The role of the Gab family of docking proteins in Met mediated membrane ruffle formation

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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Your file Votre référence
ISBN: 978-0-494-66293-9
Our file Notre référence
ISBN: 978-0-494-66293-9

Abstract

In response to extra-cellular cues, cells activate signal transduction pathways to elicit a biological response. Cell surface growth factor receptors such as the Met receptor tyrosine kinase (RTK) activate signals that result in cellular proliferation, survival, migration, as well as epithelial morphogenesis. In order for signal transduction to occur, docking proteins are recruited to the activated RTK, become phosphorylated on tyrosine residues, which then serve as docking sites for the recruitment of other signaling proteins. Docking proteins function to diversify the signal by assembling multi-protein complexes. The Gab1 docking protein is the most tyrosine phosphorylated protein upon Met receptor activation and is required for Met mediated signaling and biology.

Gab1 belongs to a family of docking proteins including the highly related Gab2 protein. Gab1 promotes signals for epithelial morphogenesis downstream of the Met receptor, however Gab2 is unable to do so. Insertion of the Gab1 Met binding Motif (MBM) which confers direct binding to the Met receptor, as well as membrane targeting of Gab2 is sufficient to switch the capacity of Gab2 to activate the morphogenic program, cell scatter and lamellipodia formation. This is achieved via activation of sustained signaling pathways, and redistribution of the Gab protein, and associated molecules to sites of lamellipodia formation at the peripheral edge of the cell.

Activation of the Met RTK, promotes the formation of dorsal ruffles on the apical surface of epithelial cells. The Met receptor, Gab1 and Gab1 associated molecules Shp2, Crk, and p85 subunit of PI3K, are localized to these structures, however only the Gab1-Crk complex is required to drive dorsal ruffle formation. Gab1 is required for Met induced dorsal ruffles as well as downstream the PDGF and EGF RTKs. These are a

signaling micro-environment which results in enhanced receptor degradation. Inhibition or enhancement of Met mediated dorsal ruffle formation correlates with receptor stability.

Dorsal ruffle formation downstream of Met requires the enzymatic activity of PI3K and PLCγ, both enzymes that metabolize PIP_2, and form complexes with Gab1 downstream of Met. PLCγ and the PIP_3 lipid product of PI3K are co-localized with Gab1 in dorsal ruffles. Gab1 engages with elements of the cytoskeleton, actin and cortactin, providing a link between growth factor signaling and remodeling of the actin cytoskeleton. Gab1 is localized to membrane protrusions of the basal surface in organoid cultures and is required for actin protrusions of the basal surface of breast cancer cells.

Résumé

Pour répondre aux changements dans l'environnement extracellulaire, les récepteurs transmembranaires présents sur la surface cellulaire, génère des réponses biologiques. Ceci est accomplis par le démarrage de la signalisation par des récepteurs à activité tyrosine kinase (RTK), par exemple le récepteur Met qui mène a des changements cellulaires comme la prolifération, la survie, la migration et la morphogenèse épithéliale. Pour transmettre les signaux, les protéines d'arrimage sont recrutées à la surface cellulaire en proximité du récepteur, où elles deviennent phosphorylées sur des acides aminées tyrosines. Sur activation du récepteur Met dans des cellules épithéliales, la protéine la plus phosphorylée est la protéine d'arrimage Gab1. Ceci crée des nouveaux sites de liaisons pour plusieurs molécules de signalisation. De cette manière, Gab1 sert à diversifier les signaux en aval des récepteurs par leur capacité de rassembler des complexes de protéines. Gab1 fonctionne pour relier les signaux du RTK Met pour la majorité de ces signaux biochimiques et de ces réponses biologiques.

Gab1 fait partie d'une famille de protéine d'arrimage dont Gab2, une protéine de très haute ressemblance à Gab1, en fait partie aussi. Gab1 active les signaux pour la morphogenèse épithéliale en aval du récepteur Met, mais Gab2 en est incapable. Par la re-introduction du motif, qui engage Gab1 directement avec Met (MBM), en conjonction avec l'attachement à la surface cellulaire, la capacité de Gab2 pour gérer la morphogenèse est activée. Aussi, ce mutant de Gab2 peut induire des colonies de cellules épithéliales de se disperser, et de former des ondulations de membranes, que l'on appelle lamellipodia. Ces effets biologiques sont acquis par l'activation des signaux

d'une manière prolongée et le changement de la localisation de Gab et les protéines associées à Gab aux lamellipodes sur le bord des cellules.

En conséquence du démarrage du RTK Met, les cellules épithéliales élaborent des extensions membranaires sur la surface apicale, que l'on nomme rides dorsale (DR). Le récepteur Met, Gab1 et les protéines associé à Gab1 dont Shp2, Crk, et la sub-unité p85 de PI3K, se retrouvent dans les DRs. Par contre, seul le complexe Gab1-Crk est requis pour la formation des DRs. Gab1 est indispensable pour la formation des DRs en aval du RTK Met et par les RTKs des ligands PDGF et EGF. Les DRs sont un microenvironnement qui amplifie la dégradation des récepteurs, des stimulis qui amplifient ou inhibent les DR ont un effet parallèle sur la stabilité du récepteur.

La formation des DRs, en aval du RTK Met, est dépendante de l'activité enzymatique de PI3K et PLCγ, des enzymes qui métabolisent le même substrat PIP_2, et qui se rassemblent en complexe avec Gab1 en aval de Met. PLCγ et PIP_3, le produit de l'enzyme PI3K, s'accumulent aussi dans des DRs, ce qui fut observé par des analyses microscopiques. Gab1 forme aussi une interaction avec actin et cortactin, des éléments du cytosquelette de la cellule, placent Gab1 au croisement des signaux en aval du RTK Met et les processus dynamiques du cytosquelette. Gab1 est aussi présent sur la surface ventrale dans des cellules épithéliale MDCK en culture de deux dimensions tant que dans les extensions membranaires dans des cultures de trois dimensions. Surtout, nous avons démontré que Gab1 est indispensable pour les structures de F-actin sur la surface basale des cellules du cancer du sein. Ceci démontre que Gab1 est une protéine instructive pour les ondulations de la membrane qui sont les structures cellulaires qui prédisposent la cellule à migrer.

Acknowledgements

I would like to thank my Ph.D. thesis supervisor, Morag Park, for providing me the opportunity to learn volumes about life and science.

I want to recognize those members of the lab that taught me invaluable lessons as a new graduate student. Christiane Maroun for her passion that inspired me, Lisa Lock whose smile and calm disposition I have only recently understood, Louie Lamorte for his sense of humor and attention to detail, and to Hanane Khoury who made me discover the freedom and excitement of scientific independence.

I would like to acknowledge Fonds de la Recherche en Santé du Québec and McGill University Health Center Research Institute for funding my research.

I want to thank Greg Paliouras for feedback during the preparation of this thesis and whose friendship I value more than he will ever know. It has been my great pleasure to share many hours of tissue culture with Monica Naujokas, with whom I was always excited to share my results. I am glad to thank Jasmine Abella for agreeing to collaborate with me, and for her friendship then and now again. I would like to thank Claire Kuzmochka for her help as a summer student setting up live cell imaging in the lab and for her friendship. I am glad to have become friends with Claire Brown whose good nature and expertise I have been fortunate to discover.

I must thank my biochemistry undergrad buddies that have become my great friends. Samar Pai for all the laughs and pep talks, and Sabine Koszegi with whom I shared many travels, and many discussions about life. I want to thank Vikas Chaubey whose genuine friendship has been such a source of happiness for me.

I also want to thank Brigitte Goulet and Stephanie Iannelli, for great food, the laughs and innumerable good times, and to Denise, Phil and Lucia DeFazio whose friendship feels like family. I must thank Sonia Rodrigues, whose friendship is invaluable to me and without whom I could never have finished this thesis.

To my grandfather whose influence remains in my heart, my grandmother for her whit and pride, to Valerie, Roy, Christopher and Amanda for gathering at their kitchen table, to Brenda and Denis for their friendship and Makarios for his love, and to my father.

Mostly, I want to acknowledge my mother whose unconditional love and endless support has molded the woman I have become, I am grateful for her care and friendship and indebted to her for making this thesis and all other achievements in my life possible.

The more I learn, the more I realize that I know nothing.

Preface

Other publications arising from this thesis

HGF converts ErbB2/Neu epithelial morphogenesis to cell invasion.

Hanane Khoury, Monica A. Naujokas, Dongmei Zuo, Veena Sangwan, Melanie M. Frigault, Stephanie Petkiewicz, David L. Dankort, William J. Muller, and Morag Park.

Molecular Biology of the Cell 16(2): 550-61, 2005.

Grb2-independent recruitment of Gab1 requires the C-terminal Lobe and Structural Integrity of Met Receptor Kinase Domain

Lisa S. Lock, Melanie M. Frigault, Caroline Saucier, and Morag Park

The Journal of Biological Chemistry 273(32):30083-90. May;2003.

Commentary: Live-Cell Microscopy: Tips and Tools

Melanie M. Frigault, Judith Lacoste, Jody Swift, and Claire M. Brown.

Submitted to the Journal of Cell Science

Contribution of authors

In Chapter 2, I performed all of the experiments with the exception of Monica Naujokas did the transfection for the generation of the stable cell lines (Figure 1), set up the scatter assays and morphogenesis assays and together we quantified the tubulogenic response and I took images represented in Figures 2 and 3.

In Chapter 3, I first demonstrated that Gab1 and p-ERK are localized to dorsal ruffles (Figure 1). I demonstrated that over-expression of Gab1 in MDCK and in HeLa cells enhances dorsal ruffle formation (Figures 2 and 4). I helped in optimization for Gab1 siRNA in MDCK cells (Figure 2).

I demonstrated the requirement of Gab1 for dorsal ruffle formation downstream of EGF and PDGF receptors and performed the rescue experiments (Figure 3 and S2).

I observed the initial effect that Met trafficking is enhanced in MDCK cells that over-express Gab1 (MDCK Met/HA Gab1) (Figure 5C). I determined the half-life of Met by fitting the densitometric data (Figure 5B).

I showed that Gab1 associated proteins Shp2, p85 and Crk are localized to dorsal ruffles (Figure S1). I demonstrated in HeLa and MDCK cells that Gab1ΔCrk is required for dorsal ruffle formation whereas Gab1Δp85 or Gab1ΔShp2 are dispensable (Figure 4).

I determined by live cell imaging that Gab1ΔPH does not accumulate into dorsal ruffles.

I determined that Gab1 does not traffic with Met into the endocytic pathway via EEA1 positive endosomes, but remains at the cell surface, both in MDCK and HeLa cells (Figure S3). I participated in the generation of the model in Figure 8.

In Chapter 4, I carried out all experiments although Monica Naujokas set up the collagen assays, and Robin Baytte (Quorum Technologies Inc.) for SDCM deconvolution.

In the publication with Hanane Khoury,

I demonstrated the extent of ERK activation in cells pretreated or not with a MEK inhibitor (UO126) with a time course of HGF stimulation (Figure 8D).

In the publication with Lisa S. Lock (Appendix 1),

I helped with the cloning of the Met C-terminal truncation mutants (Figure 3D), and the Tyrosine to Glutamic acid substitutions (Figure 5).

I performed the GST-Grb2 pulldown, Gab1 phosphorylation, and transformation experiments (Figure 5B,C).

I created the model in Figure 7.

In the Commentary on Live-Cell Imaging,

I suggested using the Leibowitz option for cell viability experiments (Figure 1).

I generated data for Figure 5 to determine the advantages of SDCM and to demonstrate the advantages of image processing.

I also participated in the generation of the manuscript.

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Chapter 1

Literature Review

1. Introduction

Cells must communicate in order for their life cycle to be maintained and so that the plethora of cell types of an organism can perform their most specialized function in a co-ordinate fashion. Striking evidence is provided in whole organism, non-invasive studies using cutting edge tools in combination with imaging techniques, which serve as a powerful way to objectively observe the intricacies of life (Spector, 2005). These observations set forth questions as to how these complex biological processes are regulated and what are the molecular events that drive them. Herein, I present work that sets out to continue the characterization of the requirements of a docking protein, Gab1, in cellular processes that regulate cell movement and function. The deregulation of these processes is considered to be a causal event in the progression of human disease, and therefore it is essential to understand the basic molecular mechanisms at work.

2. Protein Tyrosine Kinases

In the human genome, there are more than five hundred and twenty genes that encode for protein kinases. These are enzymes which function to relay intracellular signals via the addition of the γ-phosphate from bound ATP to tyrosine residues on itself and on protein substrates in a process called phosphorylation. There are more than one hundred and thirty genes that encode for another class of enzymes that mediate the reversal of phosphorylation, phosphatases (Blume-Jensen and Hunter, 2001). Together, these exert tight regulation of protein phosphorylation and hence signal transduction.

The impact of protein phosphorylation on cell biology was first evident in 1979 when Edwin Krebs determined that phosphorylation modulated the activity of metabolic enzymes and hence cell growth (Krebs and Beavo, 1979).

There are two types of protein kinases, those that transfer phosphate to the free hydroxyl of serine and/or threonine residues, and those that phosphorylate tyrosine residues. There are 90 genes that encode for protein tyrosine kinases, of which fifty-eight of them are receptor tyrosine kinases (RTK). RTKs have been classified into twenty subfamilies based on their structural similarities. All RTKs consist of an extra-cellular portion, which mediates binding to ligand, a single pass trans-membrane helix and an intra-cellular domain, which harbors the kinase and multiple tyrosine residues. RTK deregulation is a common mechanism by which cells acquire the growth advantages that confer the onset and progression of human disease. More than half of all RTKs have been characterized to drive malignancies (Blume-Jensen and Hunter, 2001).

The molecular mechanisms underlying human disease have intrigued many scientists. The onset of research to understand the mechanistic basis of cancer involved studies of the cancer induced by DNA and RNA tumor viruses. The observation that these viruses, such as the Rous sarcoma virus (RSV), were able to confer to normal cells a transformed phenotype due to one viral gene, v-SRC, (Lai et al., 1973; Martin, 1970) initiated the search for the function of this cellular counterpart's gene product. Michael Bishop and Harold Varmus won the Nobel prize in medicine for their discovery of the cellular origin of retroviral oncogenes, determining that these viruses are oncogenic by their incorporation of a cellular gene into the viral genome during replication in the host cell (Stehelin et al., 1976). The Src protein was identified (Brugge and Erikson, 1977),

and was shown to have kinase activity (Collett and Erikson, 1978; Levinson et al., 1978), to phosphorylate tyrosine residues (Hunter and Sefton, 1980) and that this phosphorylation of tyrosine was essential to the transformation potential of the oncogene (Sefton et al., 1980). With the concomitant molecular cloning and discovery that other gene products that drive the transforming potential of other tumor viruses, such as polyoma-virus middle T antigen and v-abl, and that these are associated with tyrosine kinase activity, it became clear that tyrosine phosphorylation could regulate cellular transformation, a hallmark of cancer (Hanahan and Weinberg, 2000).

3. Met Receptor Tyrosine Kinase

The Met receptor tyrosine kinase was originally identified from a chemically treated human osteogenic sarcoma cell line (Cooper et al., 1984). Cells treated with the chemical carcinogen N-methyl-N'-nito-N-nitrosoguanidine (MNNG) were found to express an oncogene that promoted transformation of murine NIH 3T3 fibroblasts. This oncogene is the product of a chromosomal translocation between the translocated promoter region (Tpr) locus on chromosome 1 and that of Met on chromosome 7 (Dean et al., 1985; Park et al., 1986). The region of Tpr which becomes fused to the cytoplasmic region of Met encodes for a leucine zipper which mediates dimerization of the translocation product and consequently, constitutively activating the tyrosine kinase portion of the molecule (Rodrigues and Park, 1993).

The *Homo sapiens MET* gene is located on chromosome 7q31 (Park et al., 1986; Park et al., 1988) and encodes for the Met RTK (Park et al., 1988). This is a single chain precursor molecule which requires post-translational proteolytic processing once the

receptor has trafficked through the synthetic pathway and is present at the cell surface (Gonzatti-Haces et al., 1988; Prat et al., 1991). In its mature form, Met is an α–β hetero-dimer with an extra-cellular α-chain disulfide linked to the membrane spanning β-chain. The extra-cellular portion of the β–chain provides a high-affinity binding site for the ligand of the receptor, Hepatocyte Growth Factor (HGF) (Basilico et al., 2008; Bottaro et al., 1991). HGF is a high molecular weight growth factor that requires proteolytic cleavage to expose binding sites for interaction with the Met receptor (Gherardi et al., 1993). HGF has an interface on the opposite surface of the structure which binds another molecule of HGF, and in this manner induces receptor clustering (Gherardi et al., 1997; Stamos et al., 2004; Wickramasinghe and Kong-Beltran, 2005).

Upon binding of HGF to the Met receptor, or constitutively in the case of Tpr-Met, *trans* activation of the receptor occurs by engaging the intracellular kinase domain. The activation loop of the kinase domain harbors residues Y1234 and Y1235 which serve as sites of trans-phosphorylation, and the addition of phosphate to these twin tyrosine residues render the kinase active (Ferracini et al., 1991; Naldini et al., 1991; Rodrigues and Park, 1994). The activated Met kinase then uses ATP to transfer phosphate to other tyrosine residues in the receptor itself as well as onto other protein substrates (Bardelli et al., 1992). The receptor contains multiple tyrosine residues that become phosphorylated upon ligand binding and subsequent kinase activation. Amongst these are tyrosine residues in the carboxyl terminal tail and the juxta-membrane region of the receptor (Figure 1). The carboxyl terminal tail harbors a two tyrosine-based motif, Y^{1349}VHVNATY^{1356}VNV, which has been shown to be absolutely required for Met induced signaling and biology (Fixman et al., 1995; Ponzetto et al., 1994; Sachs et al.,

1996). Moreover, a $DY^{1003}R$ motif present in the juxta-membrane domain serves also as a docking site for the recruitment of signaling molecules (Peschard et al., 2001).

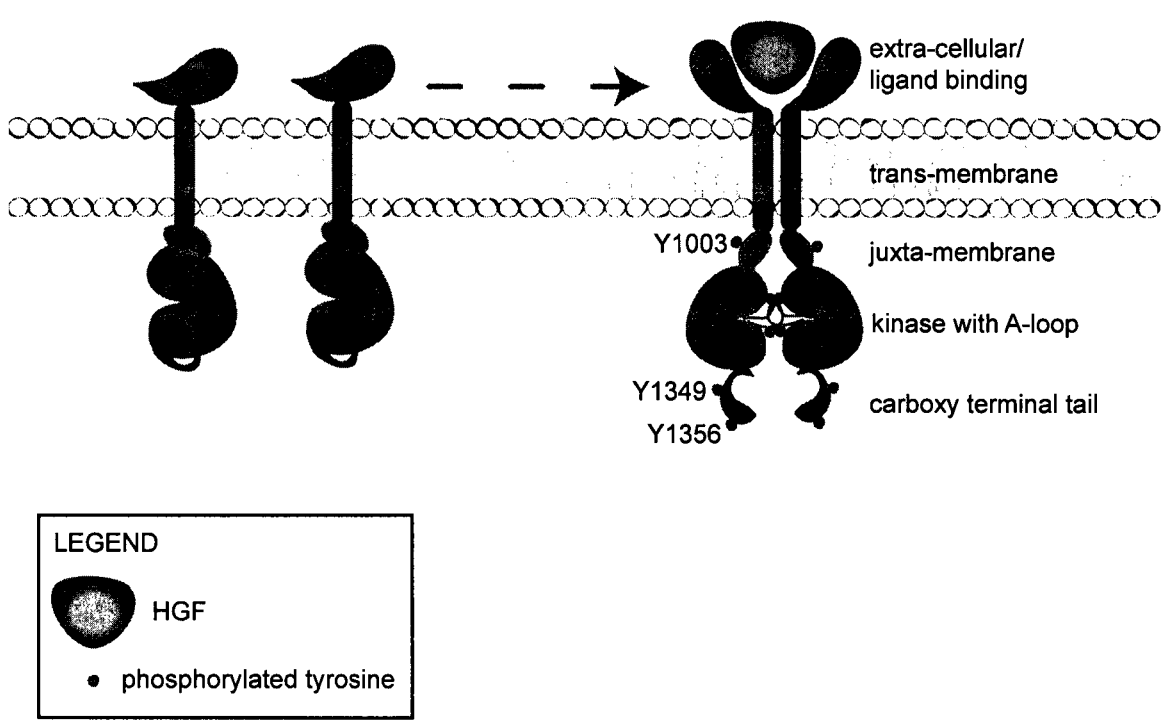

Figure 1. Model of Met receptor activation

Met resides in the plasma membrane as an inactive monomer (left hand side) and upon binding of ligand, HGF, becomes activated inducing changes in conformation of the kinase, engaging the Activation loop (A-loop) and subsequent phosphorylation of residues in the juxta-membrane region ($DY^{1003}R$) as well as in the carboxy terminal tail on the multi-substrate docking site of Met ($Y^{1349}VHVNATY^{1356}VNV$). It is thought that a conformational change in these two regions of Met exposes the tyrosine residues which serve to mediate most of Met signals. The cartoon is drawn to reflect these changes.

3.1 Met regulated biological functions

The biological consequence of the HGF-Met axis of signal transduction is briefly discussed herein.

3.1.1 Embryogenesis

During early development, the embryo becomes patterned through the temporally and spatially controlled activation of distinct sets of genes. These orchestrate the highly regulated specification of the various regions of the embryo. Once the spatial limits of the embryo have been determined, the localized establishment of various tissues is elaborated. Information for tissue patterning of the embryo occurs by coordinate signal transduction. At this stage of organ formation, the Met receptor is expressed on the cell surface of epithelial, endothelial and myogenic cells as well as in neural tissue, whereas HGF is of mesenchymal origin (Andermarcher et al., 1996; Sonnenberg et al., 1993b; Yang et al., 1996). Embryonic expression studies demonstrate that this signaling axis functions in a paracrine mode (Sonnenberg et al., 1993a). The critical function of HGF and Met in embryogenesis is demonstrated via studies of mice with targeted disruption of the HGF or Met genes. These animals die *in utero* between embryonic days 12 and 15, which is coordinate with the onset of murine organ development. Embryonic lethality is specifically due to inadequate placenta formation and hepatocyte differentiation (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995).

3.1.2 Epithelial morphogenesis

The Met and HGF signaling pathway are involved in the formation of epithelial organs by the creation of a tubular network of epithelia sheets encompassing 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. The first stage entails the conversion of a single cell into a polarized monolayer of cells, regulated primarily by an intrinsic genetically determined program. Each cell is thought to be driven to become polarized by the generation of three cell surfaces which are characteristic of a polarized cell, apical, lateral and basal domains (Montesano, 1986; O'Brien et al., 2002). This begins with several rounds of cell division and the establishment of cell-cell contacts on the lateral membrane, involving the recruitment and engagement of adherens junctions molecules and proteins of the tight junctions (Gumbiner, 1996). The ensuing cytoskeletal rearrangements engage integrin-receptor complexes at sites of cell to extra-cellular matrix (ECM) contact on the basal surface of cells. Subsequently, an elaboration of the ECM provides signals for lumen formation and the generation of an apical membrane as demarcated by tight junctions. Cellular polarity is completed by the vesicular delivery of proteins from the synthetic pathway to either the apical or baso-lateral surfaces. Those cells that are not in contact with the ECM undergo programmed cell death, which allows for the formation of a single layer of polarized epithelial cells with a hollow lumen known as a mature cyst. The second phase of epithelial morphogenesis is regulated by extrinsic growth factors, namely HGF which remodels the polarized monolayer to generate a branched tubular network of epithelial sheets encompassing a continuous lumen. This begins with the protrusion of a

single cell on the basal surface into the ECM (Figure 2). Cell migration and proliferation from the site of initial protrusion elaborates a multi-cellular chord like structure form the cyst. During migration, cells lose their apical-basolateral polarity and regain it by external cues from the ECM and from HGF (Lubarsky and Krasnow, 2003; Pollack et al., 2004). This process is repeated consecutively to elaborate a network of branching tubules, which results in the formation of viable organ capable of carrying out life sustaining vectorial transport of absorption and secretion of nutrients and biological fluids (Hammerton et al., 1991).

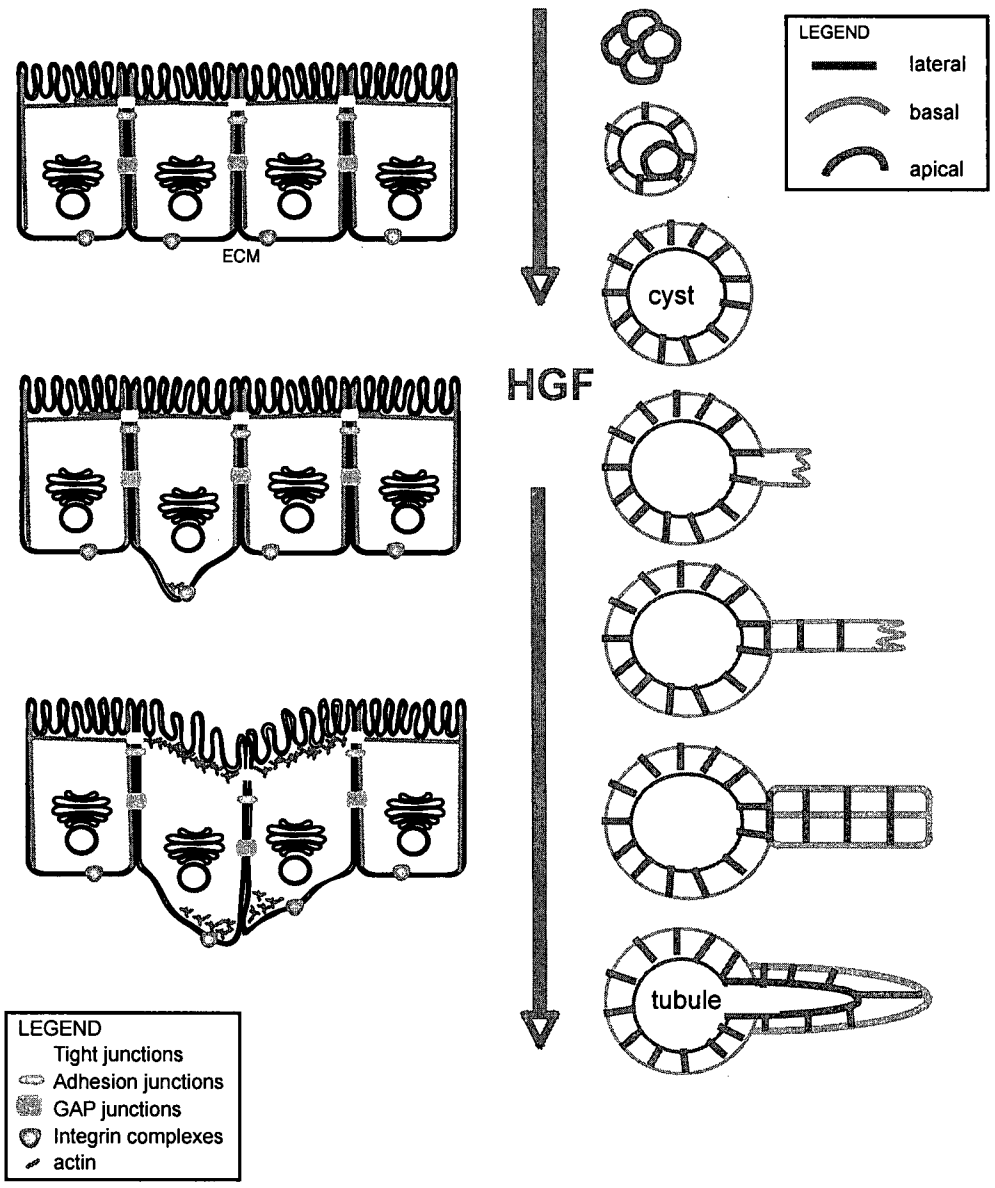

Figure 2. Model of epithelial morphogenesis.

