methanol for 20 min at room temperature. Cells on the top layer were scraped off and membranes were left to dry overnight. Images were captured using a Retiga 1300 digital camera (QIMAGING, Burnaby, BC) and a Zeiss Axioskop microscope(Carl Zeiss Canada Ltd., Toronto, ON). Image analysis and quantification was

performed using the Scion Image-NIH equivalent program for Microsoft Windows (Scion Company, Frederick, MD).

Wound Closure Assay

Confluent cell monolayers, in 6 well dishes, were carefully wounded by use of a 10-µl sterile pipette tip. Cellular debris was removed by washing with 3xPBS. The wound monolayers were then incubated in Leibovitz medium containing 10% FBS overnight and photographed with a Zeiss Axioivert 200 M inverted microscope equipped with motorized X Y stage, multi-well plate heating insert and AxioCam CCD camera (Carl Zeiss Canada Ltd., Toronto, ON). The microscope and subsequent analysis was driven by Zeiss Axiovision 4.2 acquisition software.

4.6 RESULTS

4.6.1 Gab1 associates with GEF-H1 following Met receptor activation

GEF-H1 is a microtubule associated guanine-nucleotide exchange factor (GEF), that when activated, can mediate the crosstalk between microtubules and the actin cytoskeleton (Ren, Li et al. 1998; Glaven, Whitehead et al. 1999; Krendel, Zenke et al. 2002; Ryan, Alldritt et al. 2005; Birukova, Adyshev et al. 2006; Chang, Nalbant et al. 2008). GEF-H1 forms a complex with the p21-activated kinase (Pak) family member Pak4 (Callow, Zozulya et al. 2005) during which Pak4 can promote the redistribution of GEF-H1 from microtubules to the cytoplasm and promote membrane ruffling and lamellipodia formation. We have recently established that Pak4 is recruited to the scaffold protein Gab1, following HGF stimulation and that both proteins localize at the leading edge of a cell within lamellipodia (Paliouras, Naujokas et al. 2009). To establish if GEF-H1 is present in a complex with Gab1, vectors expressing GFP-tagged-Gab1 and myc-tagged-GEF-H1 were transiently transfected in HEK293 cells in the presence or absence of Met (Figure 4.1A). Transient overexpression of Met leads to its activation in the absence of ligand (Rodrigues, Naujokas et al. 1991; Paliouras, Naujokas et al. 2009). Binding between Gab1 and GEF-H1, as established by co-immunoprecipitation, is observed in the absence of Met receptor, however, this association was significantly increased in the presence of kinase active Met (Figure 4.1A).

4.6.2 Gab1, Pak4 and GEF-H1 exist in a complex

To establish if all three proteins could exist in a complex, HEK293 cells were transiently transfected with vectors expressing HA-tagged-Gab1, GFP-tagged-GEF-H1

and increasing concentrations of myc-tagged-Pak4, in the presence of the Met receptor (Figure 4.1B). Consistent with association of Pak4 with Gab1 and GEF-H1, an increase in co-immunoprecipitation of Pak4, with GEF-H1 or Gab1 is observed with increasing concentrations of Pak4 (Figure 4.1B). Following co-immunoprecipitation with Gab1 or GEF-H1, all three proteins were observed supporting the formation of a complex. However, the levels of GEF-H1 that co-immunoprecipitated with Gab1 or vice versa were not altered with increasing levels of Pak4 (Figure 4.1B). These data suggest that Pak4 may not act as an intermediate between Gab1 and GEF-H1 in the complex.

Pak4 interaction with Gab1 is mediated through the GEF-interacting domain (GID) in Pak4 (Callow, Zozulya et al. 2005). Interestingly, the association between Gab1 and Pak4 was also dependent on an intact GID domain within Pak4 (Paliouras, Naujokas et al. 2009). This would suggest that Pak4 might bind independently to Gab1 and GEF-H1 through its GID domain. To determine if overexpression of Gab1 can compete with GEF-H1 for binding to the GID domain of Pak4, HEK293 cells were transiently transfected with vectors expressing myc-Pak4, GFP-GEF-H1, Met receptor and increasing concentrations of HA-Gab1. The association of Gab1 with GEF-H1 is enhanced with increasing concentrations of Gab1 (Figure 4.1C). Interestingly, the levels of Pak4 that co-immunoprecipitate with GEF-H1 is also increased (Figure 4.1C). These data support that all three proteins can exist in a complex together, and that Gab1 may possess a separate binding site for both Pak4 and GEF-H1.

4.6.3 HGF stimulation alters GEF-H1 localization

The impact of growth factor signaling on GEF-H1 subcellular localization has not been examined to date. Following HGF stimulation, MDCK cells expressing GFP-GEF-

H1 were subjected to live-cell microscopy. In unstimulated MDCK cells, GFP-GEF-H1 localized to microtubules, as previously shown (Ren, Li et al. 1998; Krendel, Zenke et al. 2002; Benais-Pont, Punn et al. 2003) (Figure 4.2). Following HGF stimulation of MDCK cells, membrane ruffling was observed as early as 3 min post-stimulation (Figure 4.2). While the majority of GFP-GEF-H1 remained localized to microtubules, a diffuse and cytoplasmic localization of GFP-GEF-H1 was observed in membrane ruffles by 3 min post stimulation. As membrane ruffling decreases by 9 min post-HGF stimulation, GFP-GEF-H1 is observed on microtubules that appear within the membrane ruffle (Figure 4.2). These data suggest that HFG stimulation induced a transient redistribution of GEF-H1 from a microtubule compartment to a membrane ruffling cytoplasmic compartment.

4.6.4 GEF-H1 enhances HGF stimulated migration and invasion

To address the biological relevance of GEF-H1 downstream of the Met receptor, we created stable MDCK cells lines expressing GFP-GEF-H1 (Figure 4.3A). To assess if overexpression of GEF-H1 can increase the migratory and invasive potential of single MDCK cells, Boyden chamber transwell assays, either uncoated to assess cell motility or coated with matrigel to assess cell invasion, to examine the potential of HGF to enhance the migration and invasion of MDCK cells (Paliouras, Naujokas et al. 2009). When compared to control MDCK cells stably overexpressing GFP (Figure 4.3B and 4.3C), MDCK cells stably overexpressing GEF-H1 showed no significant increase in migration or invasion in the absence of HGF. However, in response to HGF, a significant increase in HGF-induced migration (1.5-fold) (Figure 4.3C) and invasion (2.0-fold) (Figure 4.3C) of MDCK cells overexpressing GEF-H1 was observed. Hence this provides evidence

supporting a role for GEF-H1 in mediating the migratory and invasive response downstream from the Met receptor.

4.6.5 GEF-H1 enhances wound-closure

To assess the role of GEF-H1 on cell migration, sheets of MDCK cells were subjected to an *in vitro* scratch assay that examines wound closure of sheets of cells (Liang, Park et al. 2007). Confluent monolayers of MDCK cells stably expressing GFP alone (A2) or GFP-GEF-H1 (2GG2-5, 1GG3-5 and 1GG3-2) were wounded by scraping with a pipette tip, creating a space free of cells and time for closure was examined (Figure 4.4A). A scratch wound in the GFP-GEF-H1 MDCK stable cells was closed after 7 hours (Figure 4.4A), whereas a significant delay was observed in the closure of the GFP-expressing MDCK (Figure 4.4A). This correlated with migration of MDCK cells expressing GEF-H1 being significantly higher (17.5 μm/hr) than control cells (11 μm/hr) (Figure 4.4B).

4.6.6 GEF-H1 knockdown impairs wound-healing of MDCK

The above data demonstrate that GEF-H1 overexpression enhances epithelial cell migration and invasion. To assess the requirement for GEF-H1 on the migratory capacity of MDCK, the consequence of GEF-H1 knockdown was examined. MDCK cells lines in which GEF-H1 protein levels were stably reduced by two different siRNA specific against canine GEF-H1 (Benais-Pont, Punn et al. 2003) were produced (Figure 4.5A). Wound-healing assays on GEF-H1 knockdown MDCK cells showed a decrease in the migratory speed of wound closure when compared to control MDCK cells expressing scrambled siRNA (Figure 4.5B and 4.5C). Overall, significant decreases of 27% to 50%

were seen with GEF-H1 siRNA #1 and 35% to 76% were seen with GEF-H1 siRNA#2 (Figure 4.5B and 4.5C).

4.6.7 Loss of Fak positive, but not paxillin positive, focal adhesions

The dynamic assembly and disassembly of focal adhesions plays a central role in cell migration. Two proteins shown to be important in the formation and maturation of focal adhesions are the adaptor protein paxillin and the protein kinase focal adhesion kinase (Fak), respectively (Mitra, Hanson et al. 2005; Deakin and Turner 2008). To address the consequence of GEF-H1 knockdown on the reduced migratory speed seen during wound closure (Figure 4.5), GEF-H1 knockdown cells were stained for both paxillin and Fak to assess the formation of focal adhesions. Immunofluorescence against paxillin revealed no difference in the size or number of paxillin-positive focal adhesions (Figure 4.6A). Interestingly, GEF-H1 knockdown cells displayed no Fak-positive staining (Figure 4.6B), suggesting a defect in the maturation and turnover of focal adhesions. This correlates with the decrease in the migratory speed observed in GEF-H1 knockdown cells (Figure 4.5).

4.7 DISCUSSION

Our results have provided insight into a novel protein complex between the adaptor protein Gab1, the serine/threonine kinase Pak4 and the Rho GTPase GEF-H1 downstream of the Met receptor. We had previously shown Pak4 to be a downstream target of the Met receptor and provided a mechanism through which a Gab1-Pak4 complex modulates Met-dependent signals needed for cellular migration and invasion (Paliouras, Naujokas et al. 2009). We now show that the Pak4-binding partner GEF-H1, is present in the same complex together with Gab1 and Pak4 following Met receptor activation (Figure 4.1). Furthermore, HGF stimulation induced the relocalization of GEF-H1 from microtubules into membrane ruffles (Figure 4.2). The overexpression of GEF-H1 in MDCK cells increased the speed of closure in a scratch-wound healing assay (Figure 4.4) and the migratory and invasive capacity of cells in Boyden chamber assays (Figure 4.3). The ability of GEF-H1 knockdown cells to make mature focal adhesions was impaired as GEF-H1 knockdown significantly reduced the number of FAK-positive adhesions (Figure 4.6B), however the quantity of paxillin adhesions was unaltered (Figure 4.6A). These results correlated to a reduced speed of closure of MDCK cells, depleted of GEF-H1, in a scratch-wound healing assay (Figure 4.5).

We have shown that GEF-H1 can associate with both Gab1 and Pak4 and this association is enhanced in the presence of activated Met (Figure 4.1). One issue arises when discussing the potential of a multi-protein complex between Gab1, Pak4 and GEF-H1. GEF-H1 interacts with Pak4 via a GEF-H1 interaction domain (GID) present within Pak4 (Callow, Zozulya et al. 2005), which also mediates the association between Gab1 and Pak4 (Paliouras, Naujokas et al. 2009). However, when overexpressed, Gab1 is not

sufficient to compete for binding to Pak4 with GEF-H1 (Figure 4.1). This suggests that a separate binding site exists on Gab1 for GEF-H1. To examine this further, Gab1 structure function will need to be performed to determine the region/domain of Gab1 mediating the association with GEF-H1 downstream from Met receptor. To confirm that Gab1, Pak4 and GEF-H1 can exist in a complex downstream of Met activation, size exclusion chromatography should be performed to isolate protein-protein complexes and characterize their association.

At the leading edge of a migrating cell, microtubules regulate cortical polarity by delivering the necessary cues that activate small Rho GTPases such as Rac1, Cdc42 and RhoA. These, in turn, regulate actin dynamics, and lamellipodia and focal adhesion formation, the protrusive and pulling forces needed to promote migration (Wittmann and Waterman-Storer 2001). The redistribution of GEF-H1 from microtubules to the cytoplasm within a membrane ruffle (Figure 4.2) suggests a role for GEF-H1 in leading edge actin dynamics and migration. Further analysis of Figure 4.2 shows that as the membrane ruffle begins to collapse on itself and retract onto the cell cortex, GEF-H1 is redistributed back onto microtubules. This suggests that GEF-H1 is negatively regulated within the membrane ruffle. Pak1 has been shown to negatively regulate GEF-H1 by phosphorylation of residue Ser885. Phosphorylation of Ser885 induces the recruitment of 14-3-3 protein to GEF-H1 and its recruitment to microtubules (Zenke, Krendel et al. 2004). Thus, Pak1 and 14-3-3 are involved in the regulation of GEF-H1 activity and that phosphorylation of GEF-H1 by Pak1 can thus act to coordinate Rac/Cdc42- and Rho-dependent signaling pathways. The ability of Pak1 to localize to membrane ruffles and to be activated downstream of the Met receptor (Royal, Lamarche-Vane et al. 2000) suggests that one function of Pak1 following HGF stimulation is the spatial and temporal

regulation of GEF-H1 activity. However, further work deducing the negative regulation of GEF-H1 needs to be explored.