The first stage of epithelial morphogenesis results in the generation of a well-polarized monolayer of epithelial cells. The right 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 homophilic ligands or those of the ECM respectively, result in a monolayer of cells with apical, basal and lateral membrane domains. During organogenesis, HGF is secreted by the surrounding tissues and activates the remodeling of cell-cell contacts and the formation of protrusions into the ECM (bottom, right hand side). This is elaborated to form a series of cells elongated from the initial cyst during this second phase of epithelial morphogenesis for the formation of a tubule (bottom, left hand side).

The Madin-Darby Canine Kidney (MDCK) epithelial cell system is the most characterized model to study epithelial polarity and morphogenesis. MDCK cells cultured in a three-dimensional collagen matrix self-organize into a spherical cyst in the absence of HGF, however, co-culture for several days with HGF promotes the second phase of the morphogenic program and the formation of a branching tubular network (Figure 3) (Montesano et al., 1991; Pollack et al., 1998). This kidney model serves as a representative *in vitro* system to delineate the signals and dynamics of cell polarity and morphogenesis (Zegers et al., 2003). Met and HGF are expressed in the developing murine kidney (Woolf et al., 1995) as well as in overall human organogenesis (Kolatsi-Joannou et al., 1997). The Met receptor belongs to a small family of RTKs composed of Met and mammalian Ron or avian Sea. The ligand of this related RTK is macrophage stimulating protein (MSP) and the chicken ortholog. Upon binding of their cognate ligands, these RTKs activate intrinsic morphogenic programs, confirming the role of Met and closely related RTKs in guiding morphogenesis (Maffe and Comoglio, 1998; Medico et al., 1996). The process of epithelial morphogenesis of organs is based on the coordination of multiple cell functions including proliferation, survival, migration, and ECM degradation (Trusolino et al., 1998).

3.1.3 Cell scatter and migration

As epithelial cells proliferate, they form colonies of cells held together by cell-to-cell contacts and to the ECM by cell-matrix adhesions. HGF treatment induces colonies of epithelial cells cultured in a Petri dish to scatter from one another (Figure 3) (Gherardi et al., 1989; Stoker, 1989; Stoker et al., 1987). This requires breakdown of cell-cell

contacts, remodeling cell-matrix adhesions, activating signals for cell locomotion, and cell scatter. The dissociation of cells from one another, and subsequent cell movement occurs during embryonic development, where for example myogenic precursor cells migrate from the dermomyotome in a Met dependent manner to the developing limb buds, the diaphragm and the tip of the tongue to form skeletal muscle (Bladt et al., 1995; Yang et al., 1996).

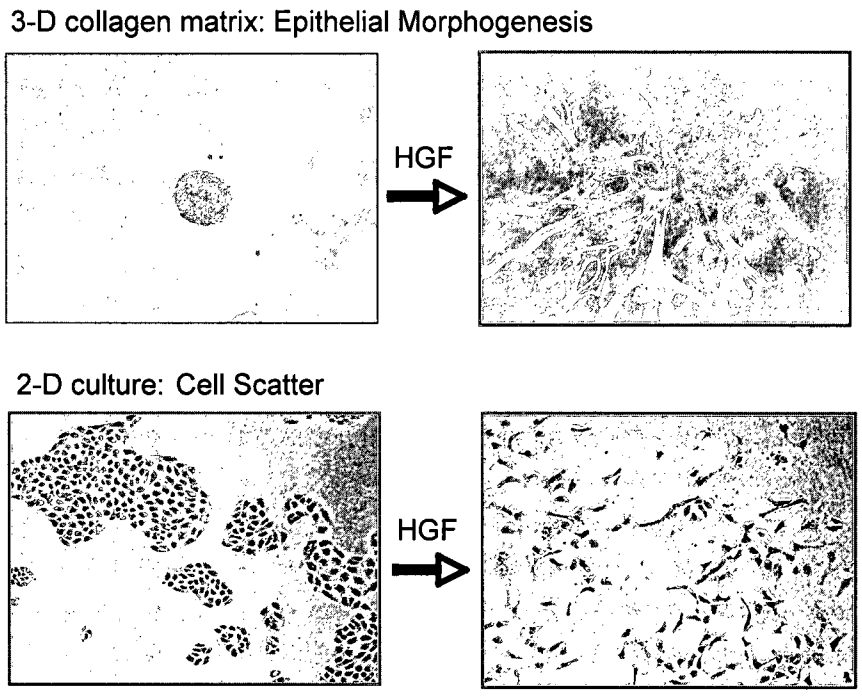

Figure 3. MDCK *in vitro* model for Met mediated biological processes.

MDCK cells seeded in a three-dimensional collagen matrix were cultured for one week to allow cells to undergo the first stage of epithelial morphgenesis resulting in the formation of a well polarized cyst. HGF treatment for an additional week induced the second phase of epithelial morphogenesis. This results in a branching network of a single layer of MDCK cells (top, right hand side). MDCK cells seeded on a Petri dish in a two-dimensional culture undergo a similar first stage of epithelial polarity via the establishment of cell colonies which have distinct apical, and basolateral membrane surfaces. When colonies of MDCK cells are stimulated overnight with HGF, they breakdown their cell-cell contacts, remodel their actin cytoskeleton and activate signals for individual cell migration resulting in cell scatter as seen in the DIC image (bottom, right hand side).

The breakdown of cell-cell contacts and subsequent migration of cells is also a mechanism used for tumor metastasis where a single cell from the primary tumor dissociates and migrates to a distant site. Hence, tumor cells likely use embryonic programs to become invasive. However, in the adult, cell migration of individual cells is not a common event. Migration of cells as a collective sheet occurs during wound healing, where their cell-cell contacts are maintained, as in epithelial morphogenesis during organ formation. Consistent with a role for the HGF/Met signaling axis, HGF mediates tissue regeneration of the liver and kidney (Matsumoto and Nakamura, 2001; Michalopoulos and DeFrances, 1997; Roos et al., 1995), as well as skin wound healing (Chmielowiec et al., 2007).

3.1.4 Cellular proliferation and survival

The activation of the HGF-Met axis of signal transduction leads to activation of biochemical signals which regulate the ability of cells to proliferate and stay alive. These are the general result of two well characterized signaling pathways, the MAPK and PI3K pathways respectively. Proliferation is the result of activation of the Ras signaling cascade resulting in ERK activation, and translocation to the nucleus in order to promote transcription of genes for cell proliferation. Signals for proliferation are activated by HGF stimulation and are required for epithelial morphogenesis (Maroun et al., 2000; Potempa and Ridley, 1998). Activation of the PI3K pathway is also required for Met mediated cell scatter and morphogenesis (Derman et al., 1995; Royal and Park, 1995). Furthermore, activation of the PI3K pathway is a general mechanism for the activation of anti-apoptotic signals to promote cell survival. Briefly, this occurs via the PI3K

dependent recruitment of AKT and subsequent regulation of BAD and caspase-9, both regulators of apoptosis (Datta et al., 1997). Notably, HGF has been shown to protect from cardiac injury as it promotes cell survival of cardiomyocytes (Nakamura et al., 2000), protects hepatocytes from cell death (Sangwan et al., 2006), and is a regulator for DNA repair mechanisms (Fan et al., 2001).

4. Modules for signal transduction

Signal transduction in response to extra-cellular cues begins with the activation of a cell surface receptor complex. This signal is relayed from the cell surface to the interior of the cell via the recruitment of cytosolic proteins that often contain modular protein domains and/or peptide motifs. Modular domains are typically between 40 and 300 amino acids in length, which autonomously fold into a compact, stable three-dimensional structure. These may have catalytic function, or serve to mediate interaction with proteins, lipids, nucleic acids, or small molecules. A single protein may contain one or more of a variety of these modular domains and/or peptide motifs that interact with other modular domains. Protein-protein interactions are mediated by modular domains which can either interact with another modular domain in a homo-philic interaction or the interaction can occur when the structured domain of one protein interacts with a linear peptide motif of another ranging from 3 to 13 amino acids in length (Pawson et al., 2001).

The modular domains contained within proteins serve to mediate interactions in a regulated manner. These interactions may result in the recruitment of a substrate to an enzyme to make the reaction favorable, promote a change of subcellular localization, or

result in a change in conformation of the protein thereby regulating its function. Moreover, the engagement of modular protein domains with their ligands is the molecular mechanism by which signaling networks are dynamically regulated. Proteins function in a lock and key fashion to mediate highly specific interactions and carry out the relay of information from one protein complex to another resulting in a signal transduction cascade. Some of the basic modular domains are described herein, however, a plethora of other modules exist and new one are constantly being characterized and new functions are being assigned to old ones (Pawson and Nash, 2003).

4.1 Phosphorylated tyrosine binding domains

The transfer of phosphate to the hydroxyl group of a tyrosine residue in a substrate protein by a kinase functions to create a binding site. Proteins that contain a modular protein domain recognize these binding sites. An overview of their function is described herein, however these are reviewed in greater detail elsewhere (Pawson et al., 2001; Schlessinger and Lemmon, 2003; Yaffe, 2002). These are the building blocks of signaling and by surveying a few examples it is clear that there is a complexity that remains to be elucidated for the mechanisms of signal transduction.

4.1.1 SH2 domains

Src Homology 2 (SH2) domains are a folded protein domain present in a plethora of adaptor molecules and enzymes. The SH2 domain was identified by Tony Pawon's group in v-Src, as well as in other viral oncogenes, and were identified as non-catalytic and adjacent to a carboxy terminal kinase domain (SH1). The functional importance of

the SH2 domain was highlighted by determination that the integrity of these modules was required for transformation (Pawson, 1988; Sadowski et al., 1986).

Phosphorylated tyrosine residues fit into the pocket of the SH2 domain where the phosphate engages residues at the base of the binding pocket (Waksman et al., 1992; Yaffe, 2002). SH2 domains from different proteins or protein classes have distinct binding preferences. To elucidate the specificity of different SH2 domains, Lewis Cantley's group used phospho-peptide libraries to determine the binding specificity of SH2 domains from various protein classes. An SH2 domain binding to a peptide ligand requires a pY-X-X-X consensus sequence: where X is any amino acid and pY a phosphorylated tyrosine (Songyang et al., 1994; Songyang et al., 1993). Consensus binding sites for specific proteins have been determined, where X at any of the three positions requires a certain amino acid. Bioinformatics tools have been developed based on this information in order to predict the recruitment of specific proteins to potential tyrosine based motifs (Yaffe et al., 2001).

SH2 domains are not present in RTKs, however, it is clear that specific SH2 domain containing proteins are recruited to specific phosphorylated tyrosine residues in the non-catalytic region of RTKs (Anderson et al., 1990; Koch et al., 1991). The concept of signal transduction and the mechanism for assembly of RTK signaling complexes, is provided by the elucidation of the phosphotyrosine-SH2 interaction.

Proteins such as the tyrosine kinase ZAP-70, contain two tandem SH2 domains adjacent to a tyrosine kinase domain. The tandem SH2 domains of ZAP-70 bind to two phosphorylated tyrosine residues known as a tandem activation motif (TAM) in the cytoplasmic tail of the T-cell Receptor complex (TCR). The affinity of phosphorylated

tyrosine binding in this manner is 500 to 1000 fold increased over the affinity of a single SH2 domain of ZAP-70 binding. Moreover, this avidity-affinity effect was observed with the tandem SH2 domains of other proteins such as those of the p85 subunit of PI3K, the protein tyrosine kinase Syk, the tyrosine phosphatase Shp2, and phospholipase C γ1 (PLCγ1). The biologically correct tandem TAM for tandem SH2 domains is 1000 to 10000 times more specific than to any other TAM (Ottinger et al., 1998; Schlessinger and Lemmon, 2003).

4.1.2 PTB domains

Another modular protein domain that binds phosphorylated tyrosine residues is the phospho-tyrosine binding (PTB) domain (Blaikie et al., 1994; Gustafson et al., 1995; Kavanaugh and Williams, 1994; van der Geer et al., 1995). Contrary to the SH2 domain, the peptide ligand that binds to the PTB domain mediates specificity by recognizing the residues amino terminal of the tyrosine residue with the consensus; N-P-X-pY (Campbell et al., 1994). Moreover, the SH2 and PTB domains differ in that the SH2 domain binds primarily to the phosphorylated tyrosine whereas the three residues carboxy terminal to are secondary for binding, whereas the PTB domain requires the flanking residues in order to bind specifically. Intriguingly, PTB domains can also bind to un-phosphorylated tyrosine consensus peptides, or even to peptides that do not contain tyrosine residues. Although the primary sequences of PTB domains are not highly conserved, the three-dimensional structure of these domains in different proteins is highly similar (Schlessinger and Lemmon, 2003; Shoelson, 1997).

The structure of many PTB domains bound to their peptide ligands revealed that they are more similar to the Pleckstrin Homology (PH) domains (described below) than to SH2 domains (Zhou et al., 1995). The PTB domain of Disabled-1 (Dab1) has been crystallized bound to phosphoinositides and the phosphoinositide binding site is required for recruitment of Dab1 to the plasma membrane and transduction of signals downstream of Reelin (Stolt et al., 2005; Stolt et al., 2003). Weak binding of PTB domains to phosphoinositide and peptide ligands, suggest that binding to protein and to phosphoinisoitides to target cytosolic proteins to activated receptors at the plasma membrane in a cooperative manner (Pitcher et al., 1995; Takeuchi et al., 1998). Furthermore, upon examination of cytosolic proteins that contain PTB domains, most of them contain a membrane targeting domain or signal sequence that mediates binding to the plasma membrane. Conversely, some PH domains require cooperative binding to other proteins for optimal function in recruitment to the membrane (Pitcher et al., 1995).

4.1.3 Others

The tyrosine kinase binding (TKB) domain of Cbl is another modular protein domain that binds to phosphorylated tyrosine residues. Crystallographic studies of the TKB domain bound to its phosphorylated peptide ligand have shown that this TKB domain consists of a four-helix bundle, a calcium binding EF hand domain, and a variant SH2 domain (Meng et al., 1999). Although SH2 domains are highly recognizable, SH2 domain of Cbl was not detected by primary amino acid sequence analysis. This suggests that other protein domains may exist that function as SH2 domains but remain to be uncovered. Cbl is a cytosolic scaffold with ubiquitin ligase activity and is recruited to

phosphorylated tyrosine residues in the juxta-membrane region of activated RTKs. Cbl functions to transfer a ubiquitin moiety to the RTK substrate to initiate down-regulation of the receptor (Sanjay et al., 2001). Specifically, Cbl is recruited to Y1003 of the juxta-membrane region of the Met RTK. This interaction is mediated by phosphorylation of the tyrosine residue, and specificity is determined by the two amino acid residues flanking the phosphorylated tyrosine, D-pY-R (Lupher et al., 1997; Peschard et al., 2001; Peschard et al., 2004).

Crystallographic studies of other protein modules are revealing more domains that are capable of mediating interaction with phosphorylated tyrosine. For example, the C2 domain of Protein Kinase C (PKC) δ is described as a modular domain which binds phospholipids in a calcium dependent manner and results in the activation of the adjacent serine/threonine kinase domain of PKC δ (Newton and Johnson, 1998). This domain was also found to bind to phosphorylated tyrosine residues and is the first example of a serine/threonine kinase that is regulated by binding to phosphorylated tyrosine suggesting a mechanism for cross talk between different signaling pathways (Benes et al., 2005).

4.2 Phosphorylated serine and/or threonine binding domains

Serine and/or threonine phosphorylation can result in the formation of multi-molecular signaling complexes through specific interactions between phosphorylated serine/threonine (S/T) binding modules and phosphorylated sequence motifs. The first identified protein to bind phosphorylated serine and threonine residues was 14-3-3. A mechanism conserved from yeast to humans for regulating the G2/M cell cycle checkpoint uses a phosphorylated serine to provide a binding site for 14-3-3 acting as a

switch into cell cycle arrest in response to DNA damage (Peng et al., 1997). Forkhead-associated (FHA) domains are modular protein domains that bind phosphorylated threonine bases motifs. These were first identified in transcription factors and are now found in a wide variety of signaling molecules. FHA domains are typically larger than other protein domains, and are evolutionarily conserved (Yaffe and Elia, 2001).

4.3 Proline rich sequence binding domains

The elucidation of yet another modular protein domain which bind to proline rich sequences, the Src homology 3 (SH3) domain, continues to elaborate the mechanisms of signal transduction. Other proline rich sequence binding modular domains include the Enabled/VASP homology (EVH1) domain named after the proteins in which it was first discovered (Zarrinpar et al., 2003) and WW domains named for their conserved tandem tryptophane sequence. Intriguingly, while most WW domains bind proline rich motifs, one group of WW domains bind to phosphorylated peptides of the consensus phosphorylated S/T-P sequence (Ingham et al., 2005; Lu et al., 1999). These domains are structurally distinct from SH2 or PTB domains and each other which suggest multiple methods have evolved for successful relay if signals.

4.3.1 SH3 domains

The molecular cloning of v-Crk by Hidesaburo Hanafusa and Bruce Mayer, resulted in the discovery of another protein domain, which mediates interaction with a proline rich peptide ligand, the SH3 domain (Mayer et al., 1988). SH3 protein domains were initially found by primary sequence homology comparisons in other unrelated

proteins such as, PLCγ1, Abl, and Src family kinases, as well as in proteins that interact with the cytoskeleton (Matsuda et al., 1992; Mayer and Eck, 1995). The small protein Grb2 was found to link RTKs to the activation of Ras by acting as a recruitment intermediate. Thus Grb2 and similar proteins became known as adaptor molecules since they link downstream signals to upstream RTKs. An SH3 domain in Grb2 mediates recruitment of Sos to the RTK complex, a guanine exchange factor (GEF) that activates Ras (Buday and Downward, 1993; McCormick, 1993).

SH3 domains are approximately 60 amino acids in length, and form a folded globular structure with a hydrophobic interaction surface. The interaction occurs whereby individual proline residues are recognized, and the sequences flanking the proline residues mediate binding specificity. A general consensus recognition site, P-X-X-P (Wu et al., 1995), has been proposed, however studies of SH3 domain containing proteins and their biologically correct recognition peptides have determined that this is not a stringent requirement for interaction (Rickles et al., 1994). Notably, SH3 domains can bind their peptide ligands in both orientations due to the nature of the symmetric P-X-X-P binding motif (Feng et al., 1994). There possibly is an avidity-affinity effect regulating SH3-proline rich binding, since full length proteins interact with a stronger affinity than to the short peptide ligand alone, as demonstrated for Abl and Grb2 SH3 domains (Ren et al., 1993; Simon and Schreiber, 1995). Moreover, homophilic SH3 domain interactions have been described and therefore provide another mechanism for signal transduction (Dimasi, 2007).

Although SH3 domains have been studied extensively for twenty years, the regulation of binding still remains unknown. Whereas SH2 domains bind to tyrosine

residues only once they have been phosphorylated, SH3 domains require no post-translational modification to regulate their interaction. Therefore it has been proposed that the SH3-proline rich peptide interaction is a constitutive one. The regulation of the SH3 domain and proline rich motif interaction remains elusive, however, SH3 domain engagement with a protein may regulate the function of the molecule. For example, the SH3 domain of Src family kinases bound to the p85 subunit of PI3K increases *in vitro* activity of this lipid kinase 5 to 7 fold, and is competed by a 15 amino acid peptide sequence representing the residues in p85 which binds the SH3 domain of Lyn and Fyn (Pleiman et al., 1994). Another regulatory role for SH3 domain may be to keep a molecule auto-inhibited in order to prevent promiscuous activation of signal transduction. In cooperation with the SH2 domain, the adjacent SH3 domain provides further contacts in an intra-molecular fashion keeping Src family kinases in a folded conformation, masking the other domains (Superti-Furga et al., 1993). The carboxy terminal SH3 domain of Crk may serve in this manner to negatively regulate Crk signaling since it is absent in the oncogene v-Crk (Reichman et al., 1992). The absence of the SH3 domain of Crk may be the mechanism by which v-Crk is transforming. This domain has been shown to inhibit the signals downstream of Crk (Ogawa et al., 1994), however, over-expression of this SH3 domain activates PI3K and pathogen entry into cells (Dokainish et al., 2007).

Interestingly, yeast *Saccharomyces cerevisiae* has SH3, but no SH2 domain containing proteins which mediate most protein-protein interaction networks (Tong et al., 2002). The evolutionary appearance of SH2 domains coincides with that of protein

tyrosine kinases in protozoa and *Dictyostelium discoideum*, which have the ability to activate directed movement in response to extra-cellular cues.

4.4 Membrane targeting domains/sequences

Upon cell surface receptor stimulation, many cytosolic signaling proteins are recruited to specific membrane micro-domains of the plasma membrane or other endo-membranes, where they are compartmentalized and function locally to activate, regulate or maintain signal transduction, trafficking, and cytoskeletal rearrangements (Hurley and Meyer, 2001). Therefore it must be emphasized that signal transduction does not only consist of intricate protein-protein interaction via modular protein domains and peptide ligands, but also that the subcellular localization of these signals adds a spatial component of regulation. A brief examination of one class of each protein domain and signal sequences that mediate recruitment to membranes is found herein.

4.4.1 PH domains

The Pleckstrin Homology (PH) domain was identified as a region of 120 amino acids duplicated in Pleckstrin, the major substrate of PKC in platelets (Tyers et al., 1988). By sequence comparison, PH domains are potentially found in a wide variety of proteins (Pearson and Lipman, 1988). These include a vast array of molecules involved in signal transduction (Haslam et al., 1993; Mayer et al., 1993). Phospholipid interaction domains bind acidic phospholipids and in mammalian cell membranes these are phosphatidylserine, phosphatidic acid, and phosphatidylinositol. Phosphatidylinositides are a minor constituent of the inner leaflet of the plasma membrane. A small portion of

phosphatidylinositides are phosphorylated on the hydroxyl groups of the inositol ring of the molecule namely, on positions 4 and 5, generating the second messenger phosphatidylinositol 4,5 bisphosphate (PIP_2) which is estimated at less than 1% abundance (McLaughlin and Murray, 2005). This very minor constituent of cellular membranes has a disproportionate role in signaling, as it is the most influential. PIP_2 functions as a ligand for PH domains to recruit and activate signaling pathways. Moreover, PIP_2 is the substrate of lipid kinases, phosphatases and lipases, which remodel PIP_2 to generate other lipid species that function in signal transduction (Figure 4).

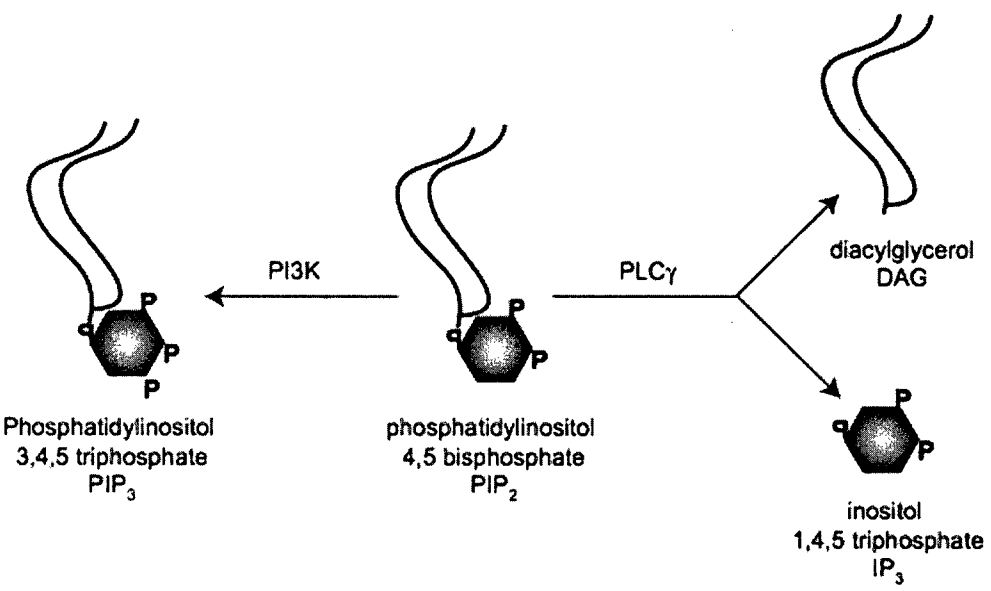

Figure 4. PIP_2 metabolism in biological membranes.

PIP_2 is phosphorylated on two hydroxyl groups of its inositol ring, and is the substrate for PI3K which adds a phosphate group to the 3' position generating a tri-phosphorylated lipid species important for biological signaling, PIP_3. PIP_2 is also a substrate of PLCγ, which hydrolyses the molecule to generate two second messengers for signaling, DAG that remains in the membrane and IP_3 which diffuses into the cytosol.