In order for cell motility to occur, cells must organize a branching actin meshwork at the leading edge forming a protrusion or lamellipodium, establish new adhesions at the leading edge of the cell, shift forward through cell body contraction, and detach cell adhesions at the rear of the cell (Raftopoulou and Hall 2004). These steps involve reorganization of the actin cytoskeleton through a dynamic process of actin assembly and disassembly in a spatially- and temporally-coordinated manner in order to generate directional movement. Data presented in this paper show that GEF-H1 overexpression can increase the migratory capabilities of MDCK cells as measured by wound closure and Boyden chamber assays (Figure 4.3 and 4.4). The mechanism through which GEF-H1 can regulate cellular motility is still unknown. GEF-H1 was initially characterized as a RhoGEF necessary for the activation of Rac1 and RhoA (Ren, Li et al. 1998), suggesting a role for GEF-H1 in both lamellipodia formation and stress fiber formation. However, subsequent studies revealed that GEF-H1 only possessed GEF activity for RhoA (Krendel, Zenke et al. 2002; Benais-Pont, Punn et al. 2003; Zenke, Krendel et al. 2004). Nonetheless, it has been shown that GEF-H1 overexpression is capable of stimulating the formation of membrane ruffles and lamellipodia (Ren, Li et al. 1998; Glaven, Whitehead et al. 1999; Callow, Zozulya et al. 2005), and proposed that GEF-H1 may modulate the localization of Rac1 within the cell and not its activation. In contrast, a kinase-dead Pak4 when co-expressed with GEF-H1 failed to promote lamellipodia formation and contained many stress fibers (Callow, Zozulya et al. 2005). We have already shown that Pak4 siRNA and uncoupling the recruitment of Pak4 to Gab1 reduced the migratory capabilities of MDCK and HeLa cells following HGF stimulation (Paliouras, Naujokas et

al. 2009). The impact of recruitment of Pak4 to Gab1 on GEF-H1 subcellular localization and activity should be examined using the Gab1ΔPak4 mutant that has been previously characterized in downstream of the Met receptor (Paliouras, Naujokas et al. 2009). This data will be important to better understand the spatial and temporal activation of GEF-H1. A Gab1ΔPak4 mutant is unable to recruit Pak4 to the leading edge (Paliouras, Naujokas et al. 2009) may impact GEF-H1 activation and its redistribution into membrane ruffles (Figure 4.2).

Gab1 acts as a scaffold protein responsible for the localized recruitment of signals downstream from the Met receptor to specific subcellular locations. Therefore, the ability of Gab1 to associate with Pak4 can play an important role in regulating the localized activation of GEF-H1. Phosphorylation of GEF-H1 by other kinases such as Pak1 (Zenke, Krendel et al. 2004), Aurora A and CDK1 (Birkenfeld, Nalbant et al. 2007), have all been reported in the down-regulation of GEF-H1 activity. In addition to Pak4 positively regulating GEF-H1 activity, phosphorylation of GEF-H1 by Erk1/2 increases its GEF activity, leading to the activation of RhoA (Fujishiro, Tanimura et al. 2008). Although not shown, this suggests that Erk1/2 phosphorylation of GEF-H1 results in the dissociation of GEF-H1 from microtubules and release into the cytoplasm. Interestingly, many growth factor receptors are known activators of the MAPK pathways. Furthermore, downstream from the Met receptor, the recruitment of Shp2 to Gab1 leads to sustained activation of the MAPK-Erk1/2 signaling pathway (Maroun, Naujokas et al. 2000), which is important in mediating multiple Met biological responses, including morphogenesis (Maroun, Naujokas et al. 2000), and lamellipodia formation (Frigault, Naujokas et al. 2008). The recruitment of Gab1 to the lamellipodia promotes localized Erk1/2 activation

at the leading edge and specific uncoupling of the Shp2 tyrosine phosphatases to Gab1 abrogates lamellipodia formation (Frigault, Naujokas et al. 2008). Therefore, the association of GEF-H1 to Gab1 may allow for the localized activation of GEF-H1 near the leading edge of a migrating cell, allowing for rearrangements in actin cytoskeletal dynamics.

The ability for a cell to form mature focal adhesions is important in generating the contractile forces necessary to promote cellular migration. The lack of Fak positive staining in GEF-H1 knockdown cells correlates with the reduction in migratory speed seen in the wound closure assays (Figure 4.5 and 4.6). A decrease in the rate of migration and spreading was also exhibited in FAK -/- MEFs (Ilic, Furuta et al. 1995), supporting the results that we observed (Figure 4.5 and 4.6). Fak is recruited to paxillin-positive focal adhesions via the LIM domains, LD2 and LD4, of paxillin. Fak mediated phosphorylation of Y31 and Y118 of paxillin is necessary for the recruitment of additional molecules necessary for focal adhesion maturation and organization of the actin cytoskeleton (Bellis, Miller et al. 1995; Schaller and Parsons 1995; Cary, Han et al. 1998; Turner, Brown et al. 1999);(Scheswohl, Harrell et al. 2008). Our studies suggest that GEF-H1 is required for Fak recruitment to paxillin-promoted focal adhesions in MDCK cells (Figure 4.6). The recruitment of Fak to focal adhesions induces the autophosphorylation of Fak at tyrosine 397, creating docking sites for the recruitment of multiple proteins, including Src (Schaller 2001). Interestingly, paxillin -/- cells exhibit reduced localization of FAK to focal adhesions and reduced phosphorylation of Fak (Hagel, George et al. 2002; Wade, Bohl et al. 2002). This suggests that paxillin binding may function in the regulation of Fak activity, in addition to its proposed role in regulating the localization of FAK. Further analysis of the phosphorylation status of

paxillin and Fak in GEF-H1 depleted cells will need to be analyzed to determine if a potential defect in recruitment or activation of Fak is responsible for lack of Fak positive adhesions.

This study describes the formation of a multi-protein signaling complex downstream from Met involving Gab1, Pak4 and GEF-H1, and propose a novel role for GEF-H1 in focal adhesion maturation. This correlates with the migratory and invasive response of sheets of epithelial cells downstream from the Met receptor. The role of Gab1 as a scaffold protein plays an important role in the invasive growth of epithelial cells downstream from Met. Deregulation of the Met receptor is associated with many human cancers (Birchmeier, Birchmeier et al. 2003) highlighting the importance to identify and deduce the molecular mechanisms regulating the spatial and temporal regulation of protein-protein interactions and protein activation and the subsequent impact on tumourigenesis, invasion and metastasis.

4.8 ACKNOWLEDGMENTS

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Figure 4.1: Gab1, Pak4 and GEF-H1 exist in a complex downstream from Met. A) Following transient transfection of GFP-Gab1, myc-GEF-H1 and Met in HEK293 cells, total cell lysate (500 μg) were immunoprecipitated with anti-myc sera. Immunoprecipitations and whole cell lysates (30 mg) were separated by SDS-PAGE and immunoblot analysis was performed with indicated antibodies. **B)** A competition assay was performed with increasing concentrations of myc-Pak4 to compete for binding with Gab1 and GEF-H1. HEK293 cells were transiently transfected with HA-Gab1, GFP-GEF-H1, myc-Pak4 and Met in HEK293 cells. Proteins were extracted and total cell lysate (500 μg) were immunoprecipitated by anti-HA, anti-GFP and anti-myc (9E10) sera, separated by SDS-PAGE and immunoblot analysis performed. Immunoblot blot analysis was performed on whole cell lysate (30 μg) and probed with antisera against HA, myc, GFP, Met and actin. **C)** A competition assay was performed with increasing concentrations of HA-Gab1 to compete for binding with Pak4 and GEF-H1. HEK293 cells were transiently transfected with HA-Gab1, GFP-GEF-H1, myc-Pak4 and Met in HEK293 cells. Proteins were extracted and total cell lysate (500 μg) were immunoprecipitated by anti-HA, anti-GFP and anti-myc (9E10) sera, separated by SDS-PAGE and immunoblot analysis performed. Immunoblot blot analysis was performed on whole cell lysate (30 μg) and probed with antisera against HA, myc, GFP, Met and actin.

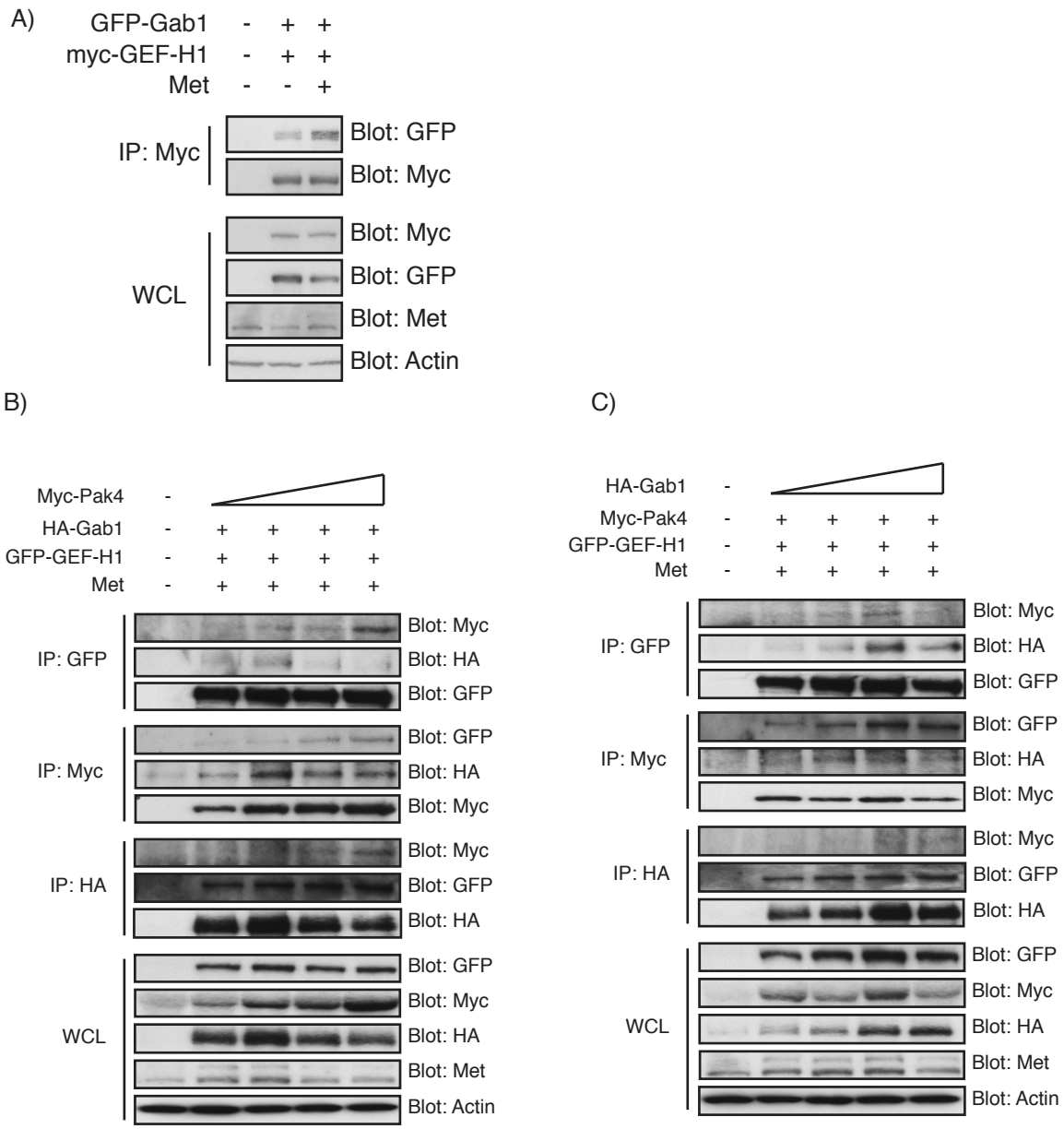

Figure 4.1: Gab1, Pak4 and GEF-H1 exisit in a complex downstream from Met.

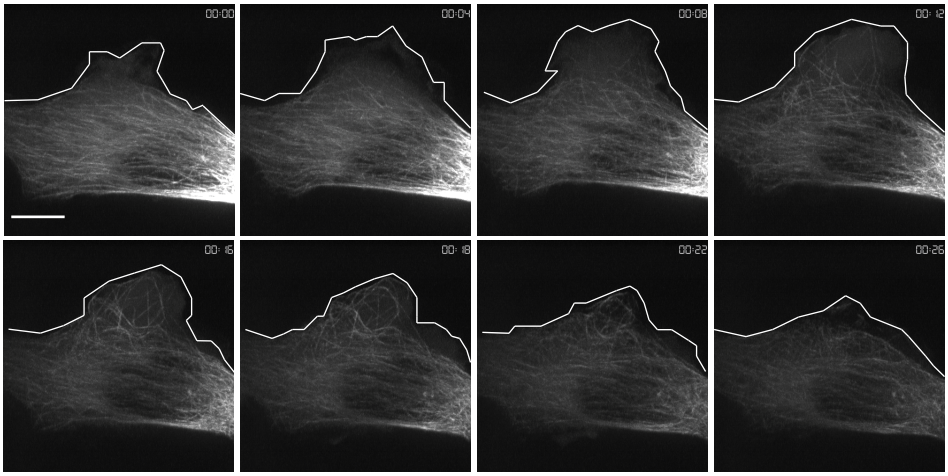

Figure 4.2: GEF-H1 localizes to cytoplasm and membrane ruffles following HGF stimulation. MDCK cells stably expressing GFP-GEF-H1 (2 x 104) were plated on glass bottom dishes, and the next day stimulated with HGF. Spinning disk time-lapse microscopy of living cells with single time frames taken at the indicated times post-HGF stimulation with a 63x objective. Images shown represent extended focus of 0.2 μm thickness slices of z-stack. Bar represents 10 μm.