Of all lipid binding protein domains, PH domains are the most abundant. There are more than 250 proteins in humans and more than 30 in yeast, that contain PH domains (Lemmon, 2008). Their abundance and conservation through evolution suggests that targeting of their host protein to a specific membrane compartment is a basic mechanism in biology. Exclusively, PH domains bind to PIP_2, phosphatidylinositol 3,4,5 triphosphate (PIP_3), and phosphatidylinositol 3,4 bisphosphate ($PI(3,4)P_2$) with high specificity and affinity (Garcia et al., 1995; Harlan et al., 1994; Kavran et al., 1998; Lemmon et al., 1995). Binding occurs by electrostatic interactions between the negative charges on the phosphate head of the lipids and hydrophobic amino acids of the binding site in the PH domain (McLaughlin and Murray, 2005).

Phosphorylation of phosphoinositides occurs at cellular membranes upon growth factor or cytokine stimulation (Cantley, 2002). Growth factor and cytokine stimulation result in activation of the lipid kinase phosphatidylinositol 3-kinase (PI3K). This lipid kinase phosphorylates the inositol ring of phosphoinositides on the 3' position producing PIP_3 (Figure 4) and $PI(3,4)P_2$. These then bind PH domain containing proteins, recruiting them to the membrane and generally either activating the intrinsic enzymatic activity of the host protein or localizing and hence regulating a signaling pathway. Modular protein domains that engage with lipid ligands rather than peptide ligands and that this interaction was regulated by phosphorylation, expands the variety of mechanisms for signal transduction (Franke et al., 1997; Klarlund et al., 1997).

One of the major pathways activated downstream of PI3K is the cell survival pathway. Briefly, both PI3K products bind to the PH domain of AKT, resulting in the recruitment of this protein to the membrane, which allows it to be phosphorylated by 3-

phosphoinositide-dependent protein kinase (PDK1). This sequence of events promotes the activation of the survival signal (Toker and Cantley, 1997). PI3K lipid products also serves to regulate the activation of molecular switches, the Rho GTPase family, by recruiting their GEFs to the plasma membrane via an interaction with these lipid ligands, hence resulting in localized signal activation of GTPases (Welch et al., 2003).

Although the PH domain and phosphorylated phosphoinositide interaction seems to be complex and well-understood, new concepts and implications continue to emerge. For example, two proteins have been shown to come together to form an inter-molecular single PH domain, which is only then functional to engage with its lipid ligand (van Rossum et al., 2005). Moreover, PH domains that bind their lipid ligands with low affinity have been also found to function as protein-protein interaction domains. The consequence of PH domain engagement with a protein remains a mechanism to re-localize the host protein (Lemmon, 2007). In all cases, it seems that PH domains serve to target proteins to specific membrane compartments.

4.4.2 Signal sequences for lipidation

Other mechanisms of targeting proteins to the inner leaflet of the plasma membrane exist, such as the covalent attachment of lipids to amino acids in cytosolic proteins. The addition of lipophilic moieties to proteins includes the addition of fatty acids such as myristate and palmitate, isoprenoids such as farnesyl and geranylgeranyl, or a glycosyl-phsophatidyl inositol (GPI) anchor. Attachment of such lipid groups to proteins modulates protein-protein interactions, membrane binding, and signal transduction (Hancock, 2003; Resh, 1996). The lipid becomes inserted into the membrane

bilayer and specificity is determined based on the constituents of the signal sequence as well as the type of lipid that is co-translationally attached to the signal sequence.

Most Src family kinases contain a ten amino acid amino-terminal consensus sequence where the first three residues are M-G-C. This signal sequence undergoes dual acylation with myristate on the glycine residue and palmitate on the cysteine residue after removal of methionine by cleavage (Liang et al., 2004; Magee et al., 1989). This co-translational modification is imperative for the function of Src and other Src family kinases (Liang et al., 2001; van't Hof and Resh, 1999). A myriad of other cellular proteins are modified by the addition of lipophilic components, or targeted to membranes via other lipid binding domains and these are more extensively reviewed in (Resh, 1996) and (Lemmon, 2008), respectively.

5. Adaptors in signal transduction

Adaptors are proteins of multi-modular composition without enzymatic activity. These organize the transient interaction of multiple proteins, and thus serve to establish and coordinate intricate signal transduction pathways. The discovery that a viral oncogene, gag-Crk (v-Crk), was derived from a molecule whose cellular gene product does not encode for a catalytically active protein, highlighted the impact of an adaptor in signal transduction (Mayer et al., 1988).

Scaffolds or docking proteins, are a subtype of adaptor that contains multiple domains and/or motifs that mediate interactions with other signaling molecules, and most also contain a lipid binding domain or a lipidation signal sequence (Csiszar, 2006).

Specifically, scaffolds are proteins that bring enzymes in the same cascade into close proximity to favor the activation of the kinase cascade (ex: kinase suppressor of Ras (KSR) organizes the Ras-Raf-MEK-ERK pathway (Kolch, 2005)). Docking proteins serve as a signaling platform by recruiting a variety of seemingly independent enzymes and proteins to propagate and amplify the signal (ex: insulin receptor substrate (IRS) and Gab families of docking proteins (Gu and Neel, 2003; White, 1998)). These provide spatial regulation of signals by targeting associated proteins to specific subcellular compartments (Pawson, 2007). In proteomic studies of cell-matrix adhesions, the most common type of protein-protein interaction is the scaffolding function, where molecule A binds to molecule B and to C, in order for B to exert a modification to molecule C (Zaidel-Bar et al., 2007). Such studies demonstrate the abundance of these adaptors as well as their indispensable function in biological signals.

5.1 Regulation of adaptors

The primary role of tyrosine phosphorylation of adaptors is to create binding sites for SH2, PTB or TKB domain containing proteins, however, phosphorylation on tyrosine has also been described to induce a conformational change, modulating the adaptor in either a "closed" or an "open" conformation. Crk is regulated in this manner such that when it is phosphorylated, the SH2 domain folds over the protein masking the SH3 domain, thus making the SH3 domain inaccessible for protein-protein interactions (Feller et al., 1994).

Another mechanism by which phosphorylation can regulate the function of adaptors is via phosphorylation on serine and/or threonine residues. The Gab family of docking proteins, provide examples for this type of regulation. Gab1 is recruited to RTKs and phosphorylated in tyrosine based motifs for the recruitment of SH2/PTB containing proteins which lead to the activation of ERK and PI3K pathways. However, the serine/threonine kinase ERK regulates Gab1 by phosphorylating serine/threonine residues in close proximity to the tyrosine-based motifs that serve to recruit and activate PI3K. Interestingly, S/T phosphorylation by ERK downstream of the EGFR interferes with the recruitment of PI3K, and subsequently turns down the PI3K pathway (Lehr et al., 2004; Yu et al., 2002). However, downstream of Met this is a positive feedback mechanism (Yu et al., 2001). Similarly, Gab2 is regulated by negative feedback loops, which are triggered by phosphorylation on various serine and threonine residues (Brummer et al., 2008; Lynch and Daly, 2002).

Adaptor proteins are platforms on which multi-protein complexes form to aggregate signals, however, these can be regulated by cleavage. One such example occurs when proteolysis of cortactin by calpain 2 regulates membrane protrusion dynamics during cell migration. Cortactin links the actin cytoskeleton with the Arp2/3 complex resulting in productive actin polymerization and hence membrane protrusion. However, cortactin is cleaved in a polarized fashion whereby only cortactin localized to the leading edge of the cell remains intact and functional (Perrin et al., 2006). Adaptor proteins are also irreversibly regulated by degradation. Cbl interacting protein of 85 kDa (CIN85) has been shown to become ubiquitinated by Cbl upon engagement of the EGFR

and subsequently degraded along with the receptor via clathrin-mediated endocytosis and lysosomal degradation resulting in signal termination (Haglund et al., 2002).

5.2 Gab family of docking proteins

Gab family of docking proteins are evolutionarily conserved and are indispensable in transmitting signals from many cell surface receptors to control cell growth and differentiation. Several other families of docking proteins exist such as the insulin receptor substrates (White, 1998), fibroblast growth factor receptor substrate-2 (Gotoh, 2008), and Downstream of kinase (Jones and Dumont, 1998). These adaptors serve as signal amplifiers of the extra-cellular signal by assembling multi-protein complexes. Docking proteins are intrinsically regulated by changes in subcellular localization by being targeted to the plasma membrane and specifically recruited to cell surface receptors directly or via protein-protein interactions described above. In this manner, docking proteins assemble multi-protein signaling complexes and serve to amplify and propagate the signal initiated at the receptor.

All Gab family members are highly similar in their domain structure. Structural studies of full-length Gab proteins have not been successfully undertaken. Although Gab family members are only 40-50% identical with respect to their amino acid sequence, their overall topology is highly similar. These contain an amino-terminal PH domain, a proline rich region, as well as multiple conserved tyrosine based motifs for the specific recruitment of SH2 or SH3 domain containing signaling molecules (Figure 5). Moreover, Gab proteins are substrates of multiple kinases, both serine/threonine and tyrosine

kinases, RTK and non-RTKs. The phosphorylation of Gab proteins and their subcellular localization is the mechanism by which cell surface signals are transmitted and amplified.

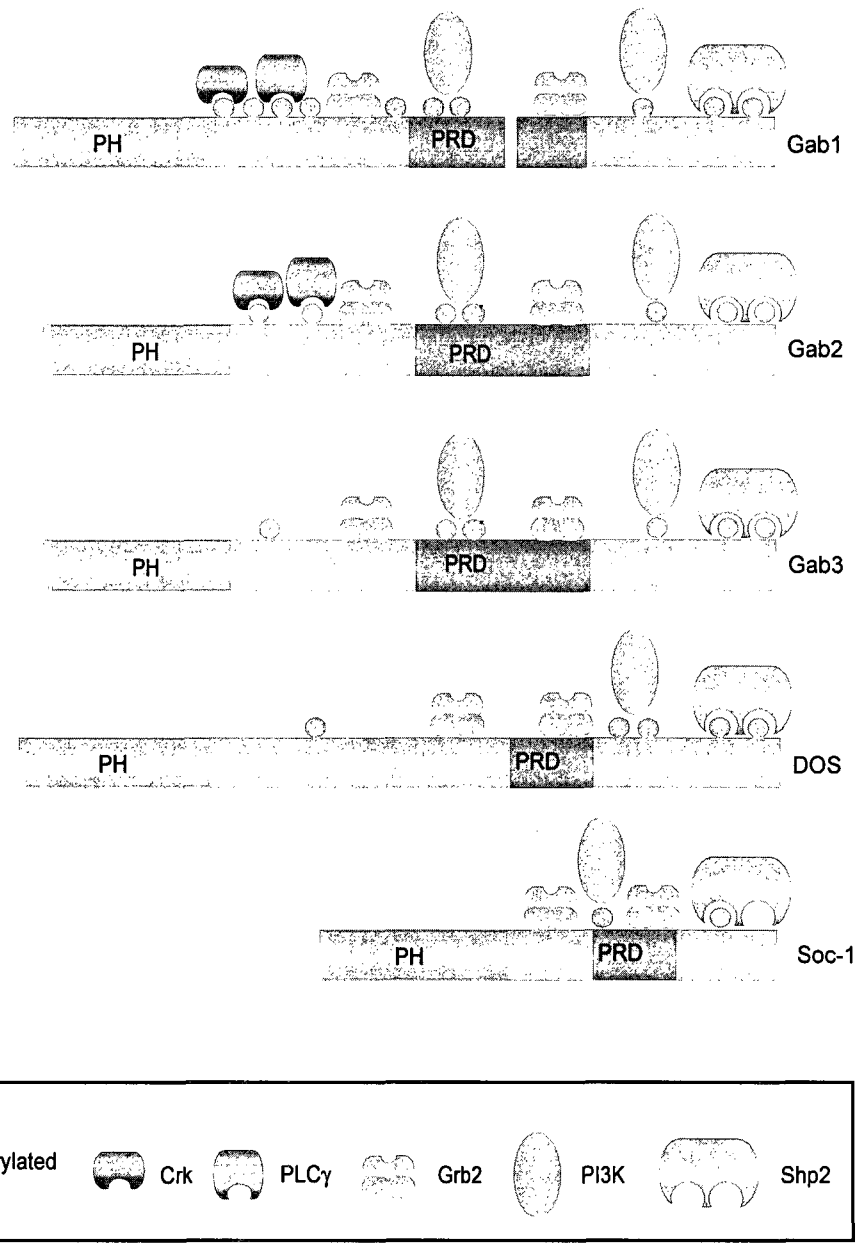

Figure 5. Gab family of docking proteins.

Gab family members have similar domain structure. Mammalian Gab1, Gab2 and Gab3 that have been identified to date. Gab family proteins are conserved through evolution, exist in *Drosophila melanogaster* as DOS and in *Caenorhabditis elegans* as Soc-1. These all contain an amino terminal PH domain and a central proline rich domains (PRD). Gab family members recruit a variety of singaling molecules which contain SH2 and/or SH3 domains to organize signaling platforms downstream of cell surface receptors.

5.2.1 Invertebrate Gab proteins

Sevenless signaling, the EGFR ortholog, is essential for *Drosophila melanogaster* eye development. The SH2 domain containing protein tyrosine phosphatase Corkscrew is also indispensable (CSW: mammalian Shp2). In a genetic screen to find the required effectors in this pathway, Daughter of Sevenless (DOS) was identified (Herbst et al., 1996; Raabe et al., 1996). DOS, the only identified Gab ortholog in *Drosophila*, was also found to be essential for DER and Torso signaling (EGFR and PDGFR orthologues, respectively) (Johnson Hamlet and Perkins, 2001). The Gab ortholog in nematodes was identified by screening *Caenorhabditis elegans* for mutations that would suppress the clear phenotype driven by EGL-15 (mammalian FGFR). Suppressor of clear-1 (Soc-1) is required for the clear phenotype and hence FGFR signaling in worms (Schutzman et al., 2001). Studies from invertebrate organisms, delineate the requirement for Gab family proteins in signal transduction and development.

5.2.2 Gab1

Gab1, the first identified mammalian Gab family member, was originally isolated as a Grb2-binding protein from a human glial tumor expression library and found to be tyrosine phosphorylated in response to EGF and insulin (Holgado-Madruga et al., 1996). Gab1 (Grb2-associated binder-1) was also independently identified in a yeast-two hybrid screen as a direct binder of the Met RTK (Weidner et al., 1996). Gab1 is the major tyrosine phosphorylated protein in cells transformed by the TPR-Met oncogene (Fixman et al., 1997), and in epithelial cell lines upon HGF stimulation of endogenous Met

(Nguyen et al., 1997). Gab1 has been reported to be tyrosine phosphorylated downstream of many cell surface receptors, such as other growth factor receptors, G protein coupled receptors, antigen receptors, and cytokine receptors (Liu and Rohrschneider, 2002). Many structure function studies have elucidated the contribution of specific Gab1 complexes in the activation of downstream signaling pathways in the context of a multitude of signals.

5.2.2.1 Gab1 recruitment to the plasma membrane

The function of Gab1 is intricately linked to its requirement to be membrane targeted. The Gab1 PH domain is known to mediate specific binding to PIP_3 via two residues in the PH domain, W^{26} and R^{29}. Substitution of these two residues for uncharged amino acids or deletion of the entire PH domain abrogates the ability of Gab1 to signal and promote morphogenesis downstream of Met (Maroun et al., 1999a; Maroun et al., 1999b). The Gab1 PH domain preferentially binds to the PI3K product PIP_3 (Isakoff et al., 1998; Maroun et al., 1999b; Rodrigues et al., 2000), and upon treatment of cells with a PI3K inhibitor, Gab1 remains cytosolic and is not localized to cellular membranes (Maroun et al., 1999a), further demonstrating that the recruitment of Gab1 to the membrane is via the PH domain-PIP_3 mechanism. Epithelial morphogenesis mediated by the Met receptor is abrogated when a Gab1 lacking the PH domain is expressed in MDCK epithelial cells, however, this defect is rescued by the addition of a myristoylation signal sequence to the Gab1ΔPH protein. This demonstrates that Gab1 must be membrane localized for productive Met signaling and biology (Maroun et al.,

2003). Although it seems from these studies that membrane targeting is the only function of the Gab1 PH domain, studies in *Drosophila* show that membrane targeting of DOS does not substitute for the PH domain (Bausenwein et al., 2000), demonstrating that their might be a more complex role for Gab family PH domains.

The function of the Gab1 PH domain is recapitulated in invertebrates where DOS and Soc-1 are not functional for signaling without functional PH domains (Bausenwein et al., 2000; Schutzman et al., 2001). Although Gab1ΔPH is not efficiently recruited to the plasma membrane upon Met activation, it is still weakly tyrosine phosphorylated and recruits many of the same signaling molecules as wild-type Gab1 (Maroun et al., 1999a). In other signaling systems, the PH domain is required for recruitment of Gab1 to the B Cell Receptor (BCR) and the Epidermal Growth Factor Receptor (EGFR) (Ingham et al., 2001; Rodrigues et al., 2000). Gab1 recruitment to these receptors functions by a positive feedback mechanism whereby Gab1 is initially recruited to the cell surface receptor, becomes tyrosine phosphorylated on docking sites for the SH2 domain of the p85 subunit of PI3K, which activates the kinase to make more PIP_3. The increased local production of PIP_3 positively reinforces the engagement of the Gab1 PH domain at the site of the receptor complex and is essential to maintain Gab1 at the receptor.

5.2.2.2 Gab1 recruitment to the Met receptor

Gab1 functions downstream of a plethora of cell surface receptors, including the Met receptor. Gab1 is recruited to cell surface receptors via several mechanisms of recruitment, which in general are mediated by protein-protein interactions. Gab1 is

indirectly recruited to receptors via another protein or set of proteins, which often include the adaptor Grb2. The central proline rich domain (PRD) of Gab1 contains multiple P-X-X-P motifs, however, two well-defined proline rich motifs engage the SH3 domains of Grb2, one extended classical P-X-X-P-X-R motif and one atypical P-X-X-X-R-X-X-K-P (Figure 6) (Lewitzky et al., 2001; Lock et al., 2002; Lock et al., 2000; Schaeper et al., 2000). Grb2 is a small adaptor molecule that consists of nearly exclusively SH2 and SH3 domains with a structure of (N)SH3-SH2-SH3(C). The carboxy terminal SH3 domain binds preferentially to the atypical proline rich sequence in Gab1 and is responsible for much of the Gab1-Grb2 interaction (Lewitzky et al., 2001; Lock et al., 2000). The SH2 domain of Grb2 binds to a phosphorylated tyrosine in the carboxy terminal tail of the activated Met receptor, mediating Gab1 indirect recruitment (Ponzetto et al., 1994). The tyrosine residue at position 1356 of Met lies in a Grb2 SH2 domain consensus binding site, pY-X-N-X, and therefore upon receptor activation, Y1356 becomes phosphorylated and engages the Grb2 SH2 domain (Bardelli et al., 1997; Fixman et al., 1997). Grb2 concurrently binds to the proline rich regions in Gab1 via its carboxy terminal SH3 domain. Gab1 also is recruited to the EGF and PDGF RTKs via the adaptor Grb2 (Kallin et al., 2004; Lock et al., 2000).

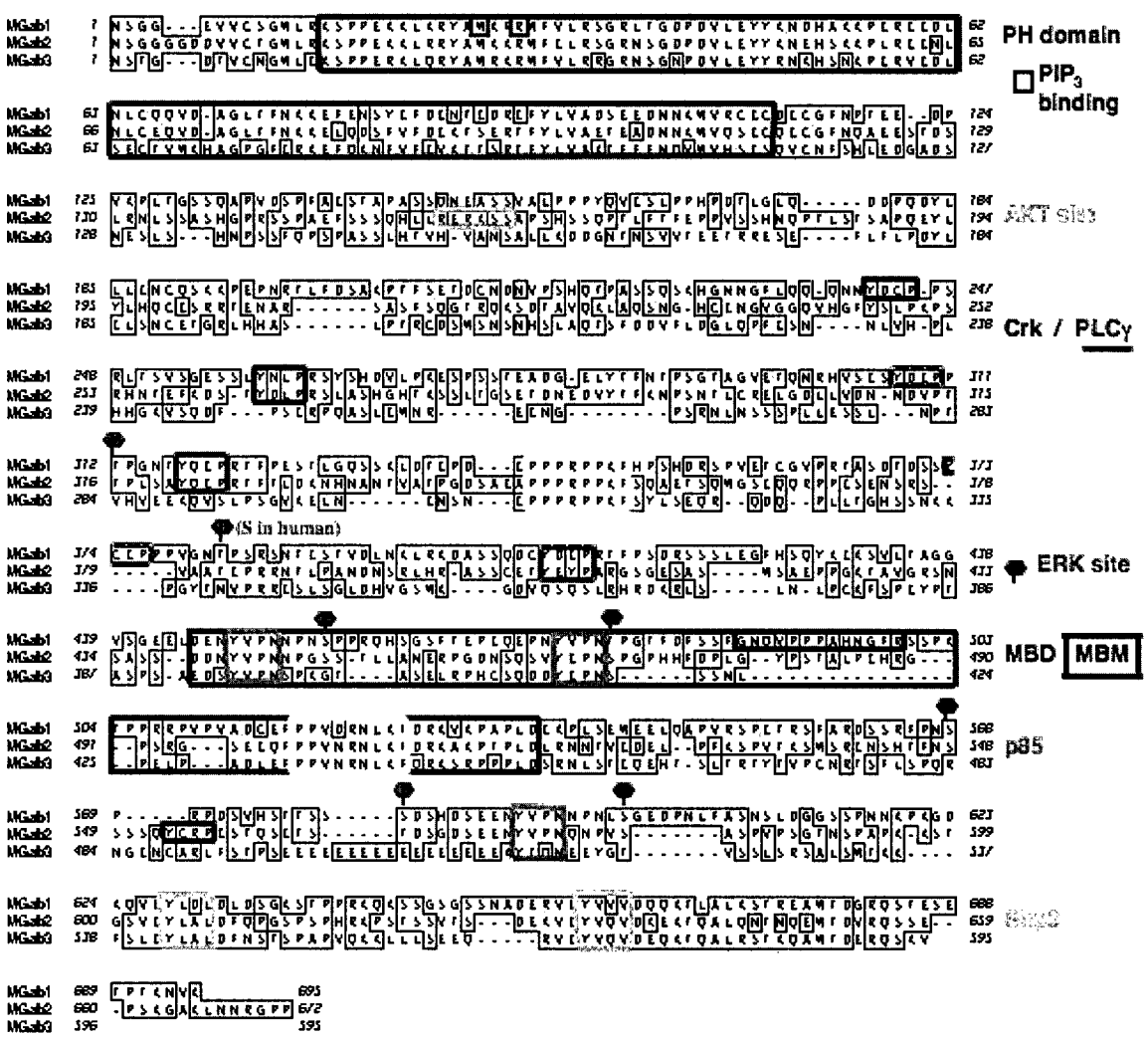

Figure 6. Annotated Gab1, Gab2, and Gab3 protein sequence alignment.

Sequences of murine Gab family of proteins were aligned using MacVector analysis. Sites of PIP3 binding and the PH domain are denoted in red (Maroun et al., 1999a; Maroun et al., 1999b). The site on Gab2 that is phosphorylated by AKT, in green (Lynch and Daly, 2002). The Crk SH2 domain binding sites, in purple (Lamorte et al., 2000), which overlap with those of PLCγ denoted by boxes in purple with white dots (Gual et al., 2000). The SH3 domains of Grb2 bind proline rich sequences in cyan (Lock et al., 2000). The original Met binding domain (MBD) and part of the proline rich domain of Gab1 mediating direct binding to Met (Weidner et al., 1996), and the short peptide of the Met binding motif in blue (Lock et al., 2002; Schaeper et al., 2000). Sites where Gab1 is phosphorylated by ERK in red hexagons marked with a P (Lehr et al., 2004). The SH2 domain of p85 binding sites in orange (Holgado-Madruga et al., 1997). The TAM sites for binding of the two SH2 domains of Shp2 in pink (Cunnick et al., 2001).

Uniquely, Gab1 is recruited to Met not only via the indirect Grb2 mechanism, but also directly. Using a yeast-two hybrid screen, the proline rich region of Gab1 (coined therein the Met Binding Domain (MBD)), was identified to interact directly with Met and not with other RTKs tested (Figure 6) (Weidner et al., 1996). The minimal region for direct interaction was later mapped to a 13 amino acid sequence, G-M-Q-V-P-P-P-A-H-M-G-F-R, known as the Met binding motif (MBM) (Lock et al., 2002; Schaeper et al., 2000). This extended peptide motif in Gab1 interacts with the residues in the Met kinase domain, and the interaction requires both a structured kinase domain and the integrity of the tyrosine residue at position 1349 in Met which is located at the intersect of the kinase domain and the carboxy tail. The requirement of Y1349 in Met was hypothesized to be for the recruitment of a yet unidentified phosphorylated tyrosine binding domain in the Gab1 MBD, however, an unstructured peptide ligand in Gab1 (MBM) is sufficient to bind Met. We demonstrated that the integrity of the Y1349 for interaction is through generation of a negative charge at Y1349, resulting in a hypothesized conformational change of the Met carboxy terminus, exposing the Gab1 binding site in the last lobe of the kinase (Figure 1). This was supported through the demonstration that substitution of Y1349 for a phospho-mimetic glutamic acid, was sufficient to recruit and phosphorylate Gab1 but not Grb2. The phospho-mimetic activated Met protein was able to induce transformation of fibroblasts demonstrating that the Grb2-independent Met-Gab1 interaction functions biologically (Lock et al., 2003). This is further supported by the crystal structure of Met, where the carboxy terminal of Met is buried in the kinase domain (Schiering et al., 2003).

In fact, Gab1 has been identified to be essential for many of Met dependant biochemical signals and biological activity. Using an *in vitro* model to recapitulate organogenesis of epithelial organs such as Met mediated morphogenesis of the kidney (Figure 3) (Brinkmann et al., 1995), recruitment of Gab1 to Met is required. A Met receptor that is impaired in its ability to recruit and phosphorylate Gab1 (CSF-Met ΔGrb2) is unable to activate signals for epithelial morphogenesis (Fournier et al., 1996). Simple over-expression of Gab1 is sufficient to rescue the defect of the mutant receptor and promote epithelial morphogenesis (Maroun et al., 1999a). Over-expression of a Gab1 protein unable to bind to Met due to a mutation in the MBM, is unable to rescue the phenotype (Lock et al., 2002). During embryogenesis *in vivo*, Gab1 functions to mediate Met signals demonstrated by the observations that *gab1* null mice pheno-copy the Met or HGF knock out animals (Sachs et al., 2000). *met* and *gab1* null embryos are both impaired in later embryogenesis, where embryos are unable to develop muscles in the limbs and the diaphragm due to a defect of the myogenic precursor cells to migrate over distances to the proper location in the embryo (Maina et al., 1996; Sachs et al., 2000).