Figure 4.3: Overexpression of GEF-H1 induced migration and invasion of MDCK cells downstream of Met receptor activation. A) Proteins from whole cell lysate (30 μg) from MDCK with stable overexpression of GFP-GEF-H1 were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-GFP and anti-actin sera. **B&C)** MDCK cells overexpressing GFP-GEF-H1 were analyzed for their migration and invasion capacity in the presence of HGF (34 ng/ml). Cells were seeded (5×10^4 cells) onto **B)** modified Boyden chambers or **C)** onto matrigel-coated Boyden chamber and assayed for their migration and invasion capacity respectively. Using a Zeiss Axioskop microscope, bottom layers of the transwell were imaged in five separate fields for each condition using a 10x objective in phase contrast. Image analysis of these assays was carried out using Scion Image. Bars represent standard error of the three experiments.

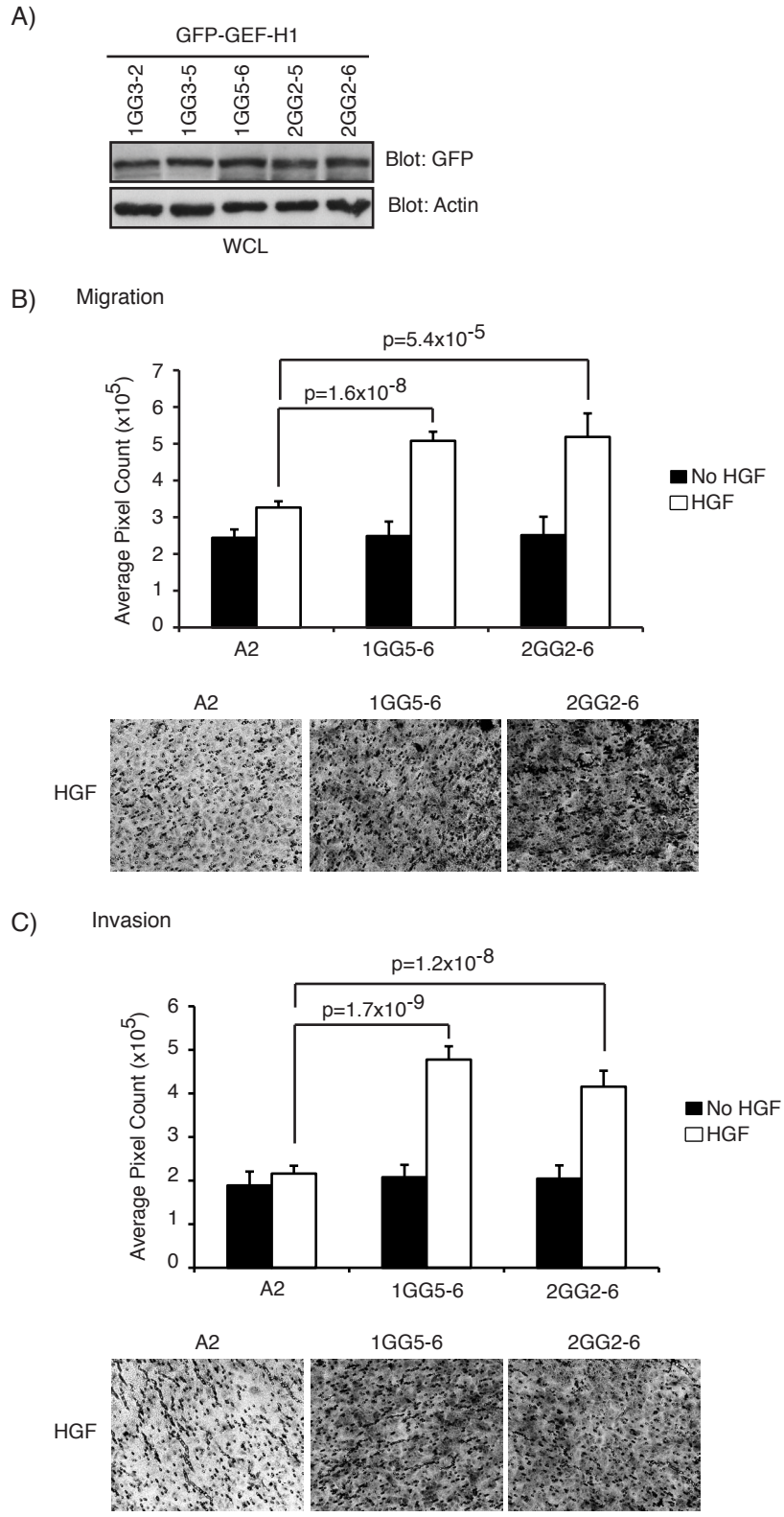

Figure 4.3: Overexpression of GEF-H1 induced migration and invasion of MDCK cells downstream of Met receptor activation.

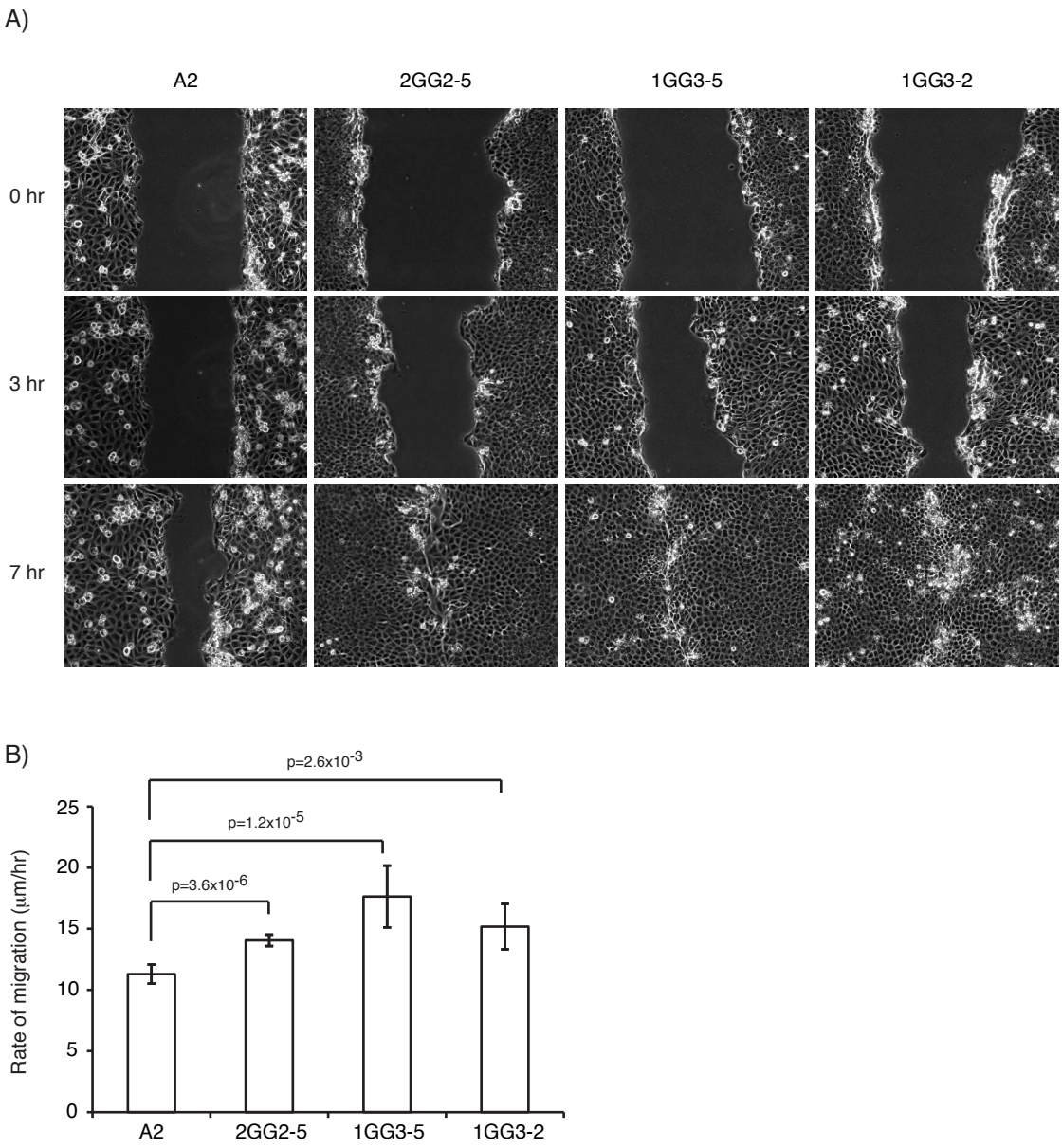

Figure 4.4: Overexpression of GEF-H1 increases migration and speed of MDCK in an in vitro scratch-wound assay: A) MDCK in vitro scratch assay. Wound was applied to cell monolayer as described in detail under "Materials and Methods". Representative fields showing the wounded area and cell migration into the wound at zero time and after 7 h, of control (A2) and GEF-H1 overexpressing MDCK stable lines. Images are 10x magnification. **B)** Quantification of speed of wound closure. Graph shows the rate of wound closure for each cell line tested. Error bars indicate standard error based on three independent experiments.

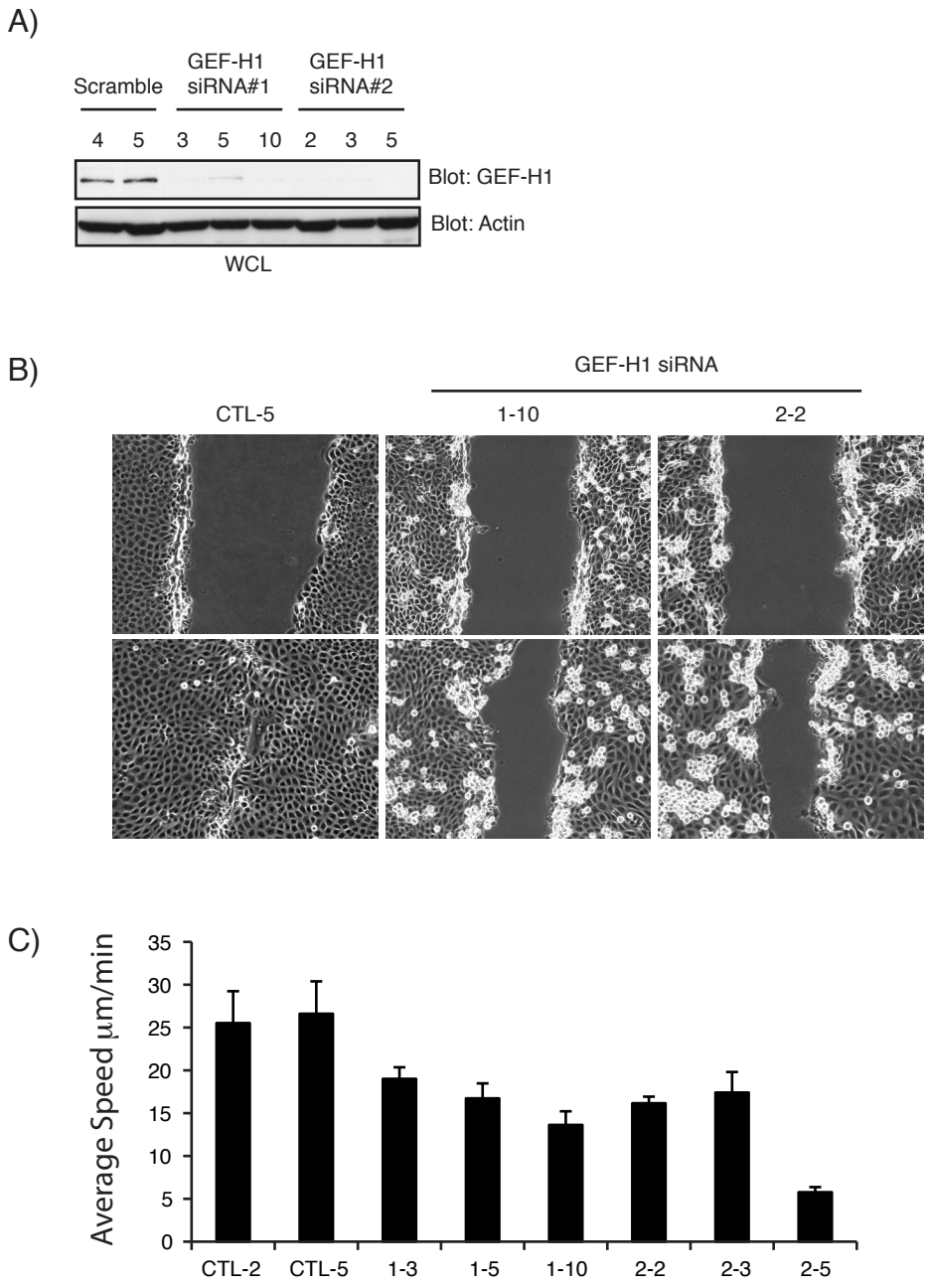

Figure 4.5: GEF-H1 knockdown reduces migration and speed of MDCK in an in vitro scratch-wound assay. A) Proteins from whole cell lysate (30 μg) from MDCK cells and MDCK with stable GEF-H1 knockdown were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-GEF-H1 and anti-actin sera. **B)** Wound was applied to cell monolayer as described in detail under "Materials and Methods". Representative fields showing the wounded area and cell migration into the wound at zero time and after 7 h, of control (CTL-2, CTL-5) and clones form 2 different GEF-H1 siRNA. Images are 10x magnification. **C)** Quantification of speed of wound closure. Graph shows the rate of wound closure for each cell line tested. Error bars indicate standard error based on three independent experiments.