5.2.2.3 The Gab1-Shp2 complex

All Gab family proteins contain one or two tyrosine residues in their carboxy terminus that are a part of the consensus binding motif; Y-X-X-I/V/L, which binds the tyrosine phosphatase Shp2 or its orthologues (Cunnick et al., 2001). Upon activation of Met, Gab1 is recruited to the receptor and becomes highly tyrosine phosphorylated (Nguyen et al., 1997). Phosphorylation of two tyrosine residues in the carboxy terminal

region of Gab1 generates a tandem activation motif (TAM) for Shp2 binding via its two SH2 domains (Figure 6) (Cunnick et al., 2001). Under basal conditions, the Shp2 protein tyrosine phosphatase (PTP) domain is auto-inhibited by an intra-molecular interaction with the amino terminal SH2 domain. Engagement of both SH2 domains of Shp2 to the TAM in Gab1, frees the PTP from inhibition and enzymatic activity is increased (Barford and Neel, 1998).

Activation of the Met receptor leads to cell proliferation. This is achieved by the activation of mitogen activated protein kinases (MAPK). The Gab1-Shp2 interaction is a regulator of the dynamic activation of the Ras-ERK pathway, considered a signal for cellular proliferation. Gab1 mutants where the tyrosine residues, which serve to recruit Shp2 are substituted for phenylalanine residues, demonstrate that the Gab1-Shp2 interaction is required for the sustained activation of ERK required for epithelial morphogenesis (Maroun et al., 2000; Schaeper et al., 2000). The duration of the ERK signal, either sustained or transient, is an instructive element for signal output. Specificity in signal transduction is attained by modulating the duration of the signal, hence providing a temporal layer of regulation. Inhibitors of ERK kinase (MEK) are known to abrogate the morphogenic program (Khwaja et al., 1998), demonstrating that ERK activation is required. Not only is the ERK signal required, but a dynamic prolonged activation is necessary for epithelial morphogenesis (O'Brien et al., 2004). Temporal differences of the ERK signal is utilized by different RTKs, which use the same signaling molecules to transduce signals, in order to provide specificity. For example, downstream of the EGFR and Met receptor, mitogenesis is activated by ERK signaling, however, only HGF stimulation promotes morphogenesis via a sustained ERK

signal (Maroun et al., 2000). Gab1 is phosphorylated for a prolonged period of time downstream of the Met receptor, where it promotes branching morphogenesis of MDCK epithelial cells, whereas Gab1 is transiently phosphorylated in response to EGF, which fails to induce a morphogenic program (Maroun et al., 1999a).

Genetic analyses in invertebrates have confirmed the essential role of the Gab-Shp2 complex in biology. The Gab1 orthologues were identified as binders and presumed substrates of their cognate phosphatases. The two tyrosine residues responsible for the DOS-CSW interaction in flies are the only tyrosine residues in the DOS proteins that serve any function in Sevenless signaling and biology (Bausenwein et al., 2000; Herbst et al., 1999). Similarly, the single tyrosine, which mediates the Soc-1-Ptp-2 complex formation in worms is essential for Egl-15 function (Schutzman et al., 2001).

5.2.2.4 The Gab1-p85 subunit of PI3K complex

All mammalian Gab proteins have three consensus tyrosine based motifs, Y-X-X-M, which when phosphorylated serve as docking sites for the SH2 domains of the p85 subunit of PI3K (Figures 4 and 5) (Holgado-Madruga et al., 1997). Invertebrate Gab proteins only have one such site and substitution of this tyrosine residue to phenylalanine in DOS or Soc-1 does not have any observable biological impact (Bausenwein et al., 2000; Herbst et al., 1999). Many receptors possess a p85 recruitment site in their carboxy terminal tail, however, many others depend on Gab family proteins for recruitment and activation of PI3K. The Gab1-p85 complex has been described to occur downstream of multiple signals, such as the B cell receptor (Ingham et al., 1998), G-protein coupled

receptors (Bisotto and Fixman, 2001; Laffargue et al., 1999), as well as osmotic shock (Janez et al., 2000) and flow shear stress signaling (Jin et al., 2005), in response to the circulating hormone Angiotensin II (Haider et al., 2005), as well as Erythropoietin (Wickrema et al., 1999) and Thrombopoietin (Miyakawa et al., 2001), many RTKs including Ret (Hayashi et al., 2000), Flt3 (Zhang and Broxmeyer, 2000), Insulin and Insulin-like growth factor receptors (Koyama et al., 2008; Lehr et al., 2000), vascular endothelial growth factor receptor 2 (Dance et al., 2006; Laramee et al., 2007), EGF and heregulin stimulation of the EGFR (Jackson et al., 2004; Rodrigues et al., 2000), the platelet-derived growth factor receptor (Rakhit et al., 2000), fibroblast growth factor receptor (Ong et al., 2001), TrkA receptor (Holgado-Madruga et al., 1997), as well as downstream of Met activation (Bertola et al., 2007; Maroun et al., 1999a).

In many cases, Gab1 recruits p85 in addition to the receptor. This is thought to function to amplify the PI3K signal. For example, the Met receptor can recruit p85 to its cytoplasmic tail (Fixman et al., 1995; Ponzetto et al., 1994), however in MDCK epithelial cells upon stimulation, the majority of PI3K is recruited to Gab1 as opposed to directly to the receptor (Maroun et al., 1999a). PI3K activation is required for Met mediated epithelial morphogenesis (Derman et al., 1995; Royal and Park, 1995), however the Gab1-p85 complex is dispensable (Maroun et al., 1999a; Schaeper et al., 2000). Since a functional PH domain is required for targeting Gab1 to the plasma membrane and for epithelial morphogenesis, PI3K may function upstream of Gab1 in generating enough of its lipid product PIP_3 to localize Gab1 to specific membrane micro-domains (Rodrigues et al., 2000).

Although the ERK and PI3K pathways are both regulated by recruitment of Shp2 and p85 respectively to Gab proteins, these pathways are not mutually exclusive. It is becoming evident that both positive and negative feedback mechanisms regulate the Gab1 mediated PI3K signal. Upon Met activation, Gab1 and ERK co-localize (Frigault et al., 2008) and Gab1 becomes phosphorylated by ERK on serine and threonine residues that are in close proximity to the p85 binding sites and these cooperate to enhance PI3K signaling (Yu et al., 2001). Conversely, downstream of EGF stimulation phosphorylation of Gab1 by ERK dampers the PI3K signal (Yu et al., 2002). Furthermore, Shp2 recruited to Gab1 has been reported to de-phosphorylate the p85 binding sites, switching off a Gab1 mediated PI3K signaling following EGF stimulation (Zhang et al., 2002). However, the Gab1-p85 complex regulates the PI3K pathway generating more PIP_3 which is a positive feedback loop to sustain Gab1 at the membrane for signaling downstream of the EGFR (Rodrigues et al., 2000). Interplay between these two pathways certainly provides another layer of signal complexity and signal specificity for different RTKs.

PI3K signaling is also required for the activation of the Rho family of small GTPases, which coordinate actin cytoskeleton reorganization, cell adhesion, membrane protrusions and cell migration. Rac and cdc42, members of the Rho family of GTPases, are both activated upon HGF stimulation and are required for cell-cell adherens junctions reorganization, lamellipodia formation, and cell motility (Royal et al., 2000). Briefly, GTPases are molecular switches that are generally bound to a GDP nucleotide and become activated by exchange of GDP for GTP by a guanine exchange factor (GEF) protein. This induces a conformational change and the GTP bound form of the protein

can then interact with effector proteins, which are often regulators of the actin cytoskeleton. GTPase activation proteins (GAP) promote the hydrolysis of GTP to GDP rendering the GTPase inactive. PI3K inhibitors impair the activation of Rac and thus abrogate the ability to cells to form a leading membrane protrusion and to scatter (Potempa and Ridley, 1998; Royal et al., 2000; Royal and Park, 1995). Moreover, PI3K is required for the down-regulation of E-cadherin, which is the molecular glue that holds neighboring epithelial cells together, and in this manner facilitates cell scatter (Royal et al., 1997). This describes another function of the activation of PI3K, complementary to the AKT-BAD cell survival pathway and to the regulation of lipid metabolism for the generation of lipid ligands. Rac activation is dependent on PI3K but has also been described to occur downstream of Crk.

5.2.2.5 The Gab1-Crk/PLCγ complexes

Gab1 contains six tyrosine containing consensus binding sites for the SH2 domains of Crk and PLCγ (Figure 6). Both the SH2 domains of Crk and PLCγ have the same specificity for binding to Y-X-X-P motifs. Both Crk and PLCγ form complexes with Gab1 in response to Met receptor activation (Gual et al., 2000; Lamorte et al., 2000).

Crk is an adaptor molecule containing an SH2 domain and two SH3 domains ((C)SH2-SH3-SH3(N)), which aggregates complexes for regulation of the actin cytoskeleton. Downstream of Met signaling, Crk is required for activation of the small GTPases Rap1 and Rac (Lamorte et al., 2003; Lamorte et al., 2002b; Sakkab et al., 2000; Watanabe et al., 2006), as well as the c-Jun amino terminal kinases (JNK) family of

MAPKs (Garcia-Guzman et al., 1999; Lamorte et al., 2000). The Gab1-Crk complex is required for epithelial morphogenesis, since a Gab1 protein where the tyrosine residues that lie in a consensus Y-X-X-P are substituted to phenylalanine is uncoupled from Crk and unable to promote the morphogenic program (Lamorte et al., 2002a).

As discussed earlier, EGF is unable to activate the morphogenic program in MDCK cells, however Crk over-expression is sufficient to activate epithelial morphogenesis downstream of EGFR. Cells that over-express Crk are also able to down-regulate E-cadherin in adherens junction in order to promote cell scatter. The signals that make this switch possible results in increased activation of Rac. These signals circumvent the requirement for PI3K activation in order to promote cell scatter (Lamorte et al., 2002a). Crk over-expression complements EGF signals to achieve an HGF-like signal promoting breaking down of cell-cell contacts, cell scatter and morphogenesis. Over-expression and knock-down studies of Crk have identified this adaptor as a threshold signal that promotes successful cell migration and invasion (Rodrigues et al., 2005).

Upon Met activation, Phospholipase C γ (PLCγ) is recruited to the receptor complex. PLCγ is recruited via binding of its SH2 domain to the phosphorylated tyrosine at residue 1356 in the multi-docking site of the receptor (Bardelli et al., 1992; Kochhar and Iyer, 1996). HGF leads to PLCγ activation in multiple cell types including epithelial cells, neuronal cells, hepatocytes, astrocytes and tumor cells (Gual et al., 2000; Kochhar and Iyer, 1996; Machide et al., 2000; Machide et al., 1998; Okano et al., 1993; Osada et al., 1992). PLCγ recruitment and activation downstream of the Met receptor can also

occur by recruitment to Gab1. The SH2 domains of PLCγ can be recruited to three tyrosine based motifs in Gab1, with the consensus sequence Y-X-I-P which share the Crk SH2 domain specificity (Figure 6) (Gual et al., 2000). The recruitment of PLCγ to Gab1 is required for epithelial morphogenesis of MDCK cells cultured in a three-dimensional collagen matrix demonstrating the requirement for this interaction for Met mediated biological responses (Gual et al., 2000). Interestingly, this interaction is not required for cell scatter and neither is the enzymatic activity of PLCγ as treatment of MDCK cells with a specific pharmacological inhibitor of the enzyme does not impair cell scatter in response to HGF. However the effect of this inhibitor on three-dimensional cultures impairs branching morphogenesis (Gual et al., 2000; Royal et al., 1997).

Upon receptor activation, PLCγ hydrolyses PIP_2 producing two second messengers, inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) (Figure 4). IP_3 rapidly diffuses to bind channels in the endoplasmic reticulum for the transient release of intracellular free Ca^{2+}, while DAG is a direct activator of Protein Kinase C (PKC) (Berridge, 1989; Nishizuka, 1986; Takai et al., 1979). Although the major effect of DAG production is the recruitment and activation of the PKC family of proteins, many other proteins bind to the DAG ligand such as RasGRP1, a regulator of the ERK pathway (Brose and Rosenmund, 2002). Calcium signaling is required to maintain epithelial polarity by regulating the assembly of cell-cell contacts and thus maintaining apical and baso-lateral cellular membranes as well as a plethora of cellular processes such as contractility and secretion, survival and apoptosis, as well as proliferation (Berridge et al., 2000; Stuart and Nigam, 1995). Downstream of Met, calcium signaling is activated (Gomes et al., 2008; Jin et al., 2003; Lail-Trecker et al., 2000; Majka et al., 2006;

Makondo et al., 2003; Osada et al., 1992; Tyndall et al., 2007), however some instances suggest that increases in calcium fluxes are dispensible for HGF signaling (Hoffmann et al., 2006).

There are two PLCγ isoforms, γ1 and γ2, however γ1 is ubiquitously expressed in all cell types whereas γ2 expression is limited to B cells (Kim et al., 2000). PLCγ has two SH2 domains, which mediate binding to phosphorylated tyrosine residues as well as an SH3 domain, which has been reported to bind to proline rich motifs in other signaling molecules, as well as a C2 domain, a PH domain and 2 catalytic lipase regions. The PLCγ PH domain preferentially binds to PIP_2, which not only mediates it's interaction with the plasma membrane, but also is its substrate.

5.2.3 Gab2 and Gab3

Gab1 is a member of a family of docking proteins, which includes mammalian Gab2 and Gab3 (Figure 5). Gab2 has a similar domain structure to Gab1 and binds many of the same signaling molecules as most of the known binding sites in Gab1 for SH2 and SH3 domain containing proteins are conserved in Gab2 (Figure 6). Gab2 was cloned as a phosphorylated protein bound to Shp2 and to the p85 subunit of PI3K (Gu et al., 1998; Nishida et al., 1999). Gab3 was identified as a protein which becomes tyrosine phosphorylated downstream of the M-CSF RTK in macrophages and forms transient complexes with p85 and Shp2 (Wolf et al., 2002). Moreover, Gab3 is important for macrophage differentiation however, *GAB3* knock-out mice have no observable defects

(Seiffert et al., 2003; Wolf et al., 2002). Notably, Gab3 lacks any of the tyrosine based motifs that mediate recruitment of the SH2 domains of Crk and PLCγ.

The three Gab family members have overlapping but also distinct patterns of tissue expression. Gab1 is expressed in most tissues, and Gab2 shares this pattern of tissue expression but is absent in the spleen and thymus where Gab3 is predominantly expressed (Wolf et al., 2002). Gab1 and Gab2 are both expressed in tissues that undergo epithelial morphogenesis, such as the kidney, lung, and pancreas, however, Gab2 is unable to promote Met mediated epithelial morphogenesis (Lock et al., 2002). Gab1 and Gab2 are both recruited to the Met receptor upon activation and both are phosphorylated however, they are differentially recruited. Gab1 is recruited both via the adaptor Grb2 as well as through its Met binding motif (MBM), whereas Gab2 is recruited to the receptor only via interaction with Grb2 (Figure 7) (Lock et al., 2002). Gab1 requires a functional MBM for morphogenesis (Lock et al., 2002) and insertion of the Gab1 MBM into Gab2 is not sufficient to switch the biological capacity of this molecule (Frigault et al., 2008).

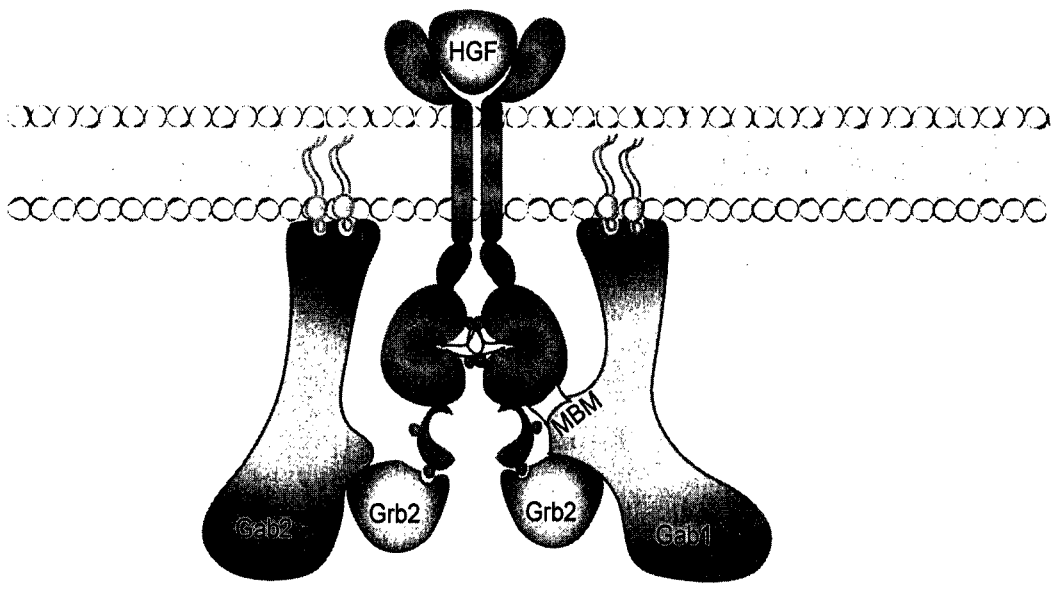

Figure 7. Model of Gab1 and Gab2 recruitment to the activated Met receptor.

Gab1 is recruited to the Met receptor by two mechanisms, a Grb2 dependant and a Grb2-independant mechanism. The Grb2 dependant mechanism occurs upon tyrosine phosphorylation of residue 1356 in the carboxy terminal tail of Met, which provides a docking site for the SH2 domain of Grb2. The SH3 domains of Grb2 mediate binding to the proline rich regions of Gab1. This is the same mechanism used by Gab2 for recruitment to Met. Gab1 has an additional mechanism of recruitment, directly via the Met Binding Motif (MBM-drawn in yellow), which requires a negative charge at tyrosine residue 1349 of Met.

Gab2 over-expression leads to increased proliferation of mammary epithelial cells in a three-dimensional culture, but requires cooperation with over-expression of ErbB2, an EGF RTK family member, to promote an invasive phenotype (Bentires-Alj et al., 2006). Gab2 is also required for v-Sea and mast cell transformation (Ischenko et al., 2003; Sattler et al., 2002) and in EGF-induced or c-Kit induced mitogenic response (Kong et al., 2003; Nishida et al., 2002). Significantly, Gab2 silencing decreases proliferation and impairs the activation of the PI3K-AKT pathway in Retinoic Acid induced neuronal differentiation (Mao and Lee, 2005). Although Gab2 can function to mediate numerous cellular processes downstream of other RTKs, only Gab1 is capable of activating signals for Met mediated biology.

6. Signal termination of receptor tyrosine kinases

RTK signaling is activated as well as switched off by elaborately regulated mechanisms. RTKs activate signals by phosphorylating tyrosine residues and these must also be switched off to maintain regulated signaling. The duration of signaling is a mechanism to regulate biological outputs, such as the differences that HGF and EGF ligands exert on Gab1 phosphorylation and signaling described above. Therefore, built into RTK activation is a mechanism to tightly regulate the termination of the signal.

6.1 Met receptor de-phosphorylation

The reversible nature of phosphorylation functions to regulate signaling such that protein tyrosine kinases transfer phosphate to a tyrosine residue in a substrate, and protein tyrosine phosphatases can remove it. This results in the elimination of the phosphorylated tyrosine residue as a docking site for the recruitment of signaling molecules and dampers the signal. The protein tyrosine phosphatase (PTP) DEP-1 de-phosphorylates the Met receptor on tyrosine residues in the carboxy terminal tail. A mutant form of the DEP-1 active site, which can trap the PTP in an intermediate complex with its substrate, suggests that DEP-1 also de-phosphorylate Gab1 terminating both recruitment of Gab1 to Met as well as Gab1 dependent signals (Palka et al., 2003). Another phosphatase that regulates the Met receptor is PTP1B. During wound healing of the corneal epithelium in the presence of HGF, PTP1B expression and activity is elevated (Kakazu et al., 2008). Moreover, in mice null for PTP1B, Met is hyper-phosphorylated following liver injury (Sangwan et al., 2006) and Met has since been identified as a substrate for PTP1B (Sangwan et al., 2008).

6.2 Met receptor endocytosis

Signals activated by growth factor binding to RTKs triggers the accumulation of the receptor into clathrin-coated pits at the plasma membrane, which eventually bud off into the cytosol removing the receptor from the cell surface in a process known as endocytosis. RTKs either recycle back to the plasma membrane or traffic down the endocytic pathway, enter mutli-vesciular bodies and eventually become degraded by the lysosome. This process requires the coordinated function of the endocytic machinery and the ubiquitination of the receptor cargo. Met undergoes ligand induced endocytosis which is required for its down-regulation (Hammond et al., 2001; Jeffers et al., 1997b). Met phosphorylates regulators of the endocytic pathway and the ubiquitination machinery such as Cbl and HRS (Abella et al., 2005; Hammond et al., 2003; Peschard et al., 2001; Petrelli et al., 2002). Moreover, the activation of PKC as well as signals from Gab1 also regulate trafficking and Met receptor down-regulation (Kermorgant et al., 2003; Kermorgant et al., 2004) (Abella and Frigault et al. submitted).

6.3 Receptor complex trafficking and signaling

Although endocytosis of RTKs is a mechanism for down-regulation of the receptor, growing evidence suggests that the intricacies of RTK trafficking provides another layer of signal specificity. The subcellular location of the receptor complex along the endocytic pathway can also provide signal specificity, and various RTKs signal differently from the plasma membrane as from an endosome (Wiley and Burke, 2001). There is evidence that Met signals from an endosomal compartment (Hammond et al., 2003). Indeed, a mutant Met receptor which is not targeted for degradation as efficiently

as the wild type receptor is endocytosed, but has a much sustained signaling capacity (Abella et al., 2005). Moreover, Met must traffic to an endosomal compartment to activate STAT3 (Kermorgant and Parker, 2008). In addition, Rac traffics on a vesicular compartment downstream of Met activation (Palamidessi et al., 2008).

Evidence for endosomal signaling for the EGFR has been reported. Careful imaging of the EGFR demonstrates that the receptor is phosphorylated at the plasma membrane as well as on an endosome (Wouters and Bastiaens, 1999). Downstream of EGFR, Grb2 and Shc, as well as SOS, a GEF for Ras, are found on endosomes (Di Guglielmo et al., 1994; Sorkin et al., 2000). PLCγ1 has also been reported to localize to endosomes downstream of the EGFR (Matsuda et al., 2001; Wang et al., 2001). Ras has been localized to endosomes and other endo-membranes such as the golgi apparatus, by many different groups and by a variety of techniques (Mor and Philips, 2006). HGF signaling utilizes many of these same signaling molecules, and their endosomal localization remains to be elucidated downstream of Met activation.

7. Actin based cellular processes

All eukaryotic cells, both unicellular and multicellular, utilize the versatility of the actin cytoskeleton to make changes in their cell shape. The coordinated polymerization of actin filaments (F-actin) against membranes provides the force for changes in cell shape during cell migration, morphogenesis, as well as endocytosis and phagocytosis. For these processes to occur, F-actin is organized into three-dimensional meshwork, which is constantly being remodeled to carry out the demands of the cellular process.

In adult mammals, certain cell types are specialized for locomotion such as neutrophils during the immune response by a process known as chemotaxis. Although cell migration of most tissue types are essential during development in organogenesis and embryogenesis (discussed above), cell movement in differentiated tissues is normally repressed, but can be activated in wound healing or oncogenic transformation. The regulation of cell movement during chemotaxis is dictated by a gradient of guidance cues from surrounding cells or tissue. Unicellular eukaryotes such as *Dictyostelium discoideum*, locomote in a similar manner to neutrophils, and have been extensively studied as a model of cell movement due to their simplicity. Therefore many of the concepts of cell migration are derived from studies of both unicellular and multicellular eukaryotes (Affolter and Weijer, 2005).

The protrusive structures found at the leading edge of a cell are called membrane ruffles, and can be of various types. There is a general consensus that when a cell receives cues for the initiation of cell movement in one particular direction, it extends initially filopodia, which are thin finger like protrusions of the membrane which consists of a linear F-actin bundle. Filopodia are thought to function as "feelers" for the new extra-cellular space adjacent to the leading edge (Mattila and Lappalainen, 2008). Once productive simple filopodial extensions have been made, signals from integrin complexes located at the tips and shaft of the filopodia results in the elaboration of a sheet like projection known as a lamellipodia (Galbraith et al., 2007). Lamellipodia are a wide but thin membrane ruffle filled with branched network of F-actin. The force used for membrane extension is via the addition of G-actin monomers to the barbed-end of the existing F-actin, resulting in elongation of the actin filament and thus pushing on the

membrane (Condeelis, 1993). New filaments arise when signaling pathways activate nucleation-promoting factors (NPF) via GTPases Rac and Cdc42. NPFs such as WASP, WAVE and cortactin, are not enzymes but scaffolds that coordinate the recruitment and stimulation of the Arp2/3 complex (Marchand et al., 2001; Miki et al., 1998; Weed et al., 2000). Furthermore, NPFs are regulated by binding other adaptors that are involved in signal transduction such as Nck and IRSp53 downstream of RTKs (Eden et al., 2002; Miki et al., 2000). The NPF and Arp2/3 complex initiates the formation of a new actin filament as a branch on the side of an existing filament (Machesky et al., 1994). The branched filament is elongated by the addition of actin monomers to the barbed end of the newly created F-actin. New branches in the actin meshwork grow rapidly at the barbed end, which push on the inner leaflet of the lamellipodial plasma membrane. The lipid ligand PIP_2 displaces capping proteins which bind to the barbed end of F-actin to stop elongation, and in this manner regulates the persistence of lamellipodia extension. This is one such example of the highly regulated dynamics of the meshwork of F-actin in membrane ruffles. These are constantly being reorganized by a plethora of factors such as capping proteins, severing proteins and modulators of the ATP bound state of monomeric actin (Pollard and Borisy, 2003). F-actin in the cell body also functions in force generation for moving forward the portions of the cell that are not involved in lamellipodia protrusion, namely the remaining of the cell including the nucleus and most other organelles. F-actin is organized into bundles, which provides a network for myosin driven generation of traction. Myosin II is abundant in motile cells and *Dictyostelium discoideum* null for myosin II cannot move (Huxley, 1973; Jay et al., 1995).