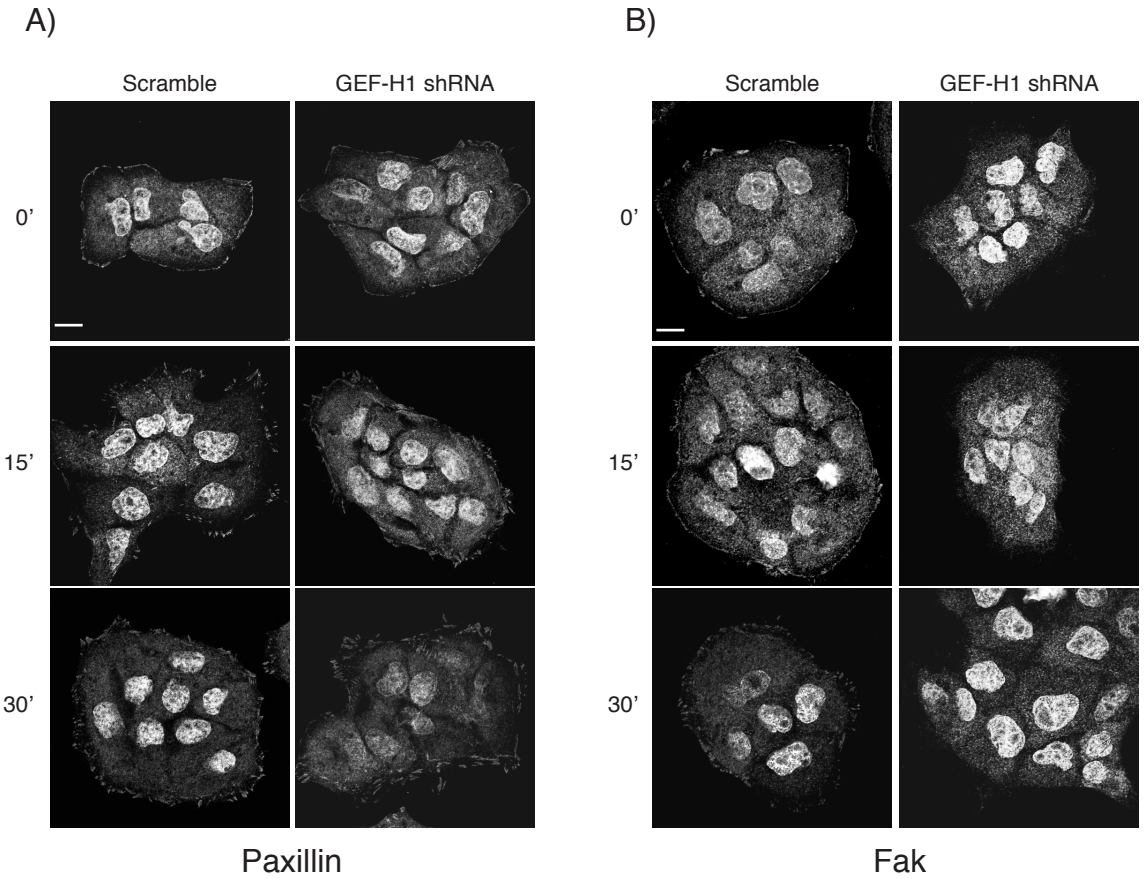

Figure 4.6: GEF-H1 knockdown cells possess Paxillin focal adhesions, but not FAK. MDCK cells stably expressing scramble or GEF-H1 siRNA were plated on coverslips. Following overnight incubation, cells were serum starved for 2 h, and stimulated with 35 ng/ml HGF at 37°C for the indicated time points. Coverslips were fixed in 3% PFA, and stained with **A)** anti-Paxillin and **B)** anti-FAK sera (second panel). Confocal images were taken with a 100x objective. Bar represents 10 μm.

CHAPTER 5

5 GENERAL DISCUSSION

5.1 Introductory Statement

The Met RTK mediates pleiotropic effects including cell growth, survival, epithelial dispersal and epithelial morphogenesis. These effects are fundamental for the diverse biological functions observed *in vivo* during embryogenesis, as well as in the adult, during organ/tissue regeneration and wound healing (Bladt, Riethmacher et al. 1995; Schmidt, Bladt et al. 1995; Uehara, Minowa et al. 1995; Birchmeier, Birchmeier et al. 2003; Ishibe, Karihaloo et al. 2009). Deregulation of these signalling pathways alters the balance and duration of the signals initiated from an activated RTK and leads to the development of human disease, such as cancer. HGF and Met are deregulated in many human cancers via multiple mechanisms including increased expression of HGF and/or Met; amplification of Met; point mutations in the Met kinase domain that leads to increased activity, as well as loss of negative regulation through the lose of the Cbl-binding site as a result of splicing mutations; and autocrine and paracrine loops. The full consequence of these alterations is still unknown. Therefore, a further understanding of the mechanisms regulating signal transduction initiation is important as we develop targeted therapeutics against the Met receptor for the treatment of cancer. In this thesis I have presented the characterization of three different proteins, the protein tyrosine phosphatases PTP1B, the serine/threonine kinase Pak4 and the guanine nucleotide exchange factor GEF-H1 and their role in regulating a biological response downstream from the Met receptor tyrosine kinase.

5.2 Dephosphorylation of the Met receptor

PTP1B actively dephosphorylates the EGFR (Flint, Tiganis et al. 1997), PDGFR (Haj, Markova et al. 2003), the Insulin receptor (Elchebly, Payette et al. 1999; Salmeen, Andersen et al. 2000), and the IGF-IR (Buckley, Cheng et al. 2002). At the start of this thesis, little was known of the role of protein tyrosine phosphatases on regulating the Met receptor tyrosine kinase or downstream signalling following stimulation with HGF. Three phosphatases had been identified to be associated with the Met receptor, DEP-1(Palka, Park et al. 2003), PTP-S (Villa-Moruzzi, Puntoni et al. 1998), and LAR (Machide, Hashigasako et al. 2006), however their impact on Met receptor signalling and biology had not been elucidated.

In Chapter II, I was the first to demonstrate that PTP1B-deficient mice and hepatocytes are resistant to Fas-induced hepatic damage and this correlated to hyperphosphorylation of the Met receptor and an increase in the pro-survival signals NF-κB, Erk and FLIP. Furthermore, inhibition of Met signalling through the use of a Met-specific inhibitor removed the protection seen in PTP1B-deficient hepatocytes. These results supported our hypothesis that the Met receptor was a physiological substrate for PTP1B activity and supported our initial *in vitro* characterization of Met as a substrate for PTP1B (Sangwan, Paliouras et al. 2008).

5.3 Mechanism of PTP1B specificity

The Met RTK contains twin tyrosine residues, 1234 and 1235, in the catalytic domain necessary for activation of the receptor. One method used to identify potential substrates of PTPs is by using substrate trapping mutants (Flint, Tiganis et al. 1997).

Substrate trapping mutants function to "trap" the substrate of interest via the interaction of the catalytic domain of the PTP with the phosphorylated tyrosine, without catalyzing the dephosphorylation of the tyrosine residue (Flint, Tiganis et al. 1997). Using a substrate-trapping mutant of PTP1B (Flint, Tiganis et al. 1997), the Met receptor was "trapped" and identified as a substrate for PTP1B (Sangwan, Paliouras et al. 2008). A requirement for the twin tyrosines was demonstrated by tyrosine to phenylalanine mutations of the twin tyrosines (Y1234F/Y1235F) of Met, which abolished the interaction (Sangwan, Paliouras et al. 2008). However, the precise means of interaction between Met and PTP1B are still undefined.

PTP1B was hypothesized to associate with the Met receptor following biochemical and crystal structure analysis of the phosphorylated tyrosine kinase domain of the insulin receptor kinase (IRK). The kinase domain of both the IR and Met contain twin tyrosine residues critical for receptor activation. The crystal structure of PTP1B and a peptide of the IRK that was phosphorylated on tyrosines Y1162 and Y1163 revealed the positioning of Y1162 in the catalytic cleft of PTP1B and Y1163 in the phosphotyrosine recognition motif that formed by a conformational change of the WPD loop (Salmeen, Andersen et al. 2000). The phosphotyrosine at position 1163 increased the peptide affinity for PTP1B by 70-fold relative to the same peptide with an unphosphorylated tyrosine residue at 1163 (Salmeen, Andersen et al. 2000). In addition to the twin tyrosines, the N- and C-terminal amino acids flanking the twin tyrosines, aspartic acid and arginine respectively, were also found to potentially contribute to the specificity of PTP1B (Salmeen, Andersen et al. 2000). Many RTKs, in addition to the IR, such as the IGF-1R, FGFR1-4, TrkA/B/C, and MuSK, also contain twin tyrosines in their activation loop. A potential consensus sequence of (D/E)YY(R/K) was hypothesized to identify

substrates of PTP1B (Salmeen, Andersen et al. 2000; Li, Depetris et al. 2005). However, this potential PTP1B consensus motif is unlikely to account for the observed *in vivo* specificity of PTP1B for the insulin receptor and other RTKs. The consensus binding motif of PTP1B as defined for RTKs only partially applies for the Met receptor as the C-terminal amino acid flanking tyrosine 1235 of Met is serine (S). Structural modeling of the kinase domain of Met and PTP1B needs to be performed to examine if Y1234 and Y1235 position themselves within the catalytic domain and recognition motif of PTP1B to promote an interaction between Met and PTP1B.

In addition a direct interaction between PTP1B and the twin tyrosines of IRK, another mechanism has been proposed that is independent of the phosphotyrosines in the IRK activation loop, but rather dependent to the opposite side of the kinase domain (Li, Depetris et al. 2005). This interaction, termed "backside binding", is mediated via tyrosines 152 and 153 within the catalytic domain of PTP1B and the β2-β3 loop of the IRK. Mutation of these two tyrosine residues reduced the interaction between PTP1B and the IR and was less efficient at dephosphorylating the IR than WT (Bandyopadhyay, Kusari et al. 1997; Dadke, Kusari et al. 2000; Dadke and Chernoff 2002). However, at the time of this study, it was proposed that the "backside binding" interaction was specific for PTP1B and the IR based on the sequence analysis and known structures of kinase domains of RTKs (Li, Depetris et al. 2005). We can not conclude the precise interface responsible for mediating the interaction between Met and PTP1B, however, it would be interesting to model the crystal structure of the Met kinase domain and PTP1B and compare it to the known modes of interaction between PTP1B and the IRK.

5.4 Met and liver regeneration

Extensive research has defined a role for the HGF-Met signalling axis in response to liver damage and liver regeneration. HGF was originally identified as a potent mitogenic factor for hepatocytes and serum levels of HGF were increased following partial hepatectomy (PHx) of rats (Nakamura, Nawa et al. 1984; Nakamura, Nishizawa et al. 1989). A requirement for HGF and Met in liver development has been established by genetic ablation studies (Bladt, Riethmacher et al. 1995; Schmidt, Bladt et al. 1995; Uehara, Minowa et al. 1995). Furthermore, direct genetic evidence for the role of HGF/Met in liver regeneration using liver specific Met-conditional knockout mouse models (Borowiak, Garratt et al. 2004; Huh, Factor et al. 2004) has been demonstrated.

Recently, the role of the Met receptor in promoting oval cell proliferation and protecting against oxidative stress have begun to shed more light on the impact of Met-dependent signalling in liver regeneration and protection against liver damage. However, what has become apparent is the similarities that are seen between the above mentioned mechanisms and the data presented in chapter II of this thesis.

5.4.1 Oval Cells, the liver stem cell

The importance of the HGF-Met signalling pathway in liver physiology is not limited only to its direct action on hepatocytes and its ability to induce their proliferative potential following hepatic injury. Hepatic progenitor cells are also responsible for regenerative growth following liver injury (Fausto and Campbell 2003; Knight, Matthews et al. 2005). Hepatic progenitor cells are also termed “oval” cells due to their distinct ovoid shape when visualized in histological cross sections (Farber 1956). Oval cells express the Met receptor and other growth factor receptors (Hu, Evarts et al. 1993; Hu,

Evarts et al. 1996; del Castillo, Factor et al. 2008), however, little is known regarding the molecular mechanisms responsible for promoting oval cell expansion and differentiation. Oval cells are normally quiescent in the liver and only become activated under certain conditions. HGF stimulation of oval cells induces their proliferation through activation of the PI3K/Akt and NF-κB signalling pathways (Okano, Shiota et al. 2003; Yao, Zhan et al. 2004). What is becoming apparent is that the regulatory signals activated in hepatocytes that mediate liver regeneration are similar to the components of the oval cell-triggered regenerative response (Santoni-Rugiu, Jelnes et al. 2005).

The importance of the Met receptor activation and signalling in oval cell biology was recently examined via generation of oval cells from liver-specific, Met-conditional knockout mice (del Castillo, Factor et al. 2008). Met-deficient oval cells displayed enhanced sensitivity to apoptosis following serum starvation and TGF-β stimulation, which is involved in the termination of liver regeneration, acting as a growth inhibitor and pro-apoptotic factor (Braun, Mead et al. 1988; Sanchez, Alvarez et al. 1996; del Castillo, Factor et al. 2008). Interestingly, both WT and Met-deficient oval cells expressed HGF mRNA. Conditioned medium collected from both Met-expressing and Met-deficient cells was capable of inducing tyrosine phosphorylation of Met and inhibiting TGF-β induced apoptosis in Met-expressing cells. These data suggest an autocrine mechanism responsible for Met-dependent oval cell survival. Furthermore, this presents another potential mechanism responsible for the protection seen in PTP1B-null hepatocytes following Jo-2 induced liver damage. Hepatic stellate cells are the main source of HGF production in the liver (Michalopoulos and DeFrances 1997). However, the ability for oval cells to produce HGF can then serve as another source of HGF to enhance

hepatocyte proliferation and inhibit apoptosis, as seen in PTP1B-null mice. No direct evidence supporting a role for PTP1B on stem cell proliferation and differentiation exists. Therefore, what impact PTP1B has on oval cell proliferation and differentiation is unknown and needs investigating.

5.4.2 Reactive oxygen species (ROS) and increased hepatic apoptosis

When levels of ROS exceed the physiological threshold, cells enter a state of oxidative stress, which can increase their sensitivity to apoptosis (Shiba and Shimamoto 1999; Tripathi and Hildeman 2004). The levels of ROS in the liver increases following liver injury, leading to increased apoptosis (Schwabe and Brenner 2006). Furthermore, some studies have shown that the levels of ROS generated upon Fas stimulation play an important role in Fas-mediated apoptosis (Devadas, Hinshaw et al. 2003). Accumulating evidence exists for HGF to function as an antioxidant factor and protect against oxidative stress (Tsuboi 1999; Kannan, Jin et al. 2004). The mechanism proposed to enhance this effect is through the PI3K/Akt pathway, leading to the activation of the redox-sensitive transcription factor, NF-κB, which regulates the expression of antioxidants, such as manganese superoxide dismutase (MnSOD) and anti-apoptotic target genes, such as FLIP (Fan, Gao et al. 2005; Schwabe and Brenner 2006).

Recent work has suggested that the apoptotic effect seen in Met-null hepatocytes treated with Jo-2 was due to an increase in production of reactive oxygen species (ROS) (Gomez-Quiroz, Factor et al. 2008). Treatment of Met-null hepatocytes with N-Acetylcysteine and glutathione (GSH) precursor, both antioxidants, significantly reduced Jo-2 induced cell death (Gomez-Quiroz, Factor et al. 2008). Interestingly, Met-null mice hepatocytes exhibited intrinsic activation of NF-κB, as well as increased levels of Bcl-2

and Bcl-xL. These results suggest the level of activation of NF-κB and protein levels of Bcl-2 and Bcl-xL were below the threshold needed to provide protection (Gomez-Quiroz, Factor et al. 2008). This was also observed in WT hepatocytes treated with Jo-2 compared to PTP1B-null hepatocytes where only and only partial degradation of IκBa is seen in WT cells (Figure 2.3). This study poses an interesting question since localized ROS elevation levels have been found to inhibit PTPs, including PTP1B (Salmeen and Barford 2005), which is correlated to an increase in RTK phosphorylation (Lee, Kwon et al. 1998; Mahadev, Zilbering et al. 2001). However, in WT animals 6 hours post Jo-2 injection, we saw no increase in the levels of phosphorylation of the IR, IGF1R or Met receptor over basal (Figure 2.6). The production of ROS within PTP1B-null and WT mice following Jo-2 injection was never examined, therefore, we cannot conclude if ROS production had an impact on PTP1B catalytic activity in WT mice.

Mice that are Stat3-deficient are also sensitive to Jo-2-induced apoptosis (Haga, Terui et al. 2003). Interestingly, Stat3 protected against hepatic liver failure through 2 independent mechanisms, a redox-dependent mechanism through increased expression of the Stat3-inducible gene, redox-associated protein redox factor-1 (Ref-1) and a redox-independent mechanism via upregulation of the anti-apoptotic proteins FLIP, Bcl-2 and Bcl-xL. In the study by Haga *et al.* they found that Ref-1 functions by suppressing oxidative stress and redox-sensitive caspase-3 activity (Haga, Terui et al. 2003). Ref-1 is a reducing agent that facilitates the DNA-binding activities of many redox-sensitive transcription factors including NF-κB. Ref-1 causes the reduction of oxidized cysteine residues within the DNA binding domains of several transcription factors. Reduction of these residues is required for DNA binding, providing a redox-dependent mechanism for

regulation of target gene expression. We can speculate then that the enhancement of NF-κB-dependent transcription, upregulates the expression of anti-apoptotic proteins, such as FLIP, inhibits the DISC complex formation and subsequent activation of the apoptotic caspase cascade. It would be interesting to examine the levels of Stat3 phosphorylation in PTP1B-null mice following Jo-2 treatment. Stat3 is a downstream substrate of Jak2, which has been identified as a substrate of PTP1B (Myers, Andersen et al. 2001), therefore, PTP1B-null hepatocytes may possess higher Stat3 activity, and thus increased Ref-1 expression.

5.5 Increased Met phosphorylation in PTP1B-null mice

To further test that Met is a physiological substrate for PTP1B, I injected HGF into the hepatic portal vein of both WT and PTP1B-null mice and examined the level of phosphorylation of Met using phospho-specific antibodies (Figure 5.1) (Sangwan, Paliouras et al. 2008). Total phosphorylation of Met and specific tyrosine residues of Met were significantly enhanced in PTP1B-null animals when compared to WT. This is similar to the IR, where livers from PTP1B-null mice exhibited higher IR phosphorylation than livers from PTP1B-WT mice in response to insulin (Elchebly, Payette et al. 1999). In agreement with the observed increase in Met tyrosine phosphorylation, the Erk and Jnk2/3 pathways downstream of Met are more highly phosphorylated in livers from PTP1B-null mice in response to HGF (Figure 5.1). This is consistent with the observed elevation of pERK downstream from insulin, EGF, and PDGF RTKs (Haj, Markova et al. 2003; Galic, Hauser et al. 2005) in PTP1B-null MEFs

What other potential consequences can dephosphorylation by PTP1B have on Met receptor signalling? RTK signalling is regulated by PTPs in both a positive and negative manner following RTK activation. One mechanism involves positive regulation of Erk signalling by the tyrosine phosphatase Shp2 following growth factor stimulation through an undetermined mechanism. In contrast, another mechanism involves downregulation of receptor signalling by dephosphorylation of the RTK by tyrosine phosphatases, such as dephosphorylation of the Insulin receptor and Met receptor by PTP1B (Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000; Maroun, Naujokas et al. 2000; Neel, Gu et al. 2003; Sangwan, Paliouras et al. 2008). Internalization of the EGF receptor from the plasma membrane is required for dephosphorylation by an ER-tethered PTP1B, demonstrating that the internalization of the EGF receptor and its dephosphorylation are coordinated processes (Haj, Verveer et al. 2002). Interestingly, the Met receptor localizes with PTP1B in peripheral puncta 5 min post-HGF stimulation, suggesting that a similar mechanism of PTP1B dephosphorylation of Met may occur (Sangwan, Paliouras et al. 2008). Furthermore, dephosphorylation of Met occurs prior to Met degradation suggesting that dephosphorylation may act to terminate the signal prior to receptor degradation (Sangwan, Paliouras et al. 2008). What fraction of PTP1B is tethered to the ER or cleaved and present within the cytosol, is unknown. What consequence PTP1B subcellular localization has on RTK activity, especially the Met receptor, is unknown. Therefore a further understanding of where PTP1B functions within the cell is needed to fully understand its impact on the dephosphorylation and or regulation of RTKs.

5.6 Positive regulation of Met receptor signalling via the adaptor protein Gab1

Gab1 is the predominant protein associated with and phosphorylated downstream of the Met receptor and functions to couple a Met signal to various downstream signalling proteins, including PI3K, PLCγ, Shp2, Nck and Crk. At the beginning of this thesis, the identification of new Gab1 binding proteins had not been addressed. Gab1 is a critical mediator of Met-receptor mediated epithelial remodeling required for the morphogenic response. Therefore, identifying novel proteins recruited to Gab1 following Met receptor activation will further our understanding of the signalling pathways regulating Met-dependent biology.

5.6.1 Identification of novel Gab1-associated binding partners

To identify Gab1-protein complexes following Met receptor activation, I generated a Gab1-Tandem Affinity Purification (TAP)-tag fusion protein that I used in a proteomic screen to identify Gab1-protein interactions. TAP was originally developed in the budding yeast *Saccharomyces cerevisiae* (Rigaut, Shevchenko et al. 1999) and has been very successfully applied in isolating and identifying protein complexes in both yeast and mammalian cells (Rigaut, Shevchenko et al. 1999; Knuesel, Wan et al. 2003). There are two major advantages of the TAP procedure over conventional chromatographic purification procedures. The TAP-tag protocol can efficiently purify high yields of protein under low stringency conditions. In addition, two purification steps are used to ensure that only a low-level of background is obtained.

To isolate Gab1-dependent protein complexes, I established HEK293 cell lines that stably expressed the Gab1-TAP-tag fusion protein (Figure 5.2A). To determine if

the Gab1-TAP-tag was functional downstream from the Met receptor, I established that it was phosphorylated following HGF stimulation (Figure 5.2B) and was capable of purifying known Gab1-associated binding proteins (Supplemental Figure 3.1). In addition to isolating the known Gab1 binding proteins Shp2 and the p85 subunit of PI3K (Figure 5.2C), additional proteins that were identified through mass spectrometry ranged from actin/microtubule cytoskeletal assembly proteins, motor proteins, degradative machinery proteins, and kinases (Figure 5.2C). Interestingly, plotting the proteins isolated ranked against their mass spectrometry score revealed that most proteins isolated fell within a score ranging between 30-150 (Figure 5.2D). The TAP method is capable of identifying proteins interacting in stable complexes (e.g. Shp2 and p85) as well as more transiently interacting partners present in nonstoichiometric amounts (e.g. Pak4 and Scribble) (Figure 5.2C and 5.2D) (Puig, Caspary et al. 2001). These results correlate directly with the function of Gab1 as a scaffold protein recruiting, responsible for mediating the biological consequences of Met activation.

One protein identified as a Gab1-associated binding protein following Met receptor activation was the Pak family member Pak4 (Chapter III). I have shown that Met receptor activation is required for association between Pak4 and Gab1 and that this interaction was dependent on protein phosphorylation of Gab1 (Figure 3.1). The association was mediated via the GEF-interacting domain (GID) of Pak4 and an uncharacterized region/domain of Gab1 located between the PH domain and first Crk binding site (Figure 3.3 and 3.5). Furthermore, both proteins co-localize within lamellipodia and Gab1 recruitment to the cell cortex was necessary for Pak4 localization to lamellipodia (Figure 3.2). Pak4 and Gab1 synergized to enhanced migration and

invasion downstream from Met and Pak4 recruitment to Gab1 was required for tubulogenesis (Figure 3.7-3.9).

5.7 Pak4 - a novel Gab1-associated protein

5.7.1 Pak4 recruitment to Gab1 and not Gab2 or Gab3

The recruitment and association between Pak4 and Gab1 presented in chapter III is mediated via a predicted unstructured region of Gab1 located between the PH domain and the first Crk binding site, which I termed the Pak4-Association Motif (Pak4-ASM) (Figure 3.5). Interestingly, the association with Pak4 can only be seen with Gab1 and not other members of the Gab1 family, Gab2 and Gab3 (Supplemental Figure 3.2). The Pak4-ASM identified within Gab1 is poorly conserved from Gab2 and Gab3 respectively, supporting a potential structural difference that may affect their association. Overexpression of the Pak4-ASM was sufficient to compete with Met-dependent recruitment of Pak4 to Gab1 (Figure 3.5) and overexpression of this domain in HeLa cells inhibited migration and invasion following HGF stimulation, in a concentration dependent manner (Figure 3.9). It would be interesting to see if insertion of the Pak4-ASM of Gab1 into Gab2 or Gab3 would promote their association with Pak4.

From two-dimensional modeling studies, the Pak4-ASM displays no similarity to known protein binding motifs. Additional characterization of the Pak4-ASM needs to be performed to accurately determine which amino acids are critical for the association of Pak4 with Gab1. Gab1-deletion constructs possessing smaller regions of the Pak4-AMS can be constructed or alanine scanning mutagenesis are two potential methods that can be used to further deduce the amino acids critical for the association of Pak4 to Gab1. One

potential mechanism that could explain the interaction between Gab1 and Pak4 can be inferred from the direct interaction between Gab1 and Met. The direct association between Gab1 and Met is dependent on a 13 amino acid sequence in Gab1 termed the Met binding motif (MBM) (Schaeper, Gehring et al. 2000; Lock, Maroun et al. 2002). The MBM interacts as a linear peptide motif with the negative charge of phosphotyrosine 1349 of Met and the residues upstream of it located in the C-terminal lobe of the kinase domain. The interaction between Gab1 and Pak4 is dependent on Gab1 phosphorylation (Figure 3.1). Therefore the GID (GEF-H1 interaction domain) domain of Pak4 may function in a similar manner to the MBM of Gab1. It would be interesting to perform NMR (nuclear magnetic resonance) with the Pak4 GID domain together with the Pak4-ASM region of Gab1 to examine if any platform for interaction can be found.