Signal transduction pathways regulate the elaboration of the actin meshwork in lamellipodial protrusions as well as the reorganization of the remaining of the actin cytoskeleton resulting in changes in cell shape, promoting the transition from a static cell to a migrating cell. Activation of the Met RTK as well as many other cell surface receptors activates these changes. HGF stimulates the remodeling of the actin cytoskeleton of MDCK cells (Ridley et al., 1995; Royal et al., 2000; Royal and Park, 1995). Moreover, HGF stimulation results in the activation of GTPases Rac and Cdc42 as well as the formation of filopodia and lamellipodia. HGF induced Ras activation leads to changes in the cytoskeleton of MDCK cells such that cells spread out and break down their cell-cell adherens junctions. Activation of the Ras pathway leads to ERK phosphorylation, and Rac activation in a manner dependant on PI3K signaling (Potempa and Ridley, 1998). Rac binds to and activates p21-activated kinase (PAK1), which is a serine/threonine kinase that localizes to lamellipodia to regulate the turnover of the actin cytoskeleton (Royal et al., 2000).

Many other cellular processes require the coordination of F-actin assembly and turnover. These include cell division, where a cell is physically divided into two in a process that requires the actin and myosin machinery (Barr and Gruneberg, 2007; Thery and Bornens, 2006). The actin cytoskeleton is an integral part of membrane trafficking in RTK endocytosis both at the invagination of the plasma membrane for accumulation of RTKs into clathrin coated pits, as well as throughout various stages of the endocytic pathway (Merrifield et al., 2002; Yarar et al., 2005). Moreover, the NPF cortactin is also an endocytic adaptor and coordinates receptor mediated endocytosis by recruiting the GTPase dynamin to sites of membrane invagination resulting in vesicle budding from the

cell surface (Cao et al., 2003). Also, requirements for actin at the golgi apparatus for vesicular budding and fission demonstrate the intricate link between membrane remodeling and actin dynamics (Egea et al., 2006).

8. Deregulation in disease

Met and Gab signaling are tightly regulated during embryonic development for the proper and intact patterning of the embryo as well as the sophisticated development of many epithelial organs. Both Met and Gab family proteins have been found to be deregulated in human disease. Therefore, the extensive knowledge of how this signaling pathway functions under normal circumstances can provide a starting point for targeted therapeutics.

8.1 Met receptor

HGF and Met are over-expressed or deregulated in many human cancers and this correlates with bad patient prognosis (Birchmeier et al., 2003). In hereditary and sporadic papillary renal cell carcinoma, point mutations in the kinase domain of Met have been identified (Schmidt et al., 1997; Schmidt et al., 1999; Schmidt et al., 1998; Schmidt et al., 2004). Moreover, somatic mutations in the kinase domain of the Met receptor in hepato-cellular and gastric carcinomas, as well as in human gliomas have also been described (Moon et al., 2000; Park et al., 1999; Park et al., 2000). These mutations in the kinase domain of Met serve as activating mutations rendering the Met kinase more active to exogenous substrates (Jeffers et al., 1997a). Other mutations or aberrations of the *MET* gene lead to the production of a receptor that harbors mutations in the juxta-

membrane region of Met and are oncogenic through their ability to uncouple the mutant Met receptor from mechanisms of down-regulation. In breast, lung, thyroid and gastric cancers these types of *MET* mutations have been reported (Kong-Beltran et al., 2006; Lee et al., 2000; Ma et al., 2003; Wasenius et al., 2005).

Met functions as a driver for tumorigenesis via up-regulation of receptor expression and/or of its ligand, HGF. This mechanism of oncogenesis provides a deregulated signal probably by altering the stringency of receptor activation, the duration and robustness of the signal, as well as the spatial regulation of the signal. Met activation by autocrine or paracrine loops with HGF expression has been reported in breast and lung carcinomas as well as sarcomas and gliomas (Camp et al., 1999; Jin et al., 1997; Koochekpour et al., 1997; Rong et al., 1993; Tsao et al., 1993). Moreover, Met gene amplification has been reported in gliomas and colon tumors (Di Renzo et al., 1995; Koochekpour et al., 1997). Met deregulation also plays a causative role in other human diseases. A common functional variant of the promoter of the *MET* gene is a contributing risk factor for autism (Campbell et al., 2007; Campbell et al., 2006). Additionally, aberrations in Met regulators and effectors may have the same effect.

8.2 Gab proteins

Breast cancer is the most common type of cancer among woman, and one out of eight Canadian women will develop breast cancer in her lifetime (Foundation, 2008). Gab2 has been implicated in this disease. In human breast cancer cell lines, Gab2 over-expression leads to increased proliferation in a three-dimensional culture, increases in cell cycle progression, a sustained activation of the ERK and AKT signaling pathways,

formation of enlarged three-dimensional acinar structures coupled with a defect in cessation of proliferation during morphogenesis (Bentires-Alj et al., 2006; Brummer et al., 2006; Daly et al., 2002). In addition, ErbB2 RTK induced mammary tumorigenesis is attenuated in a *Gab2*-null background whereas over-expression aggravates the phenotype (Bentires-Alj et al., 2006). Gab2 ablation impairs lung metastases in this mouse model of mammary tumorigenesis (Ke et al., 2007). Also, Gab2 synergizes with Src mediated tumorigenic growth and signaling as well as migration and invasion in breast epithelial cell lines *in vitro* (Bennett et al., 2008). The Gab2 locus on human chromosome 11q13 is amplified in 10-15 % of breast cancer patients (Schwab, 1998; Yamada et al., 2001).

Other models of human disease have highlighted the causative roles of Gab1 and Gab2. 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 models (Million et al., 2004; Ren et al., 2005; Sattler et al., 2002; Scherr et al., 2006). Moreover, Gab2 is hyper-phosphorylated in CML models that are resistant to imatinib (Wu et al., 2008). In rheumatoid arthritis models, Gab2 and Gab1 are hyper-phosphorylated and Gab2 is shown to be a key mediator of proliferation by siRNA and gene expression profiling studies (Batliwalla et al., 2005; Kameda et al., 2006). Certain polymorphisms in the *GAB1* gene are more permissive for infection of *H.pylori* in the gut (Goto et al., 2007). Although studies of Gab1 and Gab2 have been primarily on the elucidation of the molecular mechanisms of their function, the involvement of specific alterations of Gab proteins in human disease is emerging.

Abbreviations

ATP	Adenosine Trip-Phosphate
RTK	Receptor Tyrosine Kinase
MNNG	N-methyl-N'-nito-N-nitrosoguanidine
RSV	Rous sarcoma virus
Tpr	translocated promoter region
HGF	Hepatocyte Growth Factor
A-loop	Activation loop in a tyrosine kinase domain
ECM	extra-cellular matrix
MDCK	Madin-Darby Canine Kidney
Sea	Sarcom, Erythroblastosis and anemia
MSP	macrophage stimulating protein
HEK	Human Embryonic Kidney
MAPK	mitogen activated protein kinase
KSR1	Kinase suppressor of Ras
SH1	Src Homology 1
SH2	Src Homology 2
SH3	Src Homology 3
PLCγ1	Phospholipase C γ1
PTB	Phospho Tyrosine Binding
IRS	Insulin receptor substrate
PH	Pleckstrin Homology
TKB	Tyrosine Kinase Binding
PKC	Protein Kinase C
G2/M	cell cycle checkpoint growth to mitosis
FHA	Forkhead-associated
S/T	serine/threonine residues
Y	tyrosine residue
Crk	CT10 regulator of kinase
Grb2	Growth factor receptor bound protein 2
Sos	Son of sevenless homolog
GEF	Guanine Exchange Factor
GAP	Rho GTPase activating protein
GTP	Guanosine triphosphate
EVH1	Enabled/VASP homology domain
WW	tandem tryptophane containing modular protein domain
PIP_2	phosphatidylinositol 4,5 bisphosphate
PIP_3	phosphatidylinositol 3,4,5 triphosphate
$PI(3,4)P_2$	phosphatidylinositol 3,4 bisphosphate
PI3K	phosphatidylinositol 3-kinase
p85	regulatory subunit of PI3K
Shp2	SH2 domain containing protein tyrosine phosphatase
AKT	Protein kinase B
PDK1	protein dependant kinase 1
GPI	glycosyl-phsophatidyl inositol

ERK	Extracellular signal-regulated kinase 1 and 2
Gab1	Grb2-associated binder-1
Gab2	Grb2-associated binder-2
Gab3	Grb2-associated binder-3
DOS	Daughter of Sevenless
Soc-1	Suppressor of clear-1
PRD	proline rich domains
BCR	B Cell Receptor
EGFR	Epidermal Growth Factor Receptor
PDGFR	Platelet Derived Growth Factor Receptor
PRD	Proline rich domain
MBD	Met Binding domain
MBM	Met binding motif
TAM	Tandem activation motif
PTP	Protein tyrosine phosphatase
JNK	c-Jun amino terminal kinase
NPF	Nucleation-promoting factor
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
PAK1	p21-activated kinase

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Chapter 2

Gab2 requires membrane targeting and the Met Binding Motif to promote lamellipodia formation, cell scatter, and epithelial morphogenesis downstream from the Met receptor

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Published in the Journal of Cellular Physiology, March 2008; 214(3):694-705.

Preface

The Met receptor tyrosine kinase recruits and phosphorylates Gab1 and Gab2, however, only Gab1 is functional for the induction of Met mediated activation of sustained signaling pathways, cell scatter and epithelial morphogenesis. The mechanism of Gab1 recruitment of the Met receptor via the Met binding Motif (MBM) is essential for the induction of the morphogenic program since disruption of the MBM in Gab1 abrogates the ability of the protein to induce morphogenesis. Reintroduction of the Gab1 MBM into Gab2, however, was not sufficient to switch the capacity of Gab2 to promote morphogenesis. Another required element of Gab1 in order to function in Met mediated epithelial morphogenesis is its capacity to be recruited to the plasma membrane. The studies presented in this chapter demonstrate that both the MBM and membrane targeting are both required to confer the morphogenic program to Gab2 where each individually is not sufficient.

Abstract

Gab1 and Gab2 are conserved scaffolding proteins that amplify and integrate signals stimulated by many growth factor receptors including the Met receptor. Gab1 acts to diversify the signal downstream from Met through the recruitment of multiple signaling proteins, and is essential for epithelial morphogenesis. However, whereas Gab1 and Gab2 are both expressed in epithelial cells, Gab2 fails to support a morphogenic response. We demonstrate that Gab1 and Gab2 are divergent in their function whereby Gab1, but not Gab2, promotes lamellipodia formation, and is localized to the membrane of lamellipodia upon Met activation. We have identified activation of ERK1/2 as a requirement for lamellipodia formation. Moreover, activated ERK1/2 are localized to lamellipodia in Gab1 expressing cells, but not in cells that over-express Gab2. By structure-function studies, we identify that enhanced membrane localization conferred through the addition of a myristoylation signal, together with the addition of the direct Met binding motif (MBM) from Gab1, are required to promote lamellipodia and confer a morphogenic signaling response to Gab2. Moreover, the morphogenesis competent myristoylated Gab2MBM localizes activated ERK1/2 to the leading edge of lamellipodia in a similar manner to Gab1. Hence, subcellular localization of the Gab scaffold, as well as the ability of Gab to interact directly with the Met receptor, are both essential components of the morphogenic signaling response which involves lamellipodia formation and the localization of ERK1/2 activation in membrane ruffles.

Introduction

The process of epithelial remodeling is crucial during embryonic development and for wound healing in the adult. This requires the coordination of multiple cellular responses, including cell proliferation, survival, migration, invasion and differentiation (reviewed in (O'Brien *et al.*, 2002)). Hepatocyte growth factor, HGF, a ligand for the Met receptor tyrosine kinase is a potent epithelial morphogen and promotes epithelial remodeling and invasion (Matsumoto and Nakamura, 1997). HGF induced Met signaling initiates the intrinsic morphogenic program of epithelial cells grown in a three-dimensional matrix and regulates multiple biological responses that are required for epithelial remodeling in-vivo (Gherardi *et al.*, 1989; Montesano *et al.*, 1991; Nakamura, 1991; Weidner *et al.*, 1993; Zhu *et al.*, 1994). Dysregulation of the Met/HGF axis has been identified in many human tumors, reviewed in (Birchmeier et al., 2003; Vande Woude et al., 1997), highlighting the need to understand Met dependent signals that mediate epithelial remodeling and invasion.

Gab1 is the major tyrosine phosphorylated protein downstream of the Met receptor in epithelial cells and is essential for the invasive and morphogenic response (Maroun *et al.*, 1999a). The Gab family of proteins, Gab1, Gab2, and Gab3, belong to a larger group of scaffold proteins including the insulin receptor substrates (IRS1-4), downstream of kinases (Dok-1-5), and fibroblast growth factor receptor substrate 2 (FRS2) reviewed in (Giovannone et al., 2000; Guy et al., 2002; Liu and Rohrschneider, 2002; Nishida and Hirano, 2003; White, 1998). These proteins lack enzymatic activity, but become phosphorylated on tyrosine residues providing binding sites for multiple proteins involved in signal transduction (Gu and Neel, 2003). Gab1 and Gab2 contain

tyrosine residues within consensus sites for recruitment of p85, phospholipase Cγ, Shp2, Crk and GC-GAP (Crouin et al., 2001; Gu et al., 1998; Meng et al., 2005; Pratt et al., 2000; Zhao et al., 2003) and share conserved Grb2 SH3 domain binding sites (Lock *et al.*, 2000; Schaeper *et al.*, 2000). They are both phosphorylated upon HGF, epidermal growth factor, platelet-derived growth factor, interleukin-6, interleukin-3, Flt3 ligand, thrombopoietin, and erythropoietin stimulation, as well as T-cell receptor engagement (Bouscary et al., 2001; Gu et al., 1998; Kameda et al., 2006; Meng et al., 2005; Nguyen et al., 1997; Nishida et al., 1999; Podar et al., 2004; Wickrema et al., 1999; Zhang and Broxmeyer, 2000; Zhao et al., 1999). In this manner, they act to potentiate and diversify the signals downstream from receptors by virtue of their ability to assemble multi-protein complexes.

Gab1 and Gab2 have overlapping but distinct patterns of expression (Gu *et al.*, 1998; Nishida *et al.*, 1999; Wolf *et al.*, 2002; Zompi *et al.*, 2004). Studies of knockout mice demonstrate that both Gab1 and Gab2 have differential functions during development. Gab1 null animals are embryonic lethal and phenocopy HGF or Met null animals. During embryonic development, myogenic precursor cells are unable to migrate to the limb bud in both Met and Gab1 null embryos (Bladt et al., 1995; Sachs et al., 2000). Gab2 is expressed in epithelia, Gab2 null animals are deficient in the allergic response and in osteoclastogenesis (Gu et al., 2001; Itoh et al., 2000; Sachs et al., 2000; Wada et al., 2005). Studies of human disease have placed a specific role for Gab2 in cell proliferation. Gab2 over-expression leads to increased proliferation in three dimensional culture of human breast cancer cell lines, sustained activation of the ERK and AKT signaling pathways, and leads to formation of enlarged three-dimensional acinar

structures coupled with a defect in cessation of proliferation during morphogenesis (Bentires-Alj et al., 2006; Brummer et al., 2006; Daly et al., 2002). Gab2 is required for cell proliferation, anchorage independent cell growth, and constitutive AKT and ERK activation in the chronic myeloid leukemia BCR-ABL and Tel-ABL cell models (Million et al., 2004; Ren et al., 2005; Sattler et al., 2002; Scherr et al., 2006). Gab2 is also required for v-SEA and mast cell transformation (Ischenko et al., 2003; Sattler et al., 2002) and in EGF-induced or c-Kit induced mitogenesis (Kong et al., 2003; Nishida et al., 2002). However, only Gab1 promotes an invasive morphogenic program downstream from the Met receptor.

Upon stimulation with HGF, Gab1 couples with the p85 subunit of phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ, the tyrosine phosphatase Shp2, adapter proteins Crk and Shc (Garcia-Guzman et al., 1999; Gual et al., 2000; Hoffmann et al., 2006; Lamorte et al., 2000; Lock et al., 2002; Maroun et al., 1999a; Sakkab et al., 2000; Watanabe et al., 2006) and acts to recruit these signaling proteins to the Met receptor to propagate the activation of multiple signaling pathways (Lock et al., 2002; Maroun et al., 1999a). Epithelial remodeling downstream from the Met receptor is dependent upon Gab1-dependent recruitment of Shp2 which is required for sustained mitogen-activated protein kinase activity (Maroun *et al.*, 2000; Schaeper *et al.*, 2000). The invasive morphogenic response also requires the integrity of the Gab1 pleckstrin homology (PH) domain (Maroun *et al.*, 1999a), as well as the direct recruitment of Gab1 to the Met receptor (Lock et al., 2002). The Gab1 PH domain binds phosphoinositide 3,4,5 tri-phosphate (PIP_3) in a PI3K-dependent manner (Isakoff et al., 1998; Maroun et al., 1999b; Rodrigues et al., 2000) and is required for membrane localization of Gab1 at

cell-cell junctions in epithelial cells (Maroun et al., 1999b; Maroun et al., 2003). Gab1 is recruited to the Met receptor by both indirect and direct mechanisms. Gab1 associates constitutively with the C-terminal SH3 domain of the adapter protein Grb2, allowing for the recruitment of Gab1 via the interaction of the SH2 domain of Grb2 with Y1356 of the Met receptor (Bardelli et al., 1997; Fixman et al., 1997; Lewitzky et al., 2001; Nguyen et al., 1997). In addition, Gab1 interacts directly with Y1349 of the Met receptor through a 19 amino acid Met binding motif (MBM) in Gab1. Notably, Gab2 lacks the Met binding motif and is only recruited to Met indirectly through the Grb2 adaptor protein and is defective in promoting the invasive morphogenic program (Lock et al., 2002; Schaeper et al., 2000).

We have identified the elements of Gab1 that are required to promote epithelial remodeling and invasion downstream of Gab2. We show that membrane targeting as well as the ability of the Gab protein to interact directly with the Met receptor, are both essential to promote the formation of lamellipodia, and for Gab to promote epithelial cell scatter and an invasive morphogenic program.

Materials and Methods

Antibodies and Reagents

Anti-HA (mHA.11) was purchased from Covance (Berkeley, CA). Anti-phospho-AKT (Ser473), anti-total AKT, anti-total ERK (p44 ERK1 and p42 ERK2), anti-phospho JNK (Thr183/Tyr185), and anti-total JNK were purchased from New England Biolabs. Anti-phospho-ERK1/2 (Tyr204) and anti-Actin antibody were purchased from Santa Cruz Biotechnology for use in western blot analysis. Monoclonal anti-phospho-ERK1/2 (Thr202) was purchased from Sigma for use in immuno-fluorescence. Anti Met antibody (145) was generated by immunizing rabbits with the carboxy-terminal 16 amino acids of the human Met sequence previously described in (Rodrigues *et al.*, 1991). HGF was a gift from Dr. George Van de Woude, Van Andel Research Institute (Grand Rapids, MI), and recombinant CSF-1 was generously provided by the Genetics Institute (Boston, MA). MEK inhibitor UO126 from Promega (Madison, WI) was diluted in DMSO from Sigma (St- Louis, MO) to a concentration of 10mM and cells were pretreated for 1h at a concentration of 20μM.

Plasmids and DNA Constructs

The c-src myristoylation signal sequence was cloned at the amino terminus of both Gab2 and Gab2MBM. The myristoylation signal sequence included the translation initiation codon from chicken c-src followed by a hemaglutinin (HA) tag in pKSII (Maroun et al., 2003). BamH1 and Spe1 restriction sites were introduced at the amino and carboxy terminus of both Gab2 and Gab2MBM coding sequences using primers 5'-GTATTAGGATCCTCATGAGCGGCGGCGGCGG-3' and 5'-

GCTACTGGATCCATGAGCACTGGTGACACTG-3' respectively. PCR products were purified using the PCR purification kit (Qiagen Mississauga, Ontario, Canada) according to manufacturer's directions. BamHI/SpeI fragment of Gab2 and Gab2MBM were cloned downstream of myrHA in pKSII+ and then were sub-cloned by digestion with KpnI/NotI into the mammalian expression vector pEBB. All plasmid constructs were sequenced using SequiTherm EXCEL TM II DNA Sequencing kit (Epicentre Madison, WI) prior to use.

Cell Culture and Stable Transfections

MDCK cells were maintained in DMEM containing 10% fetal bovine serum (FBS) (Invitrogen Burlington, Ontario, Canada). MDCK cell lines expressing a chimeric CSF-Met N1358H receptor unable to bind Grb2 (G17) were generated by retroviral infection as previously described (Fournier *et al.*, 1996), and stable cell lines expressing HA tagged Gab1, Gab2 and Gab2MBM in parental G17 cells have been described previously (Lock et al., 2000; Maroun et al., 1999a). Clonal cell lines of myrGab2 and myrGab2MBM were generated by myrHAGab2/pEBB and myrHAGab2MBM/pEBB co-transfection with PLXSH by calcium phosphate precipitation and cells were selected for by culturing with 300μg/ml hygromycin (Roche). G17 Gab1ΔShp2 (clones –5 and –7) cells are MDCK cells expressing CSF-Met N1358H and Gab1 Y627Fand have been previously characterized (Maroun *et al.*, 2000). MDCK cell lines stably expressing CSF-Met N1358H and HA tagged Gab constructs were cultured in media containing 150μg/ml hygromycin and 200 μg/ml G418 (Invitrogen), and were plated in DMEM supplemented with 10%FBS without selection for each experiment. MDCK stable cell lines expressing

eGFPGab1 were generated by calcium phosphate transfection, using 400μg/ml G418 selection and FACS sorting for fluorescent cells to generate MDCK eGFPGab1 B1 FS-1.

Stimulation of MDCK cells and immuno-precipitation

Cells were seeded at 10^6 cells per 100mm Petri dish (Nunc Rochester, NY). Twenty-four hours later, cells were serum starved overnight in DMEM containing 0.02% FBS. For stimulation, 100ng CSF-1/ml DMEM was added to the cells and incubated at 37°C for indicated times. Cells were immediately harvested in ice-cold Triton X-100 lysis buffer (1% Triton X-100, 50mM HEPES pH 8.0, 150mM NaCl, 10% glycerol, 2mM EGTA, 1.5 mM $MgCl_2$, 10mg aprotinin/ml, 10mg leupeptin/ml, 1mM phenylmethylsulfonyl fluoride, 1mM sodium fluoride, 1mM sodium vanadate). For immuno-precipitation of CSF-Met N1358H (CSF-MetΔGrb2), cells were seeded at 10^6 cells per 100mm dish in DMEM containing 10% FBS and 24 h later, protein extracts were made with ice cold TDOC lysis buffer (0.5%TDOC, 50mM HEPES pH 7.4, 150mM KCl, 5mM NaCl, 1.5mM $MgCl_2$, 4mM EGTA, 10% glycerol, 10mg aprotinin/ml, 10mg leupeptin/ml, 1mM phenylmethylsulfonyl fluoride, 1mM sodium fluoride, 1mM sodium vanadate). Following a 10 minute incubation on ice, lysates were centrifuged at 12000 rpm for 15 minutes at 4°C, and total protein concentration was determined by Bradford Assay using dye reagent concentrate (BIORAD, Hercules, CA).

Scatter Assays

Stable cell lines expressing CSF-Met N1358H with or without the panel of Gab mutants described above were plated at a density of $4X10^4$ cells per well in a 24-well dish (Nunc)

on a sterilized glass coverslip (Bellco Glass Vineland, NJ) in DMEM containing 10% FBS. The cells were allowed to seed for 24 h and stimulated for 16 h with 50ng or 150ng CSF-1 per well. Photographs were taken with a Zeiss Axiovert Microscope at 20X magnification (Carl Zeiss Canada Ltd. Toronto, Ontario). Three clones of each cell line were assayed for their ability to scatter in response to CSF-1, in three independent experiments.

Collagen Assays

The ability of MDCK cells to form branching tubules was assayed as previously described with a few modifications (Zhu et al., 1994). Briefly, $5x10^3$ cells were re-suspended in 500µl of Vitrogen 100 collagen solution (now sold as Pur-Col by INAMED Biomaterials Fremont, CA) prepared following the manufacturer's directions and layered over 350µl 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. For stimulations of cysts, HGF (15 units/ml) or CSF-1 (50ng/ml) was added to Leibowitz medium supplemented with 2% FBS. The medium was changed every 5 days for the duration of the assay. Structures were visualized by light microscopy 5 to 10 days after stimulation. Six separate fields of view were scored by counting the number of structures that formed branching tubules versus cysts counting a minimum of 10 structures per field. A tubule is defined as a branching structure whose length is greater than 5 times its width. A partial response is defined as any structure that is not longer a cyst, but whose length is less than 5 times it's width and is un-branched.

Lamellipodia Assay

Cell lines were plated on glass coverslips (Bellco Glass) at a density of $2x10^4$ cells per well in a 24 well dish (Nunc) in triplicate. 24h later, cells were either left un-stimulated or treated with 100ng/ml CSF-1 or 100units/ml HGF for 15 minutes and immediately fixed with 3% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) in PBS for 20 minutes at room temperature, stained as described below in *Immuno-fluorescence*. Actin staining was visualized using Alexa 488 conjugated phalloidin (Molecular Probes Burlington, Ontario, Canada). Nuclei were counterstained using a 0.5ng/ml dapi dilution in water and cover slips were mounted onto microscope slides with immu-mount (Thermo-Shandon Pittsburgh, PA). Confocal images of the most basal z-section were taken using a Zeiss Meta confocal microscope (model LSM 510, Carl Zeiss) with a 40x oil immersion objective. Images were scored for the total number of lamellipodia at the edge of epithelial colonies, and represented as a percentage of the total number of cells at the edge of a colony in each field of view. Ten fields of view were acquired for each condition. Epithelial colonies of three or more cells were included in the scoring of the lamellipodia assay. Multinucleated or cells with condensed chromatin were not included.