5.7.2 Gab1, a regulator of actin dynamics

The recruitment of Gab1 to the Met receptor is important in regulating many of the biological activities downstream of the Met receptor. Gab1 recruitment to the plasma membrane is mediated in part through its PH domain which is critical in targeting and localizing Gab1 to PIP3 enriched membrane compartments and thus regulating the activation of signalling pathways from specific subcellular localizations. PIP3 enriched membranes are present at the leading edge of migrating cells within lamellipodia as well as other actin-rich protrusions known as a dorsal ruffles. Data published and collected in the lab demonstrate an important role for Gab1 in promoting actin rearrangement and the formation of lamellipodia and dorsal ruffles (Frigault, Naujokas et al. 2008) (Abella, JV, unpublished data). While the function of lamellipodia is to promote cell migration, the exact function of circular dorsal ruffles is still unknown and thus several theories as to

their role in cellular based activities exist. One potential function of dorsal ruffles is to function as the initial membrane necessary for promoting the actin protrusion responsible for lamellipodia formation. Multiple proteins are thought to be responsible for the formation of dorsal ruffles. These include N-WASP, WAVE1, WAVE2, the Arp2/3 complex, cortactin, and dynamin that are all thought to be critical components of dorsal ruffles (Buccione, Orth et al. 2004). However, several protein kinases have also been localized to dorsal ruffles and also participate in their formation. One protein of interest is the serine/threonine kinase Pak1, which has been found to localize to dorsal ruffles following PDGF stimulation (Dharmawardhane, Sanders et al. 1997; Dharmawardhane, Schurmann et al. 2000). Pak1 activation downstream from the Met receptor is necessary in promoting actin cytoskeletal reorganization (Royal, Lamarche-Vane et al. 2000). Interestingly, Pak4 was also found to localize to and enhance dorsal ruffle formation following HGF stimulation alone and co-localize with Gab1 in DRs (Paliouras observation, unpublished results). I established that Pak4 recruitment to lamellipodia is dependent on Gab1 as Pak4 was unable to localize to the cell cortex when expressed with Gab1ΔPH (Figure 3.2). This would support that the association of Pak4 with Gab1 is necessary for Pak4 recruitment to dorsal ruffles. Interestingly, Pak1 fails to associate with Gab1 following HGF stimulation demonstrating that this interaction is specific to Pak4 (Figure 3.1). Therefore, the localization of Pak1 to dorsal ruffles must be mediated through an unknown mechanism and highlights an important difference between Pak1 and Pak4. No evidence exists that Pak1 can associate directly with an activated RTK. However, Pak1 can associate with the adaptor protein Grb2, therefore a transient Met-Grb2-Pak1 complex may exist that will mediate Pak1 recruitment to dorsal ruffles (Puto, Pestonjamasp et al. 2003).

Recently, Pak4 was found to be important in the formation of podosomes in macrophages (Gringel, Walz et al. 2006). Podosomes are structures present on the basal surface of a "normal" cell. An equivalent structure found in cancer cells is termed Invadopodia. Gringel *et al.* found that PAK4 was a physiological regulator of podosome number and size in macrophages (Gringel, Walz et al. 2006) and shRNA against Pak4 or kinase dead mutants of Pak4 impacted the ability of macrophage podosome size and number. Podosomes and invadopodia possess many common molecular components (N-WASP-Arp2/3-cortactin-dynamin), however they do differ in some structural and functional parameters. These include the substructure of the polymerized actin (branched vs. cross-linked filaments), the depth of the membrane extension into the ECM and the location and composition of the adhesive domains (Gimona, Buccione et al. 2008). Interestingly, many of the proteins found to be present within podosomes and invadopodia are also located in dorsal ruffles (Buccione, Orth et al. 2004). How do invadopodia and dorsal ruffles differ? Invadopodia of tumour cells enable cancer invasion and tumour metastasis via local matrix-degradation via matrix metalloproteinases (MMPs) located within the actin rich structures (Buccione, Orth et al. 2004). MMPs have also been found within the tips of dorsal ruffles, therefore it is possible that dorsal ruffles might promote degradation of the extracellular matrix during three-dimensional migration (Suetsugu, Yamazaki et al. 2003). Dorsal ruffles, invadopodia and podosomes are actin-rich structures thought to exist only in cells when grown in two-dimension on plastic. Therefore, debate exists if these represent distinct structures when cells are grown in a three-dimensional or if they are truly the same structure.

5.7.3 A Gab1-Pak4-GEF-H1 signalling complex

Met receptor stimulation leads to the activation of Rho GTPases that regulate actin cytoskeletal dynamics (Royal, Lamarche-Vane et al. 2000; Wells, Ahmed et al. 2005). However, which GEFs are regulated downstream of the Met receptor is still unclear. Crk is a known activator of Rac (Abassi and Vuori 2002) and a role for Crk in Rac activation downstream from Met may involve the GEFs DOCK180 and β-PIX (Kiyokawa, Hashimoto et al. 1998; Lamorte, Rodrigues et al. 2003). The GEF known to regulate GTP-loading on Cdc42 is unknown, although we can hypothesize that the Cdc42/Rac GEF β-PIX, which associates with Crk, may mediate some proportion of Cdc42, GTP-loading. The GEF responsible for activation of RhoA downstream from Met has yet to be identified. In chapter IV, I identify the RhoA GEF, GEF-H1, as a new Gab1-associated binding partner (Figure 4.1). GEF-H1 is a microtubule bound RhoA GEF which upon microtubule polymerization or phosphorylation on specific residues, redistributes GEF-H1 from a microtubule compartment to the cytoplasm leading to stress fibre formation (Krendel, Zenke et al. 2002; Callow, Zozulya et al. 2005; Birkenfeld, Nalbant et al. 2007; Chang, Nalbant et al. 2008). The consequence of growth factor stimulation on GEF-H1 localization and activity has not been examined to date. My data shows that HGF stimulation redistributes GEF-H1 from microtubules into membrane ruffles and this correlates to increased migration and invasion downstream from Met receptor activation. One downstream effector of RhoA activation, Rho-kinase (ROCK), is translocated to membrane ruffles following HGF stimulation (Royal, Lamarche-Vane et al. 2000). This data supports a model of localized GEF-H1 activity in membrane ruffles leading to the activation of ROCK. This is supported by a recent study that revealed a role for a GEF-

H1/RhoA/ROCK/MLC signalling pathway in mediating nocodazole-induced cell contractility (Chang, Nalbant et al. 2008). Further examination of this ROCK signalling downstream of Met in GEF-H1 knockdown cells will can shed some light on a mechanism important in regulating GEF-H1 dependent migration.

5.8 Pak4 a regulator of apoptosis

In chapter II, I discovered that PTP1B-null mice were resistant to Fas-induced hepatic failure. The mechanism of protection that was proposed in chapter II relied on increased RTK signalling, especially Met receptor signalling, leading to the activation of pro-survival pathways. Increased expression levels of the anti-apoptotic protein FLIP antagonized DISC complex formation and activation of downstream initiator and effector caspases. Interestingly, Pak4 has been shown to play a role in inhibiting apoptosis and promoting survival. Pak4 overexpression is associated with protection from apoptosis following TNFα treatment, UV irradiation and serum starvation by preventing the cleavage and activation of the downstream caspases, Caspase-8 and Caspase-3 (Gnesutta, Qu et al. 2001; Gnesutta and Minden 2003; Lu, Pan et al. 2003; Li and Minden 2005). Conversely, cells lacking Pak4 are susceptible towards apoptosis (Li and Minden 2005). This supports the reported observation of constitutively active Pak4 functioning in the development of anchorage-independent cell growth, which is an important hallmark of cancer. Pak4 has been found to inhibit apoptosis via two different mechanisms, a kinase-independent and a kinase-dependent mechanism, that mimic the protection seen in chapter II.

5.8.1 Kinase-independent Pak4 inhibition of apoptosis, inhibiting apoptosis at the DISC

Fas induced hepatic damage is initiated following the formation of the DISC complex. Pak4 can inhibit the pro-apoptotic cascade by reducing the amount of caspase-8 recruited to the DISC complex formed by TNFR1 and Fas receptors (Gnesutta and Minden 2003). Caspase-8 association with FADD in the DISC complex leads to its auto-proteolytic cleavage and activation into the active heterotetramer, two p18 and two p10 subunits (Medema, Scaffidi et al. 1997). This proposes that Pak4 is functioning in a similar manner to FLIP. FLIP is capable of associating with the DISC complex and behave as a dominant-negative, preventing the association of caspase-8 to FADD and its autoproteolytic activation. I found FLIP levels to be increased in PTP1B-null mice following Jo-2 induced liver damage (Figure 2.3). Therefore, Pak4 antagonizes the DISC complex from properly recruiting Caspase-8, while other components of the DISC complex, such as FADD, are still recruited. We cannot conclude that both Pak4 and FLIP could potentially regulate the activation of the DISC complex simultaneously downstream from the Fas receptor. Isolation and characterization of the DISC complex in PTP1B-null mice compared to WT mice would further our understanding of a potential role for Pak4 in regulating the formation and activation of the DISC complex.

5.8.2 Kinase-dependent Pak4 inhibition of apoptosis

5.8.2.1 Inhibition of pro-apoptotic pathways

Interestingly, Pak4 phosphorylates Ser112 of the pro-apoptotic protein Bad. Bad belongs to the Bcl-2 family of interacting proteins, which is composed of both survival- and apoptotic-promoting proteins (Willis and Adams 2005). Normally, Bad promotes

apoptosis by forming heterodimers with the anti-apoptotic proteins Bcl-2 and Bcl-xL, inhibiting their function and promoting the release of cytochrome c from the mitochondria (Yang, Zha et al. 1995). Pak4 phosphorylation of Bad inhibits the interaction with Bcl-2 and Bcl-xL, preventing the release of cytochrome c from the mitochondria (Gnesutta, Qu et al. 2001; Gnesutta and Minden 2003). This would inhibit formation of the apoptosome and cleavage and activation of caspase 9 preventing the activation of effector caspases and the apoptotic cascade (Figure 5.3). Bad is phosphorylated by Pak1, resulting in a similar inhibition as seen with Pak4 (Schurmann, Mooney et al. 2000). A mechanism I proposed in chapter II relied on protection against Fas-mediated apoptosis in PTP1B-null mice by preventing the cleavage and activation of Bid. Bid has previously been shown to be phosphorylated by casein kinase I and II, inhibiting its cleavage by caspases, suggesting that phosphorylation was proposed to inhibit Bid's pro-apoptotic activity. It would be interesting to establish if Pak4 is capable of phosphorylating and inhibiting cleavage of Bid in response to HGF. Furthermore, the Bad phosphorylation status was not examined in PTP1B-null mice, therefore further examination of this is warranted.

5.8.2.2 Activation of pro-survival pathways

The activation of NF-κB and MAPK signalling in PTP1B-null mice correlated with enhanced survival. Treatment of primary hepatocytes with inhibitors of NF-κB and Erk, removed the protection seen in PTP1B-null hepatocytes to Fas-induced damage (Figure 2.5). Pak4 is capable of promoting cell survival through the activation of TNFα stimulated survival pathways, the NF-κB and MAPK signalling pathways (Beg and Baltimore 1996; Li and Minden 2005) (Figure 5.3). Pak4 was necessary for the

recruitment of TRADD to the TNFR1 in a kinase-dependent manner, as expression of a constitutively activate Pak4 promoted the recruitment of TRADD to the receptor. A kinase dead Pak4 was also capable of recruiting TRADD to the TNFR1 receptor, although to a significantly lesser extent (Li and Minden 2005). Furthermore, constitutive activation of the NF-κB and Erk pathways can rescue the sensitivity to apoptosis that is induced by Pak4 siRNA treatment (Li and Minden 2005).

What is intriguing is the overlap seen in PTP1B-null mice following Fas-induced liver damage and Pak4 signalling in promoting cell survival (Figure 5.4). Although speculative, the data suggest a role for Pak4 in mediating the protective effect seen in PTP1B-null mice and hepatocytes. The ability for Pak4 to antagonize DISC complex formation and enhance the activation of the NF-κB and MAPK signalling pathways, mimics the phenotype shown to inhibit apoptosis in PTP1B-null mice following Jo-2 injection. This can be addressed through the use of small molecule inhibitors of Pak4 or Pak4 knockdown in PTP1B-null hepatocytes, to examine the consequence of Pak4 activation on protection against Fas-induced apoptosis in PTP1B-null hepatocytes.

5.9 Summary

The work presented in this thesis aims to further our understanding of the mechanisms involved in the regulation of Met receptor tyrosine kinase signalling and biology. The importance of delineating the proteins necessary for the positive and negative regulation of RTKs is highlighted by the fact that in human diseases, especially in cancer, deregulations of these mechanisms promote cells to survive and become invasive. Since receptor tyrosine kinases, such as the Met receptor, are frequently

deregulated in human cancers, elucidation of their regulation will lead to therapeutic strategies to modulate the Met receptor and target invasion and metastasis of cancers.

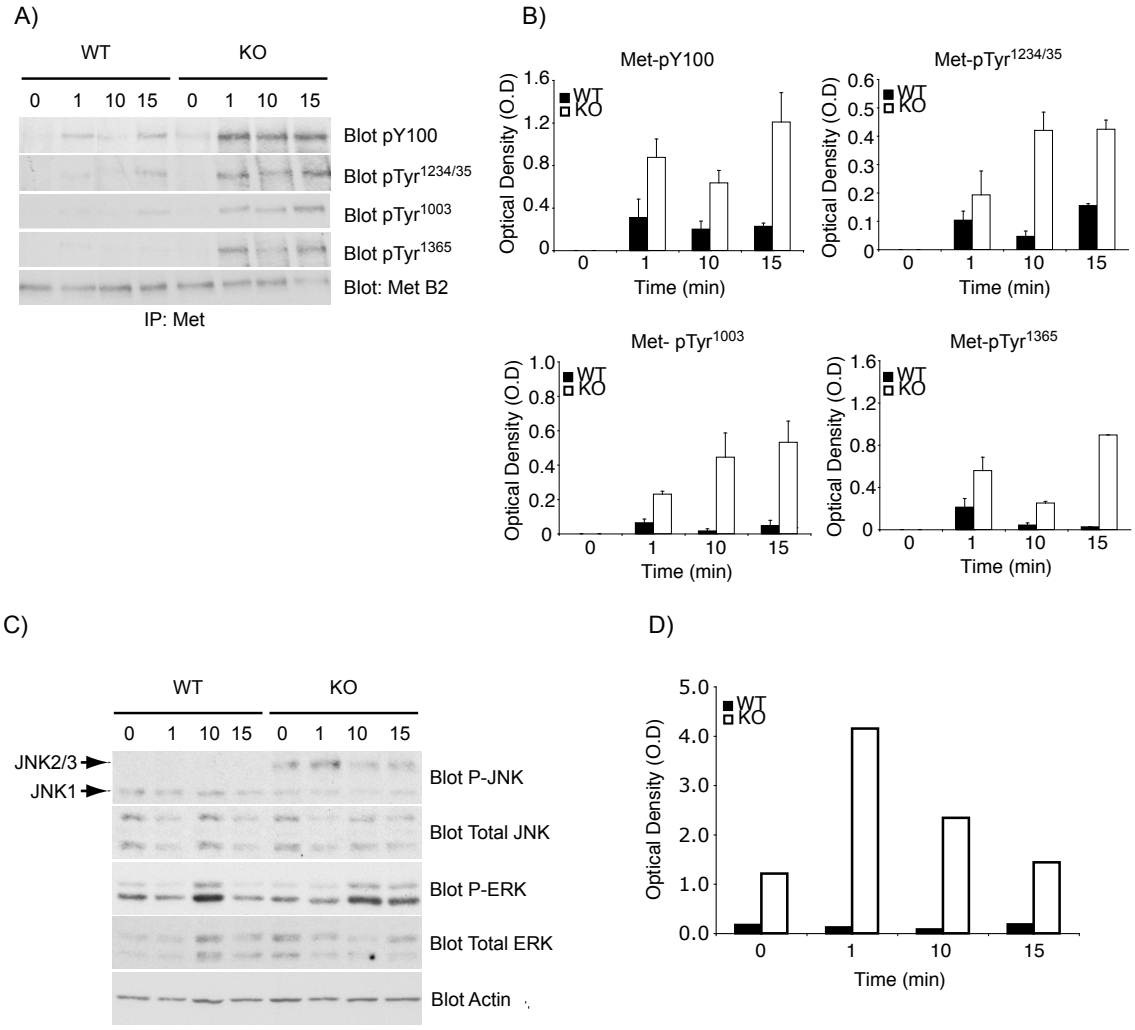

Figure 5.1: Met is hyperphosphorylated in livers from PTP1B-null mice versus WT mice, following HGF stimulation. A) PTP1B WT or null mice were injected with HGF via the hepatic portal vein at the times indicated. Protein extracts prepared from livers of the injected mice were immunoprecipitation using an anti-mouse Met antibody and probed with the anti-phosphotyrosine antibody, p-Tyr-100, or phosphotyrosine antibodies directed against specific tyrosines of the Met receptor (p-Tyr-1003, p-Tyr-1365, or p-Tyr-1234/1235). Each time point represents an individual mouse. **B)** densitometric analysis of the data presented in A measured by NIH ImageJ and normalized against total Met levels from three independent replicates. **C)** protein lysates from livers of mice injected with HGF for times indicated probed with anti-pJNK, JNK, pERK, or total ERK antibodies. **D)** densitometric analysis of the p54 JNK blot shown in C compared against total protein levels. The error bars represent S.E. Each experiment was performed a minimum of three times. Figure published in Sangwan et.al., *JBC*; 283(49);34374-83.

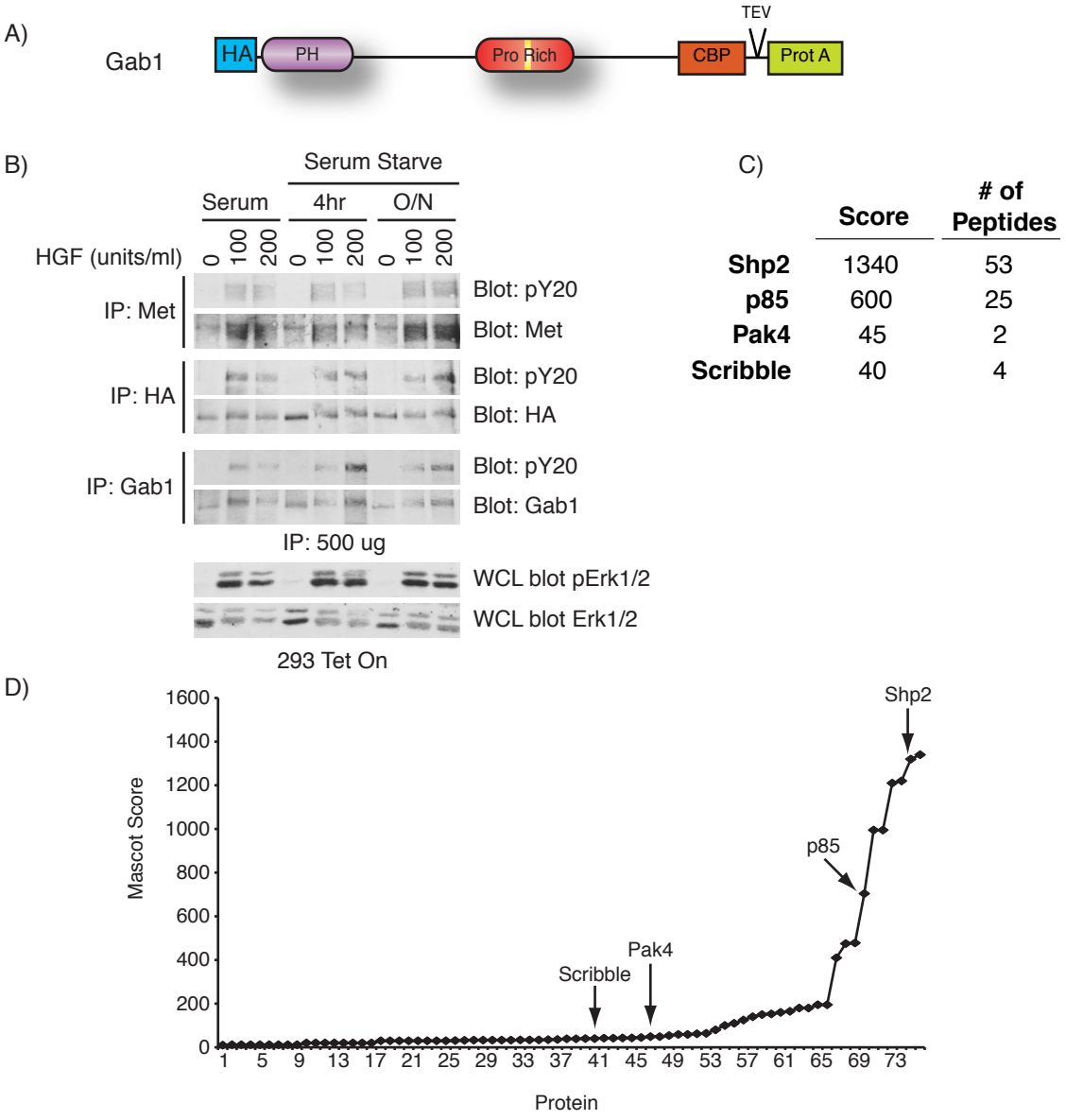

Figure 5.2: Identification of novel Gab1-associated proteins. A) Schematic representation of Gab1-TAP-tag fusion protein. **B)** Stable HEK293 Tet ON cells expressing the Gab1-TAP-tag fusion protein were analyzed for their ability to be phosphorylated following HGF stimulation. Cell lines were maintained in three different conditions to examine the consequence of 10% fetal bovine serum treatment on Gab1 phosphorylation and Erk1/2 phosphorylation. **C)** Table showing the Mascot score and number of peptides isolated for known and novel Gab1-associated proteins identified by mass spectrometry analysis. **D)** Line graph of all proteins isolated 5 mins post-HGF stimulation in relation to their Mascot score.

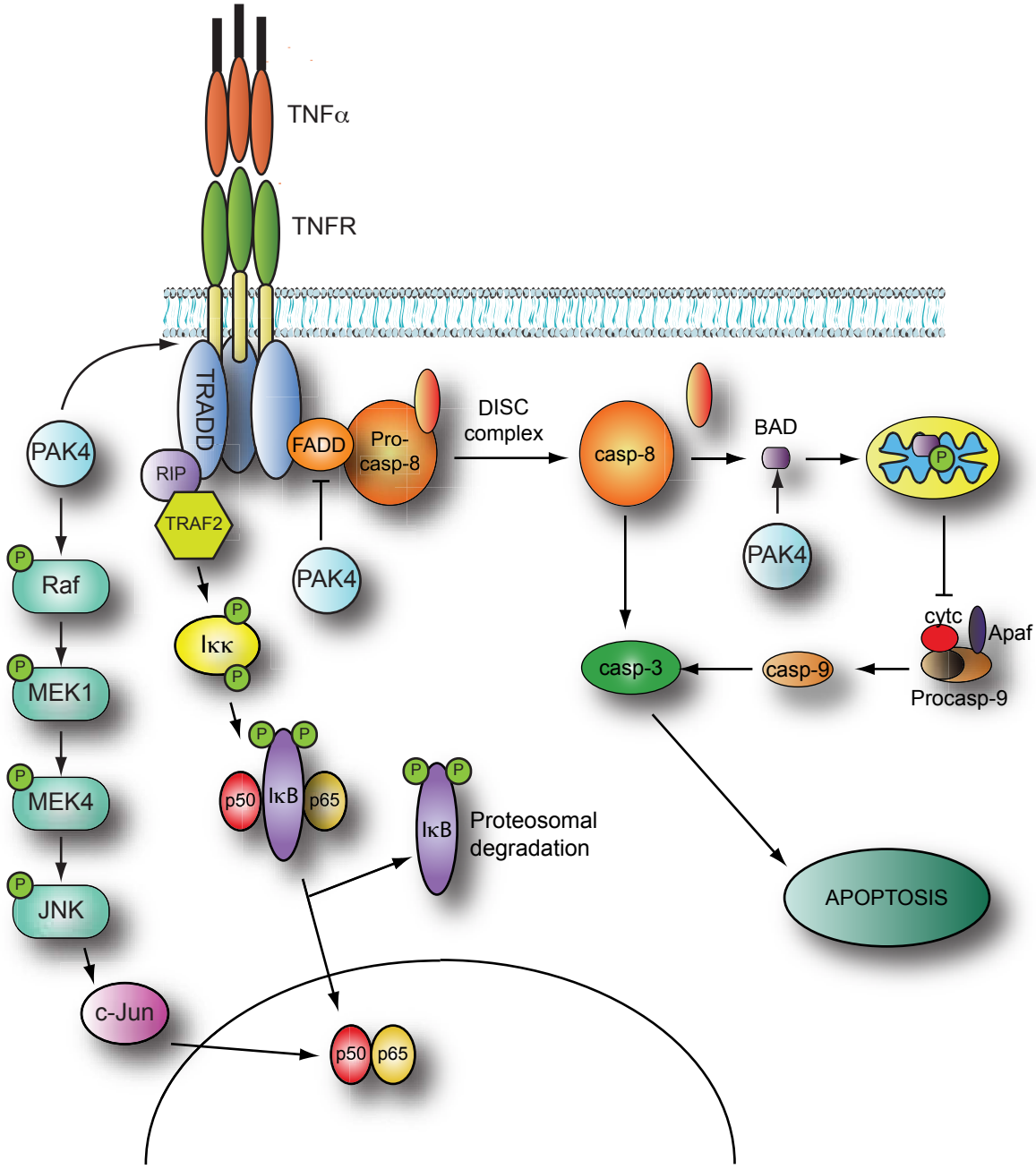

Figure 5.3: Regulation of apoptosis and cell survival by Pak4. Pak4 regulates apoptosis by two different mechanism, a kinase-dependent and kinase-independent mechanism. Pak4 phosphorylation of Bad inhibits cytochrome c release, inhibiting apoptosis. Pak4 also inhibits apoptosis by antagonizing DISC complex formation by reducing the amount of caspase-8 recruited to the DISC. Pak4 is capable of promoting cell survival through the activation of TNFα stimulated survival pathways leading to the activation of the NFκB and MAP kinase signaling pathways, both of which are important in promoting pro-survival signals.