Immuno-fluorescence

MDCK cells were plated at a density of 1 to $2x10^4$ cells on glass coverslips (Bellco Glass) in a 24-well dish (Nunc) in DMEM containing 10% FBS. 24h later, cells were either untreated or stimulated as indicated. Cells were fixed in 3% paraformaldehyde in PBS for 20 minutes at room temperature, washed three times in PBS, and incubated with gentle rocking for 10 min in PBS containing 0.3% Triton X-100. Coverslips were

washed three times for 5 minutes in 100mM glycine in PBS, followed by a 30 minute incubation with blocking buffer (2% BSA in IF buffer (0.2% Triton X-100, 0.05% Tween-20 in PBS)) at room temperature. Cover slips were incubated with primary and secondary antibodies diluted in blocking buffer for 1 h and 40 min respectively at room temperature. For localization of Gab mutants, cells were stained with anti-HA. For the localization of activated ERK1/2, cells were stained with the monoclonal anti-phospho ERK1/2 (SIGMA) antibody, followed by incubation with Alexa 555 donkey anti-mouse secondary antibody (Molecular Probes). Cover slips were mounted with immu-mount (Thermo-Shandon) and images of the most basal z-section were acquired using a Zeiss Meta confocal microscope. Image analysis was carried out using the LSM 5 image browser (Carl Zeiss).

Results

The Gab1 Met Binding Motif and membrane targeting are required for a Gab2 morphogenic response.

MDCK cells expressing a chimeric CSF-Met receptor undergo a morphogenic program in response to CSF-1. Cells expressing a CSF-Met mutant, which fails to recruit the Grb2 adapter protein (CSF-MetΔGrb2), and show reduced association with Gab1, are unable to induce branching tubulogenesis in response to CSF-1 (Fournier *et al.*, 1996). Over-expression of Gab1 but not Gab2 rescues the morphogenic program downstream of CSF-MetΔGrb2 (Lock et al., 2002; Maroun et al., 1999a). This is dependent on the direct recruitment of Gab1 to the CSF-Met receptor mediated through the MBM (Lock et al., 2002; Maroun et al., 1999a; Maroun et al., 1999b; Maroun et al., 2003). However, a Gab2 protein containing the Gab1 MBM that is robustly phosphorylated in response to Met activation and engages with the same downstream signals as Gab1, is unable to support a morphogenic program (Lock et al., 2002).

Membrane recruitment via the PH domain of Gab1 has been shown to be a requirement for the biological capacity of Gab1 (Maroun et al., 1999a). The c-Src myristoylation signal substitutes functionally for the Gab1 PH domain for epithelial morphogenesis (Maroun et al., 2003), indicating that the subcellular localization of the Gab1 scaffold is a critical aspect of the ability of Gab1 to elicit a morphogenic response. Membrane localization by myristoylation of Gab1 can rescue epithelial morphogenesis when stable cell lines over-expressing myrGab1 are assayed in our cell model ((Maroun et al., 2003) and data not shown) which indicated that membrane targeting by covalent attachment of myristate to the amino terminal glycine residue of Gab1 targets the protein

to the inner leaflet of the plasma membrane and provides efficient subcellular localization for rescue of the morphogenic defect. Although Gab1 and Gab2 have similar localizations in MDCK cells, and associate with similar signaling proteins upon stimulation, over-expression of Gab1 rescues the morphogenic defect of the mutant CSF-Met receptor where Gab2 cannot (Figure 2A and (Lock et al., 2002)). To understand whether or not Gab2 could substitute functionally for Gab1 if targeted to the plasma membrane, we generated myristoylated Gab2 and Gab2MBM (Figure 1A).

To determine their subcellular localization, Gab2, Gab2MBM, myrGab2 or myrGab2MBM were visualized by anti-HA indirect immuno-fluorescence and confocal microscopy. In colonies of MDCK cells, Gab1 localizes to cell-cell contacts (Maroun *et al.*, 1999a), whereas Gab2 is predominantly localized to the cytoplasm (Figure 1B and (Lock et al., 2002)). In contrast, Gab2MBM localizes both to the cytoplasm and at cell-cell contacts (Figure 1B), where the Met receptor localizes. Notably, although the c-src myristoylation signal targets proteins to the plasma membrane (Kubler *et al.*, 1996), myrGab2 and myrGab2MBM are predominantly localized to sites of cell-cell contacts (Figure 1B) and are not observed at the plasma membrane of cells at the edge of the colonies.

To test whether membrane targeting of Gab2 or Gab2MBM is sufficient to rescue morphogenesis, MDCK epithelial cell lines, stably expressing CSF-MetΔGrb2, as well as either myrGab2 (D2, D6, and D9), or myrGab2MBM (A1, B4, and C1), were generated and compared to cell lines expressing Gab1 (wt3) (Maroun *et al.*, 1999a), Gab2 (C9) and Gab2MBM (E5) (Lock et al., 2002). Cell lines were selected that express similar levels of the CSF-MetΔGrb2 receptor when compared to the parental G17 cell line, plus

comparable levels of Gab2 and Gab2 mutant proteins as assessed by anti Met and HA immuno-blot (Figure 1C). In three independent assays, and in three clonal cell lines, Gab2, Gab2MBM, or myrGab2 were unable to rescue the morphogenic program (Figure 2A). In contrast, the expression of myrGab2MBM efficiently rescues the morphogenic defect of CSF-MetΔGrb2. In myrGab2MBM expressing cell lines, 26% (A1), 54,5% (B4) and 75%(C1) of cysts form branching tubules in response to CSF-1, which is similar to a Gab1 expressing cell line (wt3), where 53.5% of cysts form branching tubules (Figure 2B). The structures formed in collagen in response to CSF-1 of one representative cell line expressing myrGab2 (D2) and myrGab2MBM (B4) are shown in Figure 2A. Hence, while myrGab2 expressing cell lines are deficient in their ability to form branching tubular networks in response to CSF-1, the over-expression of myrGab2MBM rescues this defect (Figure 2).

Cell scatter is rescued by myrGab2MBM.

In addition to a defect in the morphogenic response, cells expressing CSF-MetΔGrb2 (G17) fail to scatter in response to CSF-1. Colonies of G17 cells when stimulated for 16 hours, fail to completely dissociate from one another, but show some morphological changes (Figure 3 panels a and b). To understand if myristoylation of Gab2 rescues the defect in cell scatter, we tested the ability of cell lines to scatter in response to CSF-1 (Figure 3). Scatter is rescued by expression of Gab1 but not Gab2, demonstrating further that Gab2 fails to elicit a biological response in this assay (Figure 3 panels d and f respectively). Consistent with the morphogenesis assays, the insertion of the MBM into Gab2 or the addition of the c-src myristoylation signal to Gab2, are

insufficient to promote cell scatter in response to CSF-1 (Figure 3 panel h and j). However, cell lines expressing myrGab2MBM rescue cell scatter (B4; Figure 3 panel l, and A1 and C1; data not shown), further supporting a role for membrane localization of Gab2 and the MBM for both the migratory and morphogenic responses.

myrGab2MBM promotes lamellipodia formation.

One of the initial events in response to HGF stimulation is the formation of lamellipodia (Ridley *et al.*, 1995) and in general, inhibitors of lamellipodia formation also block cell scatter (Potempa and Ridley, 1998; Royal *et al.*, 2000). To assess at which stage cell scatter was inhibited, we assayed the ability of cells expressing Gab2 and Gab2 mutants to form lamellipodia within 15 minutes of stimulation with CSF-1 or HGF. All cell lines possess the ability to form lamellipodia in response to HGF and activation of the endogenous Met receptor; this serves as a positive control (Figure 4A, HGF). Visualization of polymerized actin by phalloidin staining determines the presence or absence of lamellipodia by staining the cell cortex at the limits of epithelial cell colonies (Figure 4B small arrow heads), and at the edge of lamellipodia (Figure 4B large arrow heads). Formation of lamellipodia is represented as a percentage of total number of cells at the edge of a colony. Upon 15 minutes CSF-1 stimulation, 27.4% of parental G17 cells at the edge of a colony form lamellipodia when compared to 66% of cells in response to HGF (Figure 4A). Cell lines expressing Gab2 (C9), Gab2MBM (E5), and myrGab2 (D2, D6, and D9) behave in a similar manner to parental cells. However, cell lines expressing myrGab2MBM (A1, B4 and C1) are able to form lamellipodia in response to CSF-1 to similar levels as they do in response to HGF, where 76.1%, 97.5%,

and 82.4% of edge cells form lamellipodia respectively (Figure 4). This is a similar response to cells expressing Gab1, where 77.7% of edge cells form lamellipodia (wt3, Figure 4A). Those cell lines that form lamellipodia, whereby greater than 75% of edge cells do so, also rescue epithelial morphogenesis and scatter of epithelial colonies, demonstrating that myrGab2MBM, but not Gab2, possesses the same capacity for lamellipodia formation and biological function as does Gab1.

Gab1 and myrGab2MBM localize to lamellipodia.

Since membrane targeting of Gab2MBM is required to switch the biological and biochemical responses of Gab2, we determined the subcellular localization of Gab1, Gab2 and Gab2 mutants following stimulation of the mutant Met receptor. Cells were seeded for 24 hours on glass coverslips and stimulated for 15 minutes with CSF-1, fixed, stained and visualized by indirect immuno-fluorescence using an anti-HA antibody and confocal microscopy. One representative image of three microscopic fields from three independent experiments for each cell line in response to 15 minutes CSF-1 stimulation is shown (Figure 5A). Gab1 localizes to the edge of lamellipodia as well as to the cytoplasm. In contrast, although Gab2 is recruited to the Met receptor at sites of cell-cell contacts, it is predominantly localized to the cytoplasm and does not localize to the membrane of lamellipodia. The differential localization of Gab1 and Gab2 upon stimulation provides an important difference between these two highly related family members. Although myristoylation of Gab2 leads to the redistribution of Gab2 mostly to sites of cell-cell contacts, only insertion of the MBM into myrGab2 allows for to the re-localization of myrGab2MBM to lamellipodia upon CSF-1 stimulation.

Gab1 localizes to lamellipodia downstream of Met and not EGFR.

While HGF promotes the remodeling of MDCK epithelial cells into branching tubular networks, another related growth factor receptor tyrosine kinase, EGFR, fails to do so (Maroun et al., 1999a). Activation of the EGFR in MDCK cells fails to activate a robust and sustained activation of ERK1/2 and fails to induce a morphogenic program. However, cells over-expressing a myrGab1 protein can switch the EGF response to a morphogenic response (Maroun et al., 2003), suggesting that an enhanced Gab1 localization to the plasma membrane is required.

To visualize Gab1 subcellular localization, we established that an eGFP tagged Gab1 protein is functional as it can rescue the morphogenic response downstream of the CSF-MetΔGrb2 receptor (data not shown). The subcellular localization of eGFPGab1 was examined 15 minutes post HGF or EGF stimulation, and the percentage of edge cells that make lamellipodia was also scored. Only cells stimulated with HGF induce an increased formation of lamellipodia as compared to un-stimulated cells (Figure 5B). Furthermore, eGFPGab1 localizes to lamellipodia in 32% of edge cells, whereas few edge cells (1%) localize eGFPGab1 to lamellipodia upon EGF stimulation (Figure 5B). The inset panel in Figure 5B demonstrates the localization of eGFPGab1 to lamellipodia with HGF stimulation. These data demonstrate that the localization of Gab1 to lamellipodia correlates with the formation of lamellipodia specifically upon HGF stimulation.

Membrane targeted Gab2MBM robustly activates ERK1/2 and localizes phosphorylated ERK1/2 to lamellipodia.

To establish the consequence of membrane targeting and MBM insertion into Gab2 on downstream signaling, we assessed the activation of the MEK/ERK pathway. Upon CSF-1 stimulation, Gab2 expressing cells show a weak induction of ERK1/2 phosphorylation, whereas Gab1 expressing cells activate a robust and sustained activation of ERK1/2. However, where cells expressing myrGab2MBM show robust and sustained activation of ERK1/2 following CSF-1 stimulation, myrGab2 does not (Figure 6A). This is consistent with the ability of myrGab2MBM cells and not Gab2MBM cells to promote a robust activation of other downstream signaling pathways including PI3K and JNK, and indicates that direct recruitment of Gab2 to the Met receptor and membrane targeting is essential for the sustained activation of downstream signaling pathways (Supplemental Figure 1).

In order to understand the consequence of sustained ERK1/2 activation downstream of myrGab2MBM, we determined the subcellular localization of activated ERK1/2. Following treatment with CSF-1 for 15 minutes cells were fixed and indirect immuno-fluorescence was performed using phalloidin and phospho-specific antibodies against ERK1/2. Representative confocal images of the localization of p-ERK in Gab1, myrGab2 and myrGab2MBM cells are shown with corresponding actin images to delineate the edge of colonies in Figure 6B. The results of three independent experiments were collected and represented in a histogram (Figure 6C). We observe that there is a basal level of phosphorylated ERK1/2 in parental G17 cells, which is mostly localized to the nucleus and also to a reduced level at sites of cell-cell junctions. Although a small

portion of cells display p-ERK localized to lamellipodia (1.7%), following CSF-1 stimulation, the majority of edge cells expressing myrGab2MBM (B4) and Gab1 (wt3) are positive for p-ERK staining in lamellipodia (57% and 60.5% respectively). In contrast, cells expressing Gab2 (C9), or myrGab2 (D2), as well as parental G17 cells, are unable to induce an increase of edge cells where p-ERK staining is localized in lamellipodia in response to CSF-1 (10.4%, 13.5%, and 6.3%, respectively). In conditions where ERK1/2 is activated in a sustained manner, we demonstrate that phosphorylated ERK is localized to the membrane of lamellipodia. In order to assess the contribution of the localization of activated ERK to lamellipodia in the generation of a sustained signal, we determined the localization of p-ERK on a longer time-course. We have found that cells expressing Gab1 or myrGab2 MBM, but not Gab2 or myrGab2, localize p-ERK to the membrane of lamellipodia at 30 minutes (data not shown) and 90 minutes (Figure 6B) CSF-1 stimulation. The presence or absence of p-ERK staining at the membrane of lamellipodia is determined by acquiring simultaneous actin images, which define the edge of the lamellipodia. In Figure 6B, white arrows delineating the edge of lamellipodia are also superimposed on the p-ERK image in the same position. The sustained localization of phosphorylated ERK1/2 at the membrane of lamellipodia in the majority of edge cells, which make lamellipodia only in cells expressing Gab1 or myrGab2MBM but not Gab2, demonstrates the involvement of subcellular localization as a mechanism for the differences between the function of Gab1 and Gab2.

Lamellipodia formation and activation of ERK require the Gab1-Shp2 complex.

Epithelial morphogenesis is dependent on the sustained and robust activation of MAPK. In Met mediated epithelial morphogenesis, the activation of the MEK/ERK pathway is dependent on the recruitment of the Shp2 tyrosine phosphatase to Gab1. Moreover, MDCK cells expressing CSF-MetΔGrb2 and Gab1ΔShp2 are unable to promote the morphogenic program (Maroun et al., 2000). Activation of ERK1/2 is evaluated by western blot analysis with phospho-specific ERK1/2 antibodies. Indeed, when compared with Gab1 expressing cells, cells expressing Gab1ΔShp2 are unable to activate a sustained ERK1/2 signal (Figure 7A and (Maroun et al., 2000)).

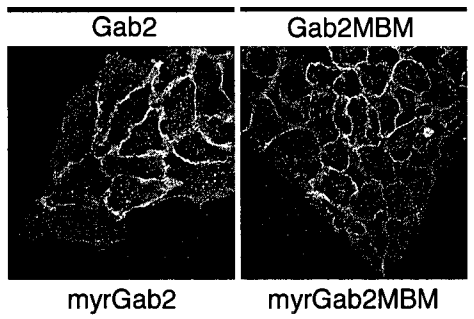
Since Gab1-Shp2 dependent ERK1/2 activation is required for epithelial morphogenesis, we assayed the ability of cells expressing Gab1ΔShp2 to promote lamellipodia formation. Two cell lines expressing Gab1 and two cell lines expressing Gab1ΔShp2, as well as the parental G17 cell line, were stimulated with CSF-1 for 15 minutes. On average, 85% of Gab1 expressing cells were able to form lamellipodia. In contrast, an average of 40% of the cells expressing Gab1ΔShp2 formed lamellipodia in response to CSF-1 as compared to 22.5% of edge cells in the parental G17 cell line. The deficiency of the two Gab1ΔShp2 expressing cell lines to promote abundant lamellipodia formation in response to CSF-1 places a role for the Gab1-Shp2 complex and subsequent sustained ERK1/2 activation for lamellipodia formation. As a positive control, Gab1ΔShp2 expressing cells efficiently formed lamellipodia in response to HGF activation of the endogenous Met Receptor (Figure 7B). Furthermore, Shp2 is localized to the membrane of lamellipodia in cells expressing Gab1 and myrGab2MBM upon 15

minutes CSF-1 stimulation providing support for the localized activation of ERK1/2 in lamellipodia downstream of Gab1.

The MEK/ERK pathway is required for the formation of lamellipodia.

Since Gab2 as well as Gab1 mutants defective in the activation of sustained ERK1/2 activation (Gab1ΔShp2) fail to induce the formation of lamellipodia, we tested the consequence of inhibiting the MEK/ERK pathway on lamellipodia formation. Parental G17 cells and cells expressing Gab1 (wt3) and myrGab2MBM (B4) were pretreated for 1h with 20μM UO126 or DMSO, and then stimulated for indicated times with CSF-1. Cell extracts and western blot analysis demonstrate that following UO126 pretreatment, ERK1/2 are not phosphorylated upon CSF-1 stimulation (Figure 7C). Moreover, UO126 pretreatment abrogates the ability of edge cells in a colony to form lamellipodia in response to CSF-1. Gab1 and myrGab2MBM expressing cells promote 89.5% and 84.7% of edge cells to form lamellipodia respectively when pretreated with DMSO. In contrast, when MEK is inhibited by pretreatment with UO126, only 52% and 43.3% of edge cells form lamellipodia (Figure 7D). Hence activation of ERK1/2 is a significant signal for the formation of lamellipodia downstream of Gab1 and myrGab2MBM.

Figure 1. Gab2 constructs and cell lines generated for structure/function analysis. A) Schematic representation of Gab1, Gab2, Gab2MBM, myristoylated Gab2 (myrGab2), and myristoylated Gab2MBM (myrGab2MBM) constructs. PH: Pleckstrin Homology domain, MBM: Met Binding Motif which confers direct binding to the Met Receptor, Pro: domain containing proline based motifs, myr (〰); myristoylation signal sequence from c-Src. All Gab constructs are HA tagged at the amino terminus, however myristoylation signal sequence is at the most amino terminal of all constructs followed by the HA tag, then the PH domain. B) MDCK cells expressing either HA tagged Gab2 (F3), Gab2MBM (A7), myrGab2 (C3), or myrGab2MBM (A3) were seeded on glass coverslips, allowed to form colonies for 24 h and fixed. Anti-HA immuno-staining and confocal images were acquired. The scale bar represents 20μm. C) MDCK cell lines expressing CSF-MetΔGrb2, stably transfected with vector (G17), or with constructs encoding, HA tagged Gab1 (wt3), Gab2 (C9), Gab2MBM (E5) or myrGab2 (D2, D6, and D9) and myrGab2MBM (A1, B4, and C1) were lysed in Triton X-100 buffer and proteins (25 μg of whole cell extracts) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and western blot (WB) analysis was carried out with anti-HA antibody. Levels of CSF-MetΔGrb2 were determined by immuno-precipitation (IP) of 0.5mg of TDOC protein extracts with anti-Met antibody and WB with anti-Met to analyze levels of receptor. Proteins from whole cell lysate were blotted with anti-actin antibody to show equal protein loading.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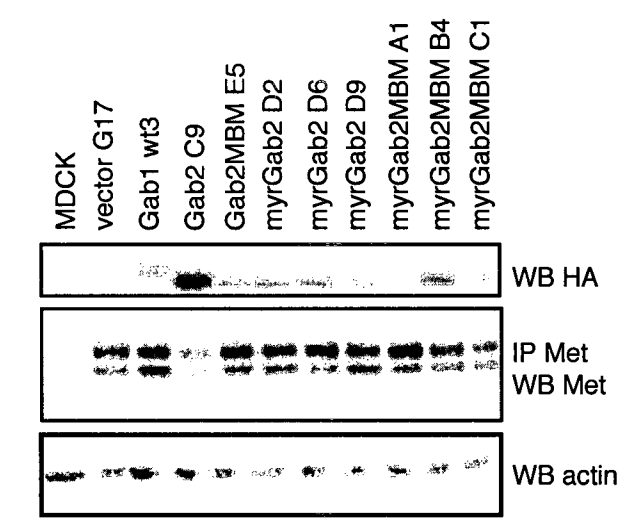
Gab2MBM
myrGab2
myrGab2MBM

C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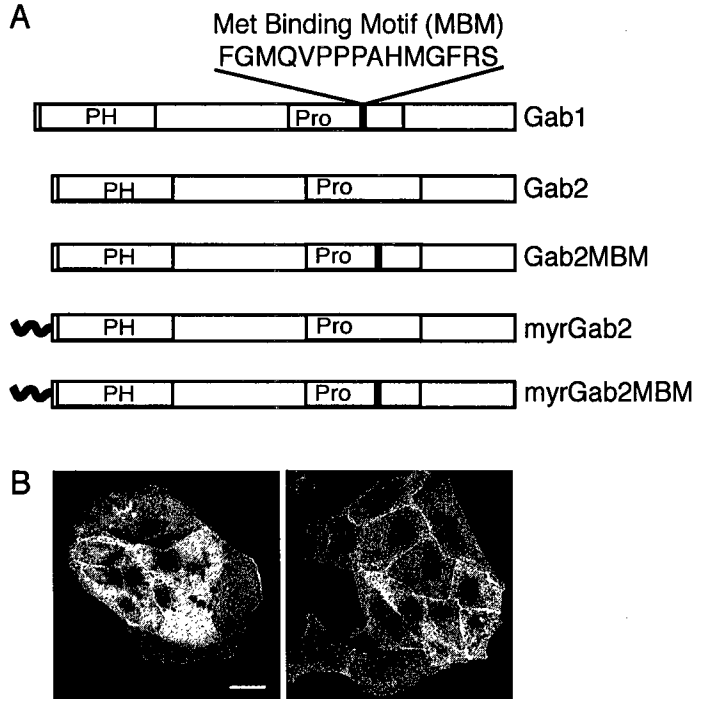
WB HA
IP Met
WB Met
WB actin

Figure 1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Met Binding Motif (MBM)
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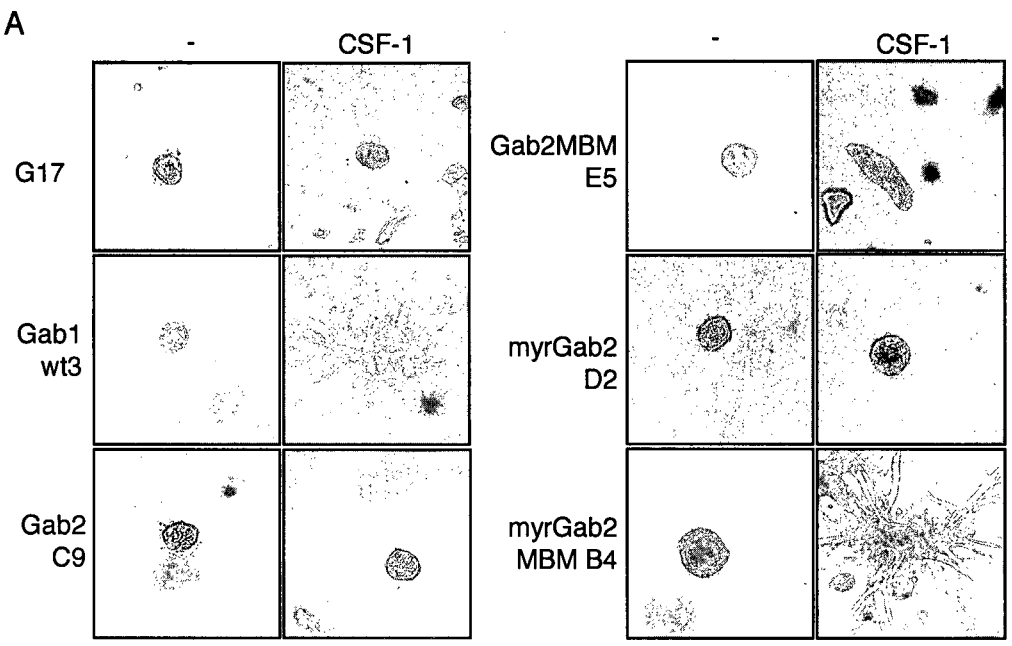
PH
Pro
Gab1
PH
Pro
Gab2
PH
Pro
Gab2MBM
PH
Pro
myrGab2
PH
Pro
myrGab2MBM
B

Figure 2. Membrane recruitment and MBM insertion to Gab2 rescues the morphogenic program. Stable MDCK cells lines were seeded in collagen and allowed to form cysts for 5 days, then were stimulated with CSF-1 (5ng/ml). A) Representative images from three independent experiments are shown. Pictures were taken at a magnification of 10x. B) Quantification of the morphogenic response is described in Materials and Methods. The response to CSF-1 stimulation for 5 to 7 days are plotted as the percentage of organoid structures that have remained as cysts (cyst) or have undergone branching tubulogenesis (tubule), or have an intermediate response (partial). The values in the table under the histogram are derived from three independent experiments with error bars representing the SEM.

Figure 2

A



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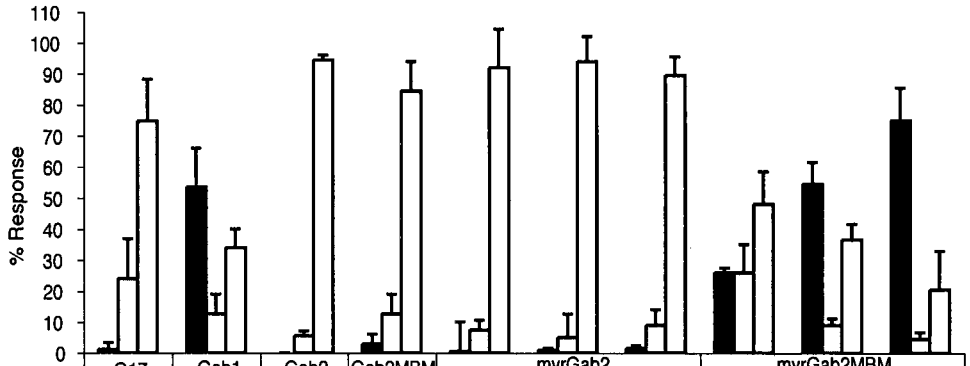
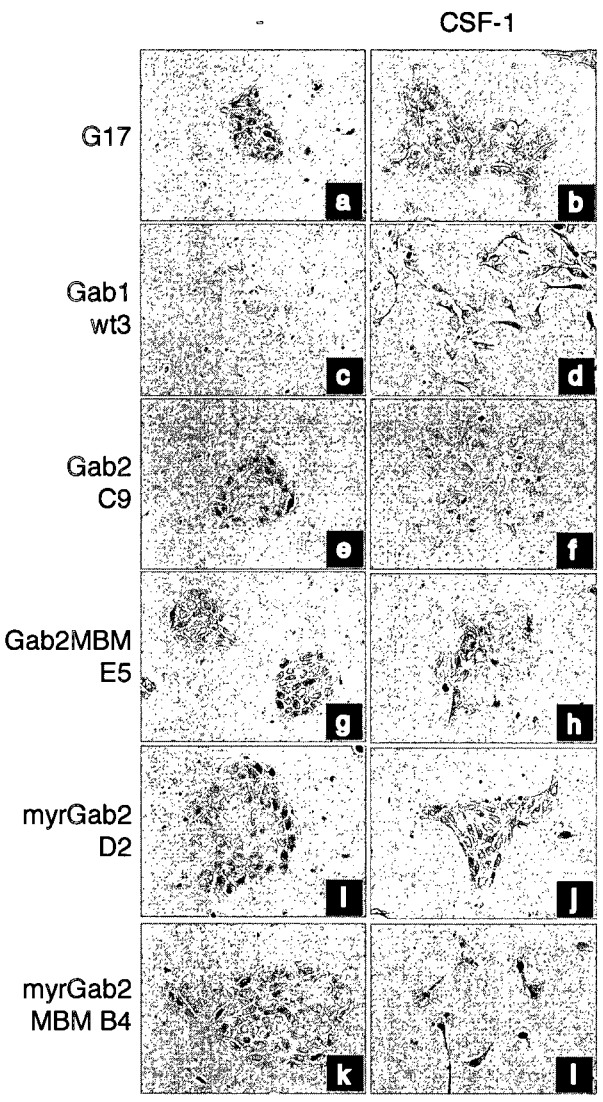


	G17	Gab1 wt3	Gab2 C9	Gab2MBM E5	myrGab2 D2	myrGab2 D6	myrGab2 D9	myrGab2MBM A1	myrGab2MBM B4	myrGab2MBM C1
■Tubule	1.25	53.5	0	3	0.5	1	1.5	26	54.5	75
□Partial	24	12.5	5.5	12.5	7.5	5	9	26	9	4.5
□Cyst	74.75	34	94.5	84.5	92	94	89.5	48	36.5	20.5

Figure 3. Membrane recruitment and MBM insertion to Gab2 rescues cell scatter. Parental MDCK cells expressing CSF-MetΔGrb2 (a and b), as well as cell lines expressing the receptor and the panel of Gab mutants including, Gab1 wt3 (c and d); Gab2 C9 (e and f); Gab2MBM E5 (g and h); three clones of myrGab2 D2 (i and j), D6, and D9; and myrGab2MBM A1 (k and l), B4, C1; were seeded in 24 well dishes in duplicate and allowed to form colonies. 24 h later, cells were left untreated (left column) or stimulated with 50ng CSF-1/ml for 16hours (right column) and phase contrast pictures were taken with a 20X objective of three microscopic fields in three independent experiments. Representative images of each condition are shown.

Figure 3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CSF-1
G17
a
b
Gab1
wt3
c
d
Gab2
C9
e
f
Gab2MBM
E5
g
h
myrGab2
D2
i
j
myrGab2
MBM B4
k
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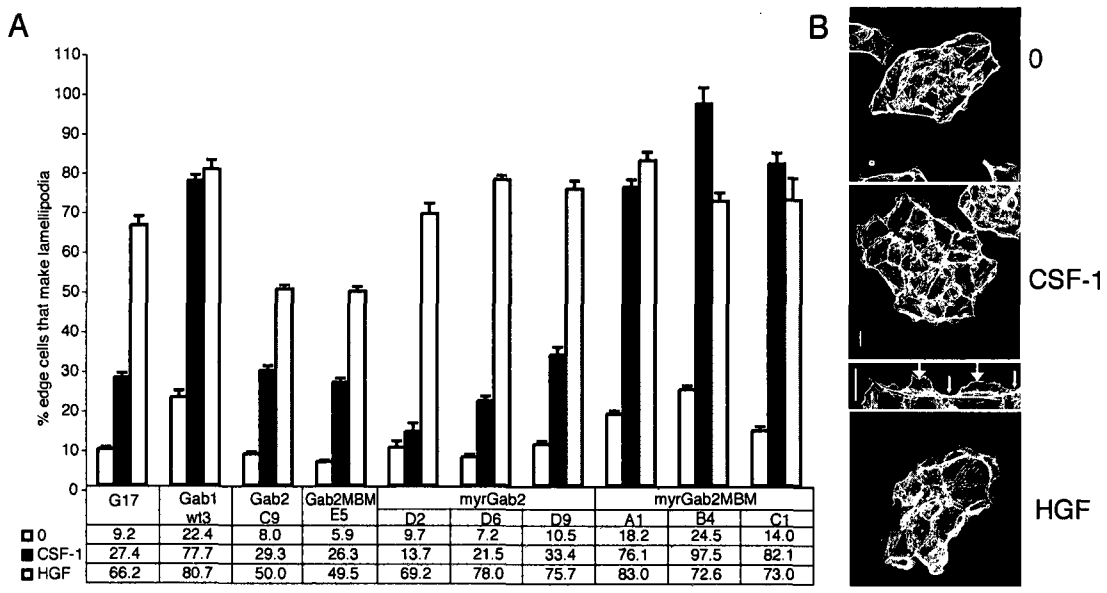
Figure 4. Lamellipodia formation occurs in cells expressing Gab1 and myrGab2MBM. MDCK cells were seeded on glass coverslips in triplicate and 24h later, cells were either left untreated, or stimulated with 100ng/ml CSF-1 or 100U/ml HGF for 15 minutes. Cells were stained with Alexa conjugated phalloidin and dapi to visualize F-actin and nuclei, respectively. Six confocal microscopic fields of the most basal z-section from two independent experiments were acquired with a 40X objective. Detailed scoring criteria are described in Materials and Methods. A) The number of lamellipodia are counted and represented as a percentage of the total number of cells at the edge of epithelial colonies. The histogram and table represent the average percentage of lamellipodia from twenty microscopic fields in the un-stimulated condition (0) or 15 minutes stimulated with indicated ligand (CSF-1 or HGF) with SED. B) Representative images of cells expressing Gab1 (wt3) untreated (0), stimulated with CSF-1 or HGF. The narrow panel is an enlargement of the CSF-1 stimulated condition where Actin staining delineates lamellipodia. Small headed arrows demonstrate the limit of the colony as defined by a cortical Actin ring. Large headed arrows demonstrate the edge of lamellipodia.

Figure 4



	G17	Gab1 wt3	Gab2 C9	Gab2MBM E5	myrGab2 D2	myrGab2 D6	myrGab2 D9	myrGab2MBM A1	myrGab2MBM B4	myrGab2MBM C1
0	9.2	22.4	8.0	5.9	9.7	7.2	10.5	18.2	24.5	14.0
CSF-1	27.4	77.7	29.3	26.3	13.7	21.5	33.4	76.1	97.5	82.1
HGF	66.2	80.7	50.0	49.5	69.2	78.0	75.7	83.0	72.6	73.0

Figure 5. Gab proteins that localize to lamellipodia promote morphogenesis.

A) Cells expressing CSF-MetΔGrb2 (G17) as well as Gab1 (wt3), Gab2 (C9), myrGab2 (D2), or myrGab2MBM (B4) were allowed to form colonies for 24 h and then stimulated for 15 minutes with 100ng/ml CSF-1. The localization of Gab proteins by indirect immuno-fluorescence with anti-HA and confocal microscopy is determined (100X objective). B) Lamellipodia assay of MDCK cells expressing a Gab1 protein fused to enhanced green fluorescent protein (MDCK eGFPGab1 B1 FS-1) with two different growth factor stimulation conditions, 100ng/ml EGF and 100U/ml HGF. The percentage of edge cells in epithelial colonies that form lamellipodia was determined (lamellipodia). The localization of eGFPGab1 in these cells was scored based on the absence or presence of Gab1 in lamellipodia (Gab1 localized to lamellipodia). The inset image is representative of the localization of eGFPGab1 with 15 minutes HGF stimulation. The mean of two independent experiments from 6 microscopic fields using a 40X objective are represented in the histogram with SED and data labels.

Figure 5

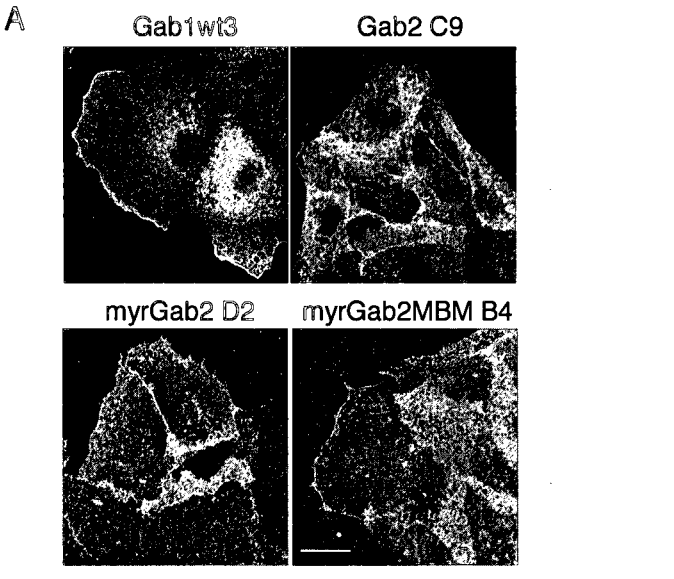
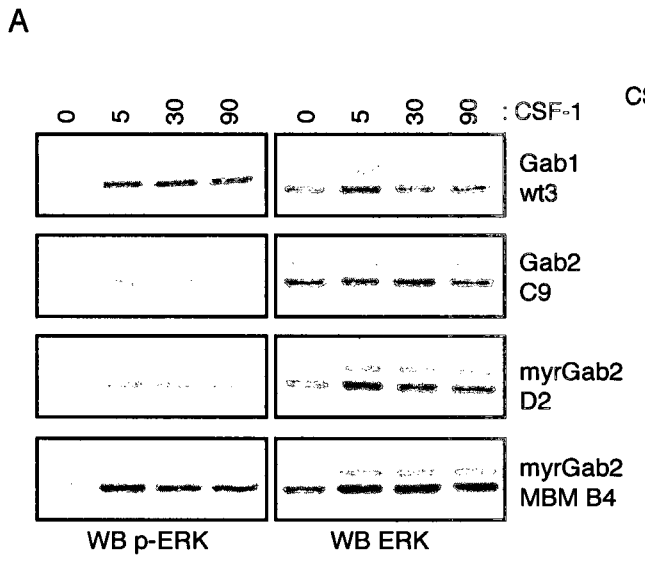
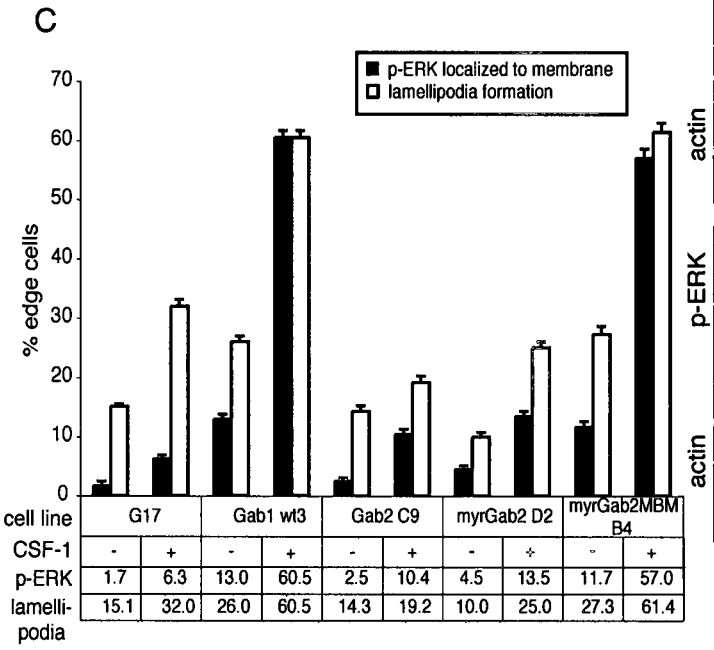

A
Gab1wt3
Gab2 C9
myrGab2 D2
myrGab2MBM B4
B
lamellipodia
Gab1 localized to lamellipodia
% edge cells
50
45
40
35
30
25
20
15
10
5
0
9
7
12
0
44
32
0
15 min EGF
15 min HGF
Growth Factor Stimulation

Figure 6. Phosphorylated ERK1/2 localizes to lamellipodia downstream of Gab1 and myrGab2MBM. A) Gab1 wt3, Gab2 C9, myrGab2 D2, and myrGab2MBM B4 cell lines were stimulated for indicated times with 100ng/ml CSF-1 and lysed in Triton X-100 buffer. 20μg WCL was resolved by SDS-PAGE, transferred to nitrocellulose and probed to determine the activation status of ERK1/2 using phospho-specific ERK1/2 and total ERK1/2 antibodies. B) Cells were seeded on glass coverslips in a 24 well dish for 24 hours and left either untreated or stimulated for 15 or 90 minutes with 100ng/ml CSF-1. Confocal sections were acquired of the most basal slice of cells stained for phospho-ERK1/2, as well as phalloidin and dapi counterstained nuclei. Representative images acquired for p-ERK staining and corresponding phalloidin staining (actin) in Gab1 wt3, myrGab2 D2 and myrGab2MBM B4 expressing cells are shown. Arrows demonstrate the membrane of lamellipodia as defined by actin staining. Arrows in the same position for the corresponding p-ERK image demonstrate the presence or absence of phosphorylated ERK1/2 at the membrane of cells on the edge of a colony. C) Cells were scored based on the presence or absence of p-ERK staining at the membrane of lamellipodia (p-ERK) without (-) or with 15 minutes (+) CSF-1 stimulation. Total number of lamellipodia formed was also scored (lamellipodia). All results are represented as a percentage of total number of edge cells. These results are gathered from two independent experiments each with observation of 10 different microscopic fields using a 100X objective.

Figure 6





cell line	G17		Gab1 wt3		Gab2 C9		myrGab2 D2		myrGab2MBM B4	
CSF-1	-	+	-	+	-	+	-	+	-	+
p-ERK	1.7	6.3	13.0	60.5	2.5	10.4	4.5	13.5	11.7	57.0
lamelli-podia	15.1	32.0	26.0	60.5	14.3	19.2	10.0	25.0	27.3	61.4

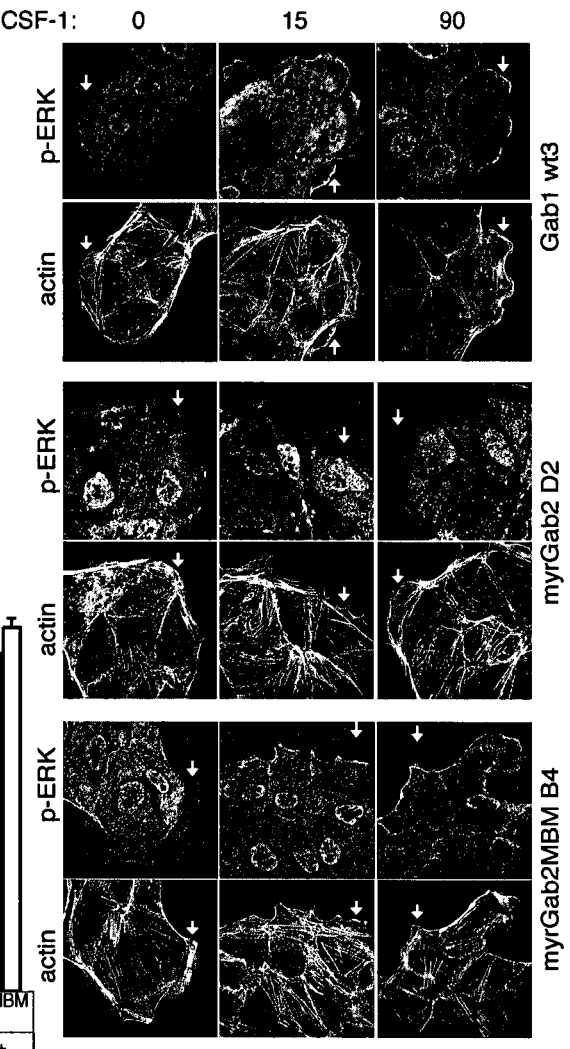
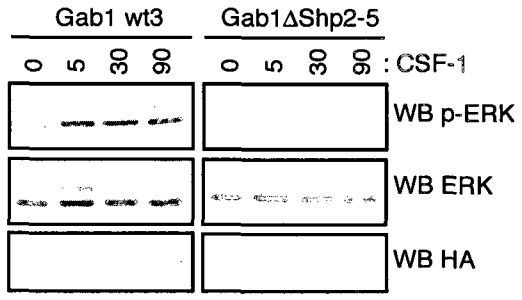


Figure 7. The MEK/ERK pathway is required for lamellipodia formation. A) Gab1 wt3 and Gab1ΔShp2-5 cells were stimulated for indicated times with 100ng/ml CSF-1 and the activation of ERK1/2 was determine by western blot with p-ERK and total ERK antibodies. Levels of Gab1 and Gab1ΔShp2 are determined by HA western blot analysis. B) Parental G17 cells as well as two clones of each Gab1 (wt2 and wt3) and Gab1ΔShp2 (-5 and –7) are assayed for their ability to form lamellipodia in three independent experiments where 6 microscopic fields using 63X objective were counted. Results are represented in the histogram as the mean average with SED. C) The localization of Shp2 is determined by immuno-fluorescence of endogenous protein in cells expressing either Gab1, myrGab2, or myrGab2MBM stimulated with CSF-1 for 15 minutes. The edge of the cell colony and lamellipodia is determined by co-staining with phalloidin to visualize F-Actin and demonstrated by arrows. Corresponding arrows in the same position of the Shp2 image delineate the presence or absence of Shp2 to lamellipodia D) Cells expressing CSF-MetΔGrb2 and vector, Gab1, or myrGab2MBM are pretreated or not for 1h with 20μM UO126 and stimulated for indicated times with 100ng/ml CSF-1. Triton X-100 protein extract (25μg) are resolved by SDS-PAGE, transferred to nitrocellulose and western blot analysis for p-ERK, and re-probed for total ERK1/2 levels. Anti HA western blot analysis determines the protein levels of Gab1 and myrGab2MBM. E) Lamellipodia assays are carried out with cells pretreated for 1 h with DMSO or UO126, and left untreated or stimulated with CSF-1 for 15 minutes. The mean average and SED is plotted in the histogram with data from two independent experiments where 6 microscopic fields of each condition were taken using a 40X objective.

Figure 7

A



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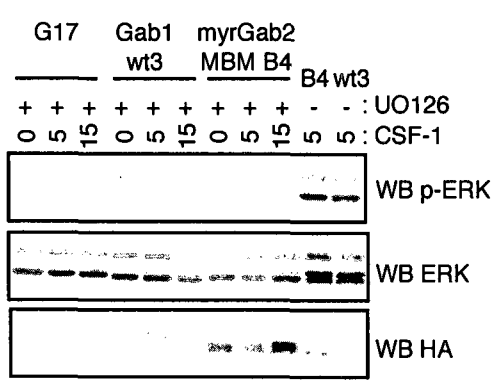
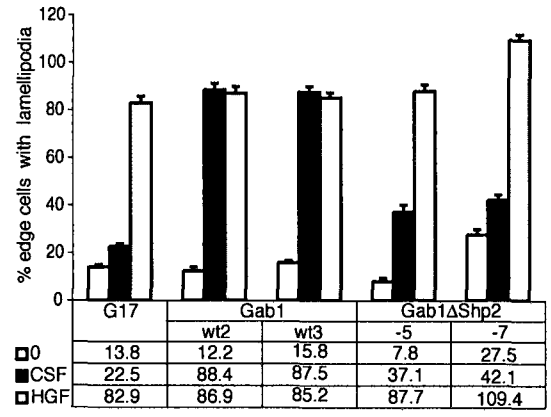
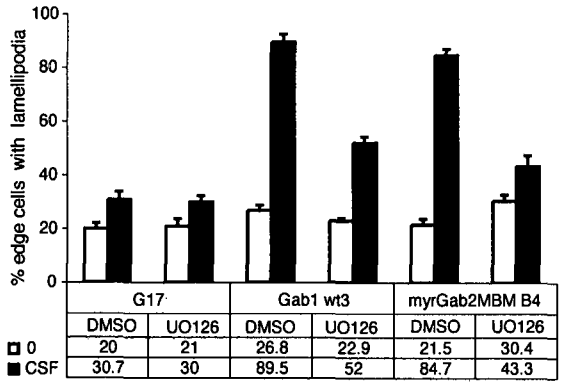


B



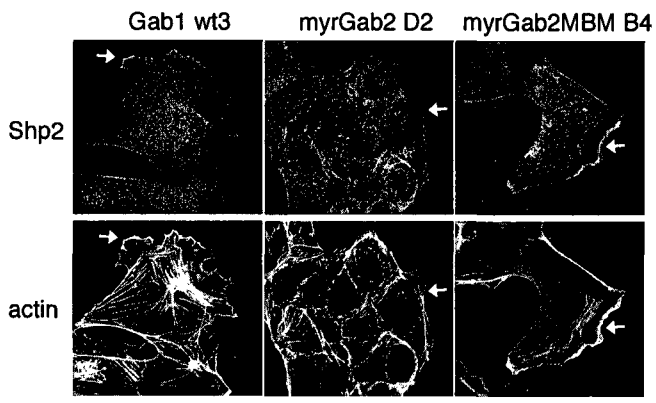
	G17	Gab1		Gab1ΔShp2	
		wt2	wt3	-5	-7
□0	13.8	12.2	15.8	7.8	27.5
■CSF	22.5	88.4	87.5	37.1	42.1
□HGF	82.9	86.9	85.2	87.7	109.4

E

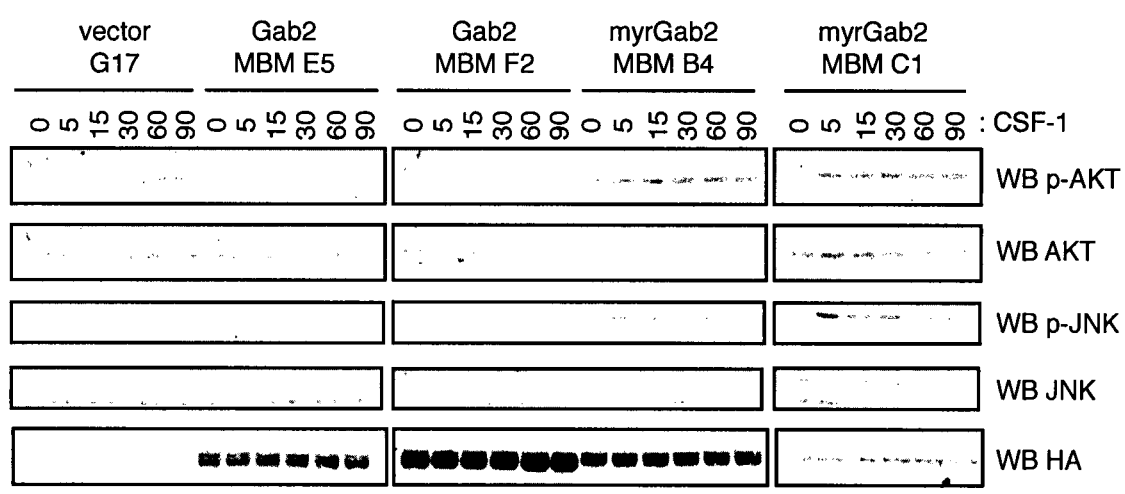


	G17		Gab1 wt3		myrGab2MBM B4	
	DMSO	UO126	DMSO	UO126	DMSO	UO126
□ 0	20	21	26.8	22.9	21.5	30.4
■ CSF	30.7	30	89.5	52	84.7	43.3

C



Supplementary Figure 1. MyrGab2MBM expression switches a transient Gab2MBM AKT and JNK activation to a robust and sustained response. MDCK cells stably expressing CSF-MetîGrb2 and vector (G17) or two clones of HA tagged Gab2MBM (E5 and F2) or myrGab2MBM (B4 and C1) are serum starved in medium containing 0.02% FBS for 18 hours and stimulated with CSF-1 (100 ng/ml) for the indicated times. Cells are lysed in Triton X-100 buffer and 20°g WCL resolved by SDS-PAGE, transferred to nitrocellulose and probed with either phospho-specific AKT antibodies or phospho-specific JNK antibodies. Blots were stripped and re-probed with an anti-total AKT or JNK antibody. WCL were also probed with anti HA (bottom panel) to show protein expression levels of Gab2MBM or myrGab2MBM in these same extracts.



Discussion

Gab1 and Gab2 are highly related scaffolding proteins, they are both expressed in epithelia, both engage the Met receptor with similar downstream signaling pathways (Lock et al., 2002). However, whereas Gab1 promotes epithelial morphogenesis, cell scatter and lamellipodia formation downstream from the Met receptor, Gab2 fails to promote these biological responses (Figures 2, 3 and 4). Through structure-function studies, we establish the requirements that confer on Gab2 the ability to promote a Gab1 dependent biological response. We show that enhanced membrane localization of Gab2, through addition of the c-Src myristoylation signal, as well as direct recruitment to the Met receptor (myrGab2MBM), promotes a morphogenic response, cell dispersal as well as lamellipodia formation to the same extent as Gab1 (Figures 2, 3 and 4). These data demonstrate that differences in the subcellular localization of Gab1 and Gab2, and not differences per-se in downstream signaling proteins, promote distinct biological responses downstream from the CSF-MetΔGrb2 receptor.