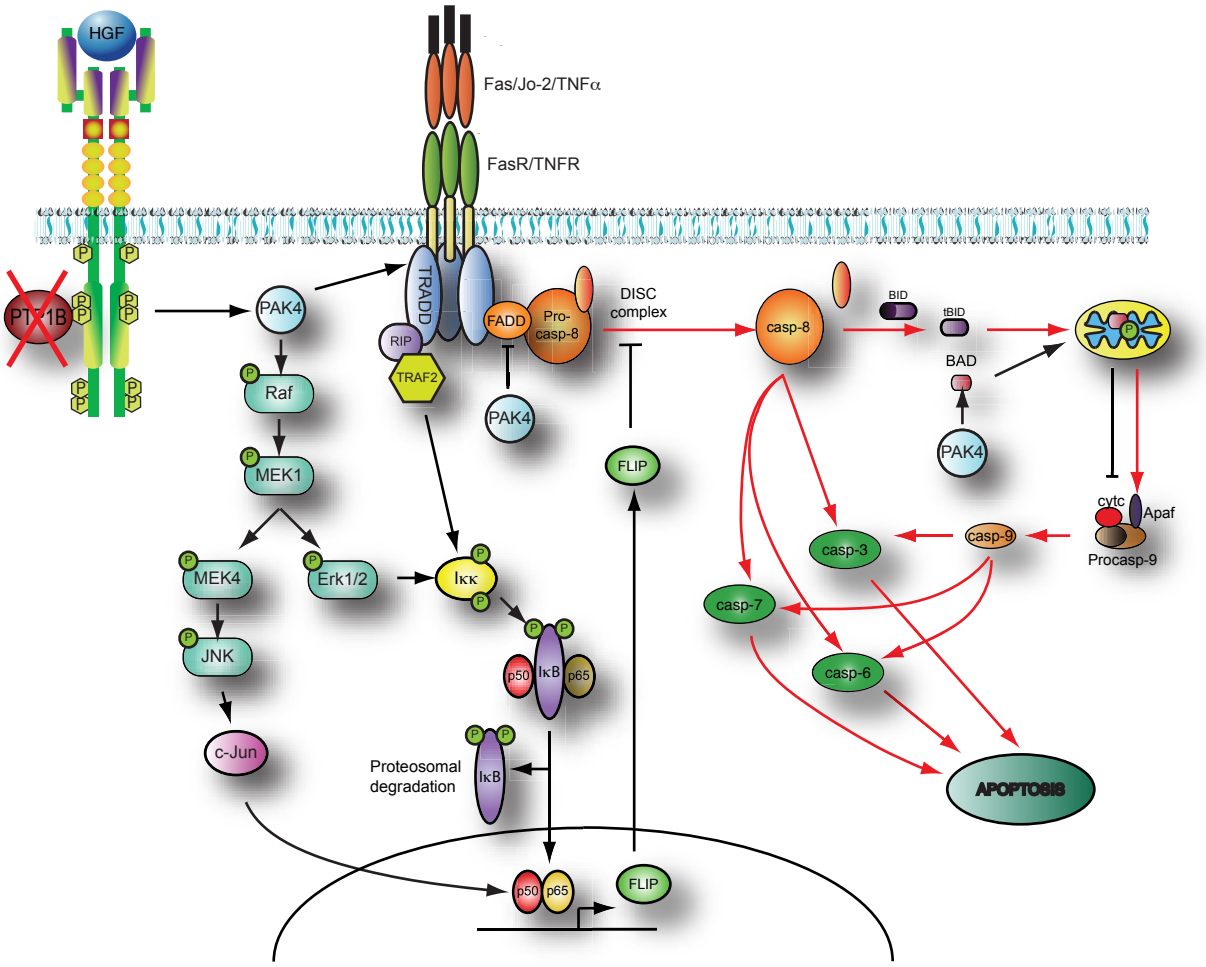

Figure 5.4: Potential mechanism involving Pak4 in the protection against Jo-2 induced liver damage in PTP1B-null mice. In PTP1B-null mice, following injection of the agonistic Fas antibody, Jo-2, enhanced phosphorylation of the Met receptor is observed. This is correlated with enhanced activation of the Raf-MAPK pathway, NFκB pathway and an increase in FLIP expression. FLIP antagonizes DISC complex formation, resulting in an inhibition of the apoptotic cascade. Pak4 is activated following activation of the Met receptor and promotes the activation of the Raf-MAPK pathway through an unidentified mechanism. Pak4 also promotes the activation of TNFα survival signals by recruiting TRADD to the TNFR1 receptor, enhancing the activation of the NFκB pathway. Pak4 phopshorylation of the pro-apoptotic protein Bad, prevents it to inhibit the anti-apoptotic proteins Bcl-2 and Bcl-xL. Pak4 can also antagonize the activation of the DISC complex by preventing the complex from recruiting Caspase-8, inhibiting apoptosis. Red lines represent activation of the apoptotic cascade. Black lines represent inhibition of the apoptosis.

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7 APPENDIX I

A key protein that associates with the Met receptor following HGF stimulation is the scaffold protein Gab1. Gab1 recruitment to and phosphorylation by Met leads to the subsequent recruitment of multiple signaling proteins to the activated Met receptor complex.

The importance of Gab1 in Met signaling is evident from the observation that mice null for Met or Gab1 are embryonic lethal and have similar pathological defects (Bladt, Riethmacher et al. 1995; Schmidt, Bladt et al. 1995; Sachs, Brohmann et al. 2000). One defect is the failure of myogenic muscle precursor cells to migrate into the tongue, diaphragm, and limbs. This highlights an important role for Gab1 in the regulation of actin cytoskeletal dynamics required for cell motility and invasion through a foreign matrix. Studies performed in our lab have established that in response to HGF, Gab1 localizes to the leading edge of a migratory cell within lamellipodia as well as on the apical surface of the cell within dorsal ruffles. Both of these processes require remodeling of the actin cytoskeleton and are dependent on Gab1 downstream from Met. Two assays that are used within the lab to study Met biological outputs, 2D scatter assays and 3D tubulogenesis assays are both dependent on remodeling of the actin cytoskeleton and Gab1 and provide a mechanism to perform structure function studies to identify key Gab1-associated proteins required for biological response (Gual, Giordano et al. 2000; Lock, Royal et al. 2000; Maroun, Naujokas et al. 2000; Lamorte, Rodrigues et al. 2002). Multiple proteins had been identified that associate with Gab1, however, these could not

fully explain the mechanisms mediating many of the actin cytoskeletal changes that were dependent on Gab1 downstream from Met.

To address this, I performed a proteomic screen to identify new proteins that are recruited to Gab1 following Met receptor activation. The approach undertaken involved generating a Gab1-Tandem Affinity protein (TAP)-tag fusion protein. TAP was originally developed in the budding yeast *Saccharomyces cerevisiae* (Rigaut, Shevchenko et al. 1999) and has been very successfully applied in isolating and identifying protein complexes in both yeast and mammalian cells (Rigaut, Shevchenko et al. 1999; Knuesel, Wan et al. 2003). There are two major advantages of the TAP procedure over conventional chromatographic purification procedures. The TAP-tag protocol can efficiently purify high yields of protein under low stringency conditions. In addition, two purification steps are used to ensure that only a low-level of background is obtained. A Gab1-TAP-tag fusion protein was used as bait to affinity purify Gab1-associated proteins following activation of Met.

7.1 Generation of Gab1-TAP-tag fusion construct.

The Gab1-TAP tag fusion protein was generated by PCR amplification of Gab1 and subsequently subcloned into a retroviral vector generated and generously donated by the laboratory of Dr. Alain Nepveu (McGill University) that contains a Calmodulin-protein A TAP-tag (pRev/Kozak/TAP-tag). A HA-tagged Gab1 was amplified from pcDNA1.1 HA-Gab1 (Maroun, Holgado-Madruga et al. 1999) using high-fidelity *Taq* polymerase (Roche Diagnostics, Laval, Quebec, Canada), as per the manufacturer's instructions. The cloning of HA-Gab1 into pRev/Kozak/TAP-tag was performed in 2 steps. HA-Gab1 was first amplified and cloned into the mammalian TAP-tag expression

vector pLS43 (generously donated by Dr. Xiang-Jiao Yang, McGill University), using the following primers: Gab1_EcoRI/NruI_FWD – CATGGAATTCGCGAATGGCTTACCCATACGATG TTCCAG and Gab1_SmaI_REV – GCATCCCGGGCTTCACATTCTTTGTGGGTGTCTCGG. The HA-Gab1-TAP-Tag was subsequently subcloned into pRev/Kozak/TAP-tag following restriction digestion with NruI and NotI (New England Biolabs, Pickering, Ontario, Canada) and agarose gel purification (Qiagen, Mississauga, Ontario, Canada). The HA-Gab1 pcDNA1.1 fragment was then ligated into pRev/Kozak/TAP-tag using T4 DNA ligase (New England Biolabs, Pickering, Ontario, Canada).

7.2 Retroviral Production using 293 VSVG cells (Tet-off system) to generate retrovirus expressing Gab1-TAP-tag construct

To generate stable cell lines expressing the Gab1-TAP-tag fusion protein, retroviruses were generated using the VSVG-293 cell line. VSVG-293 cells were grown in Dulbecco's Modified Eagles Medium (DMEM) containing 10% FBS, 5 mg/ml Tetracycline (TET), 400 μg/ml geneticin and 200 μg/ml Puromycin. TET was always added fresh to media on day of plating. The day prior to infection, VSVG-293 cells were split 1:2 and replated in 7ml 10% FBS with only TET. Transfection of VSVG-293 cells was performed using Lipo2000 (Invitrogen, Carlsbad, CA) following manufacturer's instruction with the following modifications. A ratio 3:1 of Lipo2000 to DNA and 4:1 ratio of DNA of interest to helper DNA plasmid was used. The final concentrations used were 20μg Gab1-TAP-tag or pRev/Kozak/TAP-tag vector DNA, 5μg vPak VGV Helper plasmid and 75μl Lipo2000. The day after transfection, media was removed and 10% Heat-inactivated FBS without TET was added to initiate virus production. Virus was

collected 48 hrs post-transfection for 5 days. The pooled supernatant was applied on 293 Tet-On cells ($2x10^4$ cells) at an equivalent titer, along with 8 μg/ml of polybrene (Roche Diagnostics, Laval, Quebec, Canada), and plates were centrifuged at 300 x g for 1 hr. Virus was removed 48 hrs post-infection and stably infected cells were established by selection with hygromycin (300 μg/ml) (Invitrogen, Carlsbad, CA). 293 Tet-On cells were grown in 10% FBS DMEM + 400 μg/ml geneticin prior to infection. Individual clones were selected and expanded and screened for expression of the HA-Gab1-TAP-tag.

7.3 Gab1-TAP-tag Pulldown

HA-Gab1-TAP-tag expressing and pRev/Kozak/TAP-tag expressing 293 Tet-On cells ($1x10^6$ cells/ml) were plated in 10x 15cm plates (Nalgene NUNC, Rochester, NY, USA) per time point (0 min, 5 min, 15 min and 60 min), in DMEM containing 10% fetal bovine serum (FBS), 400 μg/ml geneticin (Invitrogen, Carlsbad, CA), 75 μg/ml hygromycin (Invitrogen, Carlsbad, CA), 50 μg/ml gentamicin (Invitrogen, Carlsbad, CA) and 25 ng/ml doxycyclin. Cells were grown to 85% confluency and then serum starved overnight prior to stimulation with HGF (135 ng/ml). Cells were lysed in 1% NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, pH 8.0, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4). Each time point was pooled and the homogenates were centrifuged at 13,000 rpm for 15 min to remove debris. Protein lysate was added to 50 μl of IgG beads in a 2 ml siliconized eppendorf tube and nutated for 4 hrs at 4°C. Following binding to IgG, beads were centrifuged for 1 min at 13,000 rpm. Supernatant was removed and IgG beads were washed 2x with 1% NP-40 lysis buffer without protease inhibitors and 1x with TEV-100

buffer (10 mM Tris pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.1% Triton 2.5 and 10% Glycerol). Following washes, 600µl of TEV-100 and 80 units of TEV protease were added to Gab1-TAP-tag coupled IgG beads and digested overnight at 4°C. The following day, 50 µl of Calmodulin beads were washed 2x with CBB-100 buffer (10 mM Tris pH 8.0, 100 mM NaCl, 1 mM Immidazole, 1 mM Magnesium Acetate, 2 mM $CaCl_2$, 10 mM ß-Mercaptoethanol, 0.1% Triton X100, 10% Glycerol) in a 2 ml eppendorf tube. The IgG beads were transferred to a 1 ml Bio-Spin column and the flow-through was collected in the 2ml tube containing washed calmodulin beads. Calmodulin beads pooled with IgG flow-through were rotated for 4 hr at 4°C. Gab1-TAP-tag protein lysates coupled to calmodulin beads were transferred to a 2 ml Bio-spin column and washed with 1 ml CBB-100 buffer. Gab1-TAP-tag was then eluted from the calmodulin beads by 3x washes with 200µl of CEB-100 buffer (10 mM Tris pH 8.0,100 mM NaCl, 1 mM Immidazole, 1 mM Magnesium Acetate, 2 mM EGTA, 10 mM ß-Mercaptoethanol, and 10% Glycerol). Eluted protein was frozen in liquid nitrogen and lyophilized. Protein was denatured by boiling for 5 min in Laemmlli sample buffer and resolved on a 8-15% gradient SDS-PAGE gel. SDS-PAGE gel was stained with Colloidal Coomassie Blue (Bio-Rad) following manufacturer's instructions and gel fragments were removed with a scalpel and sent for mass spectrometry analysis. All mass spectrometry analysis was performed in collaboration with the Proteomic Core Facility within the McGill University Life Science Complex.

8 CERTIFICATES