Gab1 localizes to lamellipodia upon activation of the receptor, while Gab2 does not (Figure 5). The addition of the c-Src myristoylation signal to Gab2 or Gab2MBM promotes the re-localization of Gab2 from the cytoplasm to sites of cell-cell contacts in un-stimulated colonies of epithelial cells (Figure 1), however only myrGab2MBM localized to lamellipodia in response to receptor activation (Figure 5). The localization of Gab1 to lamellipodia 15 minutes following activation of the receptor is an early event that correlates with the ability of Met to modulate migratory and morphogenic responses following activation (Figures 2 and 3). In support of this, although Gab1 has been shown to function downstream of the EGFR, it fails to promote the formation of lamellipodia

and also fails to localize the membrane of the few lamellipodia that are formed in response to EGF (Figure 5). This is in agreement with the inability of MDCK cells to migrate and undergo a morphogenic response downstream of the EGF receptor (Maroun et al., 2003).

The MBM allows a direct recruitment of Gab2 to a MetΔGrb2 receptor, and this along with membrane targeting by myristoylation, is required to convert a Gab2 signal from a transient activation of downstream signaling pathways to the sustained activation of downstream ERK, AKT and JNK (Figure 6 and Supplementary Figure 1). Consistent with a sustained signal downstream form Gab1 being required for a morphogenic response (Maroun *et al.*, 2000), the sustained activation of ERK1/2 downstream from myrGab2MBM is required for cell scatter and morphogenesis. A sustained ERK signal is localized to lamellipodia and provides evidence to support that the sustained and compartmentalized ERK signal may function to support cell scatter and morphogenesis. Moreover, activation of the MEK/ERK pathway is required for efficient formation of lamellipodia in response to Met activation (Figure 7). Pretreatment of cells with a MEK inhibitor (UO126 20μM for 1h) decreases the ability of cells to induce lamellipodia in response to Met activation (Figure 7), identifying ERK1/2 activation as a requirement for lamellipodia formation and/or maintenance in response to Met activation.

In addition to sustained activation, phosphorylated ERK1/2 is compartmentalized and is localized to the membrane of lamellipodia, 15 minutes following CSF-MetΔGrb2 activation (Figure 6). Gab1 and myrGab2MBM but not myrGab2 or Gab2MBM, promote the localization of phospho-ERK1/2 to lamellipodia (Figure 6). The exact mechanism by which activated ERK localization to lamellipodia occurs in a sustained manner is not yet

known. It is possible that activated ERK localized to lamellipodia is due to the localized complex formation with MEK, the upstream kinase, as well as a MAPK scaffold that brings these two in close proximity to allow for specific ERK activation. In addition, localization of activated ERK to lamellipodia may provide a mechanism through which ERK is sequestered away from MAPK phosphatases. However, we have demonstrated that downstream from Gab1, sustained activation of phospho-ERK1/2 is dependent on the formation of a Gab1-Shp2 complex and that a Gab1ΔShp2 protein is defective in the induction of lamellipodia (Figure 7). This is consistent with a role for Gab1-Shp2 complexes in the chemotactic response to PDGF (Kallin *et al.*, 2004). Moreover, although Gab1 and Gab2 both activate the ERK signaling pathway, they vary in the duration and localization of the ERK signal downstream, which is a known mechanism for signal specificity (Ebisuya et al., 2005).

Changes in subcellular localization of ERK is a mechanism by which it's activity is regulated. Following sustained activation, ERK1/2 can shuttle from the cytoplasm to the nucleus to activate changes in transcription required for cell proliferation downstream of integrins and growth factor receptors (Aplin et al., 2001; Renshaw et al., 1999). Compartmentalization of ERK1/2 within the cytoplasm and/or in colonies of epithelial cells as we have described herein may also function to regulate the ERK signal. The localization of phospho-ERK1/2 to lamellipodia has been reported in single motile Caco-2 cells as well as in keratinocyte wound healing assays (Pullar et al., 2006; Yu and Basson, 2000). Moreover, phospho-ERK has been shown to be present in epithelial cell sheets only in cells that are proximal to the wound edge in corneal and MDCK cultures in response to HGF (Matsubayashi et al., 2004; Sharma et al., 2003). This is consistent with

our data where only edge cells that form lamellipodia localize Gab1 or myrGab2MBM and phospho-ERK to this same location, further supporting a role for the compartmentalization of activated ERK1/2 to lamellipodia in cell motility.

A requirement for ERK1/2 activation for cell migration in wound healing is supported by several studies. Cell migration is inhibited upon treatment with MEK inhibitors in invasive cancer models as well as normal motile cells and following scratch assays (Brahmbhatt and Klemke, 2003; Hong and Grabel, 2006; Honma et al., 2006; Matsubayashi et al., 2004; Sharma et al., 2003; Vaidya et al., 2005). Similarly, ERK activation is required for HGF mediated epithelial tubule formation (O'Brien *et al.*, 2004). The exact mechanism by which ERK1/2 activation regulates lamellipodia protrusion and cell migration is not well understood. Our data identifies the inherent capacity of Gab1 to transduce the required signals for the localization of activated ERK to lamellipodia. Mechanisms through which Gab1 dependent ERK1/2 activation leads to Gab1 localization and/or lamellipodia formation downstream of Met, remain to be elucidated.

Gab1 has been identified as a substrate of ERK1/2 (Lehr *et al.*, 2004) and this has been shown to function as a switch to terminate the positive feedback loop of PI3K activation (Yu et al., 2002; Yu et al., 2001) which is thought to regulate the lipid microenvironment. Cytoskeletal effectors of ERK1/2 such as myosin light chain II kinase (Klemke *et al.*, 1997), cortactin (Martinez-Quiles *et al.*, 2004), calpain (Glading *et al.*, 2001), and paxillin (Ishibe *et al.*, 2004) have been identified. These candidate proteins may function at the intersection of cytoskeletal networks and signaling pathways to regulate the localization of Gab1 and ERK to lamellipodia.

The localization of phosphorylated ERK to lamellipodia may provide a signaling compartment from which a sustained signal can be maintained, that is essential for the early cell protrusions that occur during epithelial morphogenesis (O'Brien et al., 2004). The localization of Gab1 associated complexes to the protruding edge of motile cells indicates that Gab1 has a mechanistic role as a scaffolding protein to localize and recruit signaling proteins involved in cell extension and migration.

Acknowledgements

We would like to thank Dr. Vande Woude for HGF and the Genetics Institute for recombinant CSF-1. We are grateful to the members of the Park lab, past and present for helpful discussions. Funding for this research was supported by an operating grant from the National Cancer Institute of Canada (NCIC) with money from the Canadian Cancer Society to M.P., and a studentship from the Fonds de Recherche Scientifique du Quebec (FRSQ) to M. F. M.P. is a senior scientist of the CIHR.

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Chapter 3

The Gab1 scaffold is required for RTK signal polarization to dorsal ruffles

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Submitted manuscript

Preface

The subcellular localization of Gab1 via membrane targeting is an essential element of Gab1 for Met signals and biology. By observation of changes of localization of Gab1 in MDCK epithelial cells upon stimulation of the Met receptor with its ligand hepatocyte growth factor (HGF), we discovered that Gab1 quickly accumulates into membrane protrusions on the apical surface of cells into dorsal ruffles. This type of membrane protrusion differs from the lamellipodia described in the previous chapter. However the function of dorsal ruffles is poorly understood. In collaboration, Jasmine V. Abella and I provide the Gab1 dependent mechanistic requirements for dorsal ruffle formation, data that suggest that this is a specialized signalling micro-environment and also provide data that demonstrates that dorsal ruffles are a mechanism of receptor degradation.

Abstract

How signalling and biological response to the hepatocyte growth factor (HGF) receptor tyrosine kinase (RTK) Met are coupled to Met trafficking is largely unknown. The Gab1 scaffold protein modulates Met signals involved in cell dispersal and morphogenesis. We show that Gab1 is indispensable for a form of RTK-induced actin remodelling, called dorsal ruffles, in response to HGF, epidermal and platelet derived growth factors. Localization of Gab1 and activated Met to dorsal ruffles is accompanied by signalling proteins recruited to Gab1. Structure-function analysis demonstrates a requirement for Gab1-Crk complexes for dorsal ruffle formation. Gab1 induced dorsal ruffles promote a polarized signalling micro-environment from which Met is bulk internalized and degraded. Ablation of dorsal ruffles delays Met degradation, but diminishes biological responses. We demonstrate an essential role for Gab1 in dorsal ruffle formation by multiple RTKs and provide direct evidence that dorsal ruffles act as a biologically relevant signalling microenvironment and mechanism for receptor down-regulation.

Introduction

Hepatocyte growth factor and its receptor tyrosine kinase, Met, are potent activators of epithelial cell dispersal, morphogenesis and invasive growth (Birchmeier et al., 2003). This biological activity is principally due to the recruitment and phosphorylation of the scaffold protein Gab1 (Grb2-associated binder-1) by the Met receptor (Lock et al., 2002; Maroun et al., 1999b; Sachs et al., 2000; Weidner et al., 1996). Phosphorylation of Gab1 by Met, generates numerous phosphotyrosine docking sites for signalling molecules that includes the p85 subunit of PI3K, the Crk adaptor protein and the tyrosine phosphatase, Shp2 (Gual et al., 2000; Lamorte et al., 2002b; Maroun et al., 1999b). Dispersal of colonies of epithelial cells in response to HGF requires the breakdown of cell-cell junctions and reorganization of the actin cytoskeleton. These are associated with morphological changes in the plasma membrane including formation of ruffles and lamellipodia (Royal et al., 2000; Royal and Park, 1995). Unlike lamellipodia, ruffles are sheet-like membrane protrusions, which lack adherence to the substratum. Two distinctive forms of membrane ruffling events have been reported, peripheral ruffles and circular dorsal ruffles or waves (Abercrombie et al., 1970).

Stimulation of colonies of epithelial Madin-Darby Canine Kidney (MDCK) cells with HGF, promotes rapid formation of dorsal ruffles, which are F-actin rich circular membrane protrusions (Dowrick et al., 1993). Dorsal and peripheral ruffles form in response to stimulation by growth factors such as EGF, PDGF and HGF (Buccione et al., 2004; Dowrick et al., 1993). However, dorsal ruffles are distinct from peripheral ruffles in their temporal regulation and localization (Buccione et al., 2004) and peripheral ruffles do not appear to convert into dorsal ruffles (Araki et al., 2000). While peripheral ruffles

can occur continuously upon growth factor stimulation, and are required for macro-pinocytosis and cell migration (Ridley et al., 1992; Suetsugu et al., 2003), dorsal ruffles form only transiently within the first 20 minutes of stimulation (Buccione et al., 2004) and their specific function is poorly understood.

In response to growth factor stimulation, dorsal and peripheral ruffle formation is dependent on Rac activation (Krueger et al., 2003; Lanzetti et al., 2004; Palamidessi et al., 2008; Ridley et al., 1992; Suetsugu et al., 2003). Each utilize common signals involving the Arp2/3 complex, cortactin and palladin, which are required for actin polymerization, as well the GTPase dynamin (Goicoechea et al., 2006; Krueger et al., 2003; Liu et al., 2007; McNiven et al., 2000). Specific activation of the Arp2/3 complex in the context of dorsal ruffles can be mediated by Rac effectors, N-WASP and WAVE (Krueger et al., 2003; Suetsugu et al., 2003; Westphal et al., 2000), where WAVE-1 is required for dorsal, but not peripheral ruffle formation (Suetsugu et al., 2003). Over-expression of activated Rac alone is not sufficient to induce dorsal ruffle formation, although it can promote the formation of peripheral ruffles, indicating that proteins which regulate the localization of activated Rac such as the small GTPase Rab5 are critical (Lanzetti et al., 2004; Palamidessi et al., 2008). The serine/threonine kinase Pak1, an effector of Rac has also been demonstrated to induce dorsal ruffles downstream from the PDGF receptor (Dharmawardhane et al., 2000). Similarly, inhibitors of PI3K, Src and PLCγ disrupt dorsal ruffle formation in response to growth factors (Dharmawardhane et al., 1997; Mettlen et al., 2006; Veracini et al., 2006). However, how RTKs coordinate these processes is poorly understood.

Dorsal and peripheral ruffles formed in response to growth factors are thought to function as sites for macro-pinocytosis, meditating nutrient uptake as well as a mechanism to recycle plasma membrane and membrane components (Dharmawardhane et al., 2000; Dowrick et al., 1993; Jones, 2007; Mettlen et al., 2006). However, fibroblasts deficient for WAVE-1 protein, form peripheral, but not dorsal ruffles in response to PDGFRβ activation and still support macro-pinocytosis, indicating that dorsal ruffles are not essential (Suetsugu et al., 2003). In response to EGF, the EGFR localizes to dorsal ruffles and the subsequent internalization of the EGFR from these structures has lead to the proposal that dorsal ruffles may also provide a mode for bulk internalization of the EGFR (Orth et al., 2006). Internalization of RTKs into the endosomal trafficking compartment is required for their subsequent degradation in the lysosome and provides one of the major mechanisms to regulate RTK signalling, stability and hence biological activity. In general, this is mediated through the entry of RTKs through clathrin coated pits, although other mechanisms for RTK entry have been proposed (Mayor and Pagano, 2007). However, the possibility that dorsal ruffles may provide a specialized membrane micro-domain in which RTK signalling and activity are regulated remains poorly understood. Here we have addressed the molecular requirements for RTK induced dorsal ruffles and have analyzed their impact on receptor signalling and stability. We demonstrate that the Gab1 scaffold protein is a common requirement for RTK mediated dorsal ruffles. We show that Gab1 associated signalling molecules localize to these structures with activated Met receptors to form a biologically relevant signalling micro-environment which in turn modulates receptor down-regulation.

Materials and Methods

Reagents and Antibodies

Antibody 147 was raised against a carboxy-terminal peptide of the human Met protein (Maroun et al., 1999a; Rodrigues et al., 1991). Commercial antibodies, Met AF276, R&D Systems (Minneapolis, MN), Met clone 14G9, NanoTools (Teningen, Germany), Gab1 and cortactin, Upstate Biotechnology (Lake Placid, NY), Anti-ubiquitin (P4D1), actin, polyclonal EEA1 and p85, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), GFP antibody, Phalloidin Alexa-Fluor 488 and 546, and Alexa 488 and 555 conjugated secondary antibodies, Molecular Probes (Eugene, OR). Total and phospho-specific Erk1/2 (pThr202/pTyr204), pY1234/35 Met and pY992 EGFR, Cell Signalling Technology (Mississauga, ON), monoclonal EEA1 and Shp2, BD Biosciences (Mississauga, ON), HA.11 and Myc 9E10 monoclonal antibodies, Covance (Berkeley CA) and Babco (Richmond, CA). Gab1 siRNAs (Duplex1: CAGAUGUCUUGGAAUACUA; Duplex2: CGAACAUUUCCAGAAGGAA; Duplex3: GAGCGAACUGAUUCACAAA) and scrambled siRNA duplex (AllStars negative control # 1027281), Qiagen (Mississauga, Ontario, Canada). Cycloheximide and SITS (4-acetamido-4'-isothiocyabatostilbene-2'2-disulfonic acid) were purchased from Sigma, the former was reconstituted in water to 10mg/ml (100x) and the latter in DMSO to a concentration of 0.5mM (200x). HGF was a generous gift from Genentech (San Fransico, USA), EGF was purchased from Roche Diagnostics (Laval, Quebec, Canada) and PDGF-bb was purchased form Calbiochem (Mississauga, Ontario, Canada). The following constructs were described previously; pcDNA1.1 pcDNA1.1-HA-Gab1, pcDNA1.1-HA-Gab1ΔPH, GFP-Gab1 (Maroun et al., 1999b). PRK5myc vector, PRK5myc-Pak1 wt and

PRK5myc-Pak1 H83-86L were a generous gift from Dr. G. Bokoch (Dharmawardhane et al., 1997).

Cell culture and Transfections

Madin-Darby canine kidney (MDCK) and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). MDCK cell lines expressing, GFP-Gab1, GFP-Gab1ΔPH, HA-Gab1, HA-Gab1ΔCrk, HA-Gab1ΔShp2 and HA-Gab1Δp85 were established as described previously (Frigault et al., 2008; Lamorte et al., 2002a; Maroun et al., 1999a; Maroun et al., 2000). MDCK stable lines expressing human Met (MDCK Met (population) and MDCK Met/HA-Gab1 (clone 7A10)) were generated via calcium phosphate transfection of pXM Met. Transient transfections for siRNA duplexes in MDCK cells were performed using Hiperfect (Qiagen) according to manufacturer's instructions. Cells were consecutively transfected and experiments were performed 96 hours post-initial transfection. Transient transfection of HeLa cells was performed using Lipofectamine Plus (Invitrogen) reagent according to manufacturer's instructions. Wild-type and *Gab1*-null MEF were a generous gift from Dr. M. Holgado-Madruga and previously characterized (Holgado-Madruga and Wong, 2003). Transient expression of GFP-Gab1 in *Gab1*-null MEF cell lines was done by using Superfect (Quiagen) as per manufacturer's instructions.

Growth factor stimulation, Immuno-precipitation and Western blotting

HeLa and MDCK cells were harvested in TGH lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM

phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium vanadate, 10 μg/ml aprotinin and 10 μg/ml leupeptin). To detect Met receptor ubiquitination in HeLa cells, cells were lysed 24 hours post transfection in RIPA lysis buffer (0.05% SDS, 50mM Tris pH8.0, 150nM NaCl,1% Nonidet P-40, 0.05% sodium deoxycholate) supplemented with 1 mM PMSF, 1 mM sodium vanadate, 1mM sodium fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Cells harvested under boiling lysis conditions were lysed in 200 μl boiling buffer (2% SDS, 1 mM EDTA). Lysates were boiled for 10 min and diluted to 1 ml with a buffer containing 2.5% Triton, 12.5 mM Tris pH 7.5, 187.5 mM NaCl. For immunoprecipitation, lysates were incubated with the indicated antibody for 2 hours at 4°C with gentle rotation. Proteins collected on either protein A- or G-Sepharose were washed three times in their respective lysis buffers, resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in 3% BSA in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 2.5 mM EDTA, 0.1% Tween-20) for 1 hour, incubated with primary and secondary antibodies in TBST for 2 hours and 1 hour respectively. Met 147 antibody was used to detect Met in HeLa, MDCK Met and MDCK Met/HA-Gab1 cells and antibody 14G9 was used to detect endogenous Met in MDCK cells. After four washes with TBST, bound proteins were visualized with an ECL detection kit (Amersham Biosciences). Receptor degradation assays were carried out using HGF (0.46nM or 48ng/ml) in the presence of cycloheximide (100ng/ml). Where indicated, cells were pre-treated with DMSO or SITS (0.5mM) for 20 minutes prior to stimulation. MEF wt and *Gab1*-null were serum starved overnight and stimulated with EGF (5nM or 30ng/ml) or PDGF-bb (11nM or 10ng/ml) for 5 minutes. HeLa and MDCK cells were harvested in TGH lysis buffer, to detect Met receptor ubiquitination in HeLa

cells, cells were lysed 24 hours post transfection in RIPA lysis buffer (for details see supplemental Materials and Methods). Cells harvested under boiling lysis conditions were lysed in 200 µl boiling buffer (2% SDS, 1 mM EDTA). Lysates were boiled for 10 min and diluted to 1 ml with a buffer containing 2.5% Triton, 12.5 mM Tris pH 7.5, 187.5 mM NaCl. MEF wt and *Gab1*-null cell lines were lysed in RIPA lysis buffer. Densitometric analysis of western blots were performed using NIH Image J software. Where indicated, data from densitometric analysis was processed using Prism 4.9 to generate a one-phase exponential decay best fit curve in order to determine the receptor half-life ($t_{1/2}$) with an error using a 95% confidence interval.

Confocal Immuno-fluorescence microscopy

MDCK and HeLa cells were seeded at $2x10^4$ on glass cover slips (Bellco Glass Inc. Vineland, NJ) in 24 well plates (Nalgene NUNC, Rochester, NY) and 24 hours later stimulated with 0.46nM HGF or 5nM EGF. Where indicated, cells were pre-treated with DMSO or SITS (0.5mM) for 20 minutes prior to growth factor stimulation. Wild-type and *Gab1*-null MEF were seeded at 1 $x10^4$ on glass cover slips and 24 hours later were serum starved for 16 hours prior to stimulation with 5nM EGF or 11nM PDGF-bb. To study Met trafficking, MDCK and HeLa cells were serum starved for 2 hours in the presence of cycloheximide prior to HGF stimulation. Cover slips were washed twice with PBS, fixed with 2% paraformaldehyde (PFA, Fisher Scientific). Staining procedures have previously been described in (Abella et al., 2005). Images were taken using a confocal laser scanning microscope model Zeiss 510 Meta (Carl Zeiss, Canada Ltd, Toronto, ON) with 100X or 40X objective. Image analysis was carried out using the LSM 5 image

browser (Empix Imaging, Mississauga, ON). Live cell imaging was performed either using a Zeiss 510 Meta laser scanning confocal microscope or with Spinning disk confocal microscope from Quorum Technologies. Image analysis from data acquired using the LSM510 microscope was performed on LSM 5 image browser. Data from the spinning disk microscopy was analysed using Volocity 4.1 software.

Dorsal Ruffle Assays

Cells plated on coverslips were stimulated with growth factor for 5 minutes, fixed in 2% PFA and stained with Phalloidin Alexa-Fluor 488 or 546. Using a confocal microscope with a 40x objective, the number of cells which formed dorsal ruffles were counted and represented as percent of the total number of cells counted. At least 10 fields of view were counted for each experiment. For Gab1 siRNA experiments, the number of cells forming dorsal ruffles was scored from a minimum of 12 different fields of view (>650 cells) were scored and represented in a histogram as fold change in dorsal ruffle formation compared to mock transfected cells.

Results

HGF induced dorsal ruffles form in cell colonies and contain Met signalling complexes.

Regulators of dorsal ruffle formation downstream of growth factor receptor tyrosine kinases have been characterized in fibroblasts or tumor cells that grow as single cells (Krueger et al., 2003; Lanzetti et al., 2004; Orth et al., 2006; Suetsugu et al., 2003). To gain insight into the biological consequence of dorsal ruffles, we examined the regulation of dorsal ruffle formation and their requirement for signalling and biological response downstream from the Met receptor in colonies of well-polarized epithelial sheets, which reflect a more physiological environment. Stimulation of colonies of MDCK cells with physiological concentrations of HGF (Funakoshi and Nakamura, 2003), induces the formation of dorsal ruffles in cells throughout the colony; visualized as concentric actin rich rings on the apical cell surface (Figures 1A, solid arrows and S1A) (Dowrick et al., 1993). Indirect immuno-fluorescence reveals that Met and the Met substrate, Gab1, are present on HGF induced dorsal ruffles (Figure 1B-C). Live cell imaging of MDCK cells over-expressing GFP tagged Gab1 (MDCK GFP-Gab1), demonstrates that in response to HGF, Gab1 is rapidly recruited to dorsal ruffles, where each dorsal ruffle persists for approximately 8 minutes. These structures can be initiated as early as 1 minute post stimulation, and can continue to form up to 20 minutes after HGF treatment, in cells both within and at the edge of a colony (data from live cell experiments, and not shown here). Notably, each cell within the colony can produce more than one dorsal ruffle at any time (Figure 1A, solid arrows), and these can occur

independently of the ability of the cells to form lamellipodia (Figure 1A, dashed arrow), which occur only at the edge of a colony.

The prolonged localization of both Met and Gab1 in dorsal ruffles (up to 8 minutes), suggests that these structures are an active signalling microenvironment in which Gab1 can couple activated Met receptors with downstream signalling molecules. Using phospho-specific antibodies that reflect activation of the Met receptor (pY1234/35), we show the presence of activated Met and Gab1 in dorsal ruffles (Figure 1C). Signalling molecules recruited to Gab1 following Met activation, including phospho-ERK1/2, Crk, Shp2 and p85, also localize to dorsal ruffles in response to HGF stimulation consistent with this structure reflecting an active signalling compartment (Figures 1C-D and S1B-D).

Gab1 is required for, and enhances HGF dependent dorsal ruffle formation.

To determine if Gab1 dependent functions contribute to the formation of dorsal ruffles we examined the consequence of Gab1 knock down using siRNA, as well Gab1 over-expression. Using three independent siRNA duplexes to knock down Gab1, we show a statistically significant decrease (*) in the formation of dorsal ruffles in MDCK cells in response to HGF. Up to 4 fold fewer dorsal ruffles were observed with a knock down of 62% in Gab1 protein levels (Figure 2A and S1E-F). In contrast, over-expression of Gab1 in MDCK cells increases the incidence of dorsal ruffle formation in response to HGF by more than two fold, where approximately 60% of MDCK GFP-Gab1 cells form dorsal ruffles when compared to approximately 25% of wild-type MDCK cells at any one time (Figures 2B-D). Notably, in HeLa cells, which do not readily form circular dorsal ruffles in response to low levels of HGF (Figure 2E, cell denoted by *), transient over-

expression of Gab1 promotes the formation of dorsal ruffles in response to HGF (Figure 2E). Together, these results demonstrate that Gab1 dependent signals mediate dorsal ruffle formation downstream from the Met receptor.

Gab1 is essential for dorsal ruffles downstream from EGF and PDGF receptors.

To examine whether Gab1 is required for dorsal ruffle formation downstream from other RTKs which also phosphorylate Gab1 (Gu and Neel, 2003), we determined the ability of mouse embryonic fibroblast (MEF) cells, isolated from *Gab1* knock out or wild-type embryos, to form dorsal ruffles (Holgado-Madruga and Wong, 2003) (Figure S2A). Stimulation of wild-type (wt) MEF cells with either EGF or PDGF promoted dorsal ruffle formation as visualized by staining for Actin and cortactin, established markers for dorsal ruffles (McNiven et al., 2000) (Figure 3A). In response to PDGF, approximately 40% of wt MEF cells form dorsal ruffles whereas only 15% (7C) and 10% (2B) of cells null for *Gab1* form dorsal ruffles (Figures 3A-B). Similarly, EGF induced dorsal ruffle formation was drastically impaired in the absence of Gab1, where approximately 30% of wt MEF cells form dorsal ruffles in response to EGF whereas only 8% (7C) and 7% (2B) of *Gab1*-null MEF cells form EGF dependent dorsal ruffles (Figures 3A-B). Importantly, stable re-expression of GFP-Gab1 in *Gab1*-null MEF cells rescued the dorsal ruffle response to both EGF and PDGF (Figure 3C). In addition, expression of Gab1 in HeLa cells promoted EGF induced dorsal ruffles (Figure S2B). Together, this demonstrates that Gab1 is required for dorsal ruffle formation downstream from the HGF, EGF and PDGF receptors.

A Gab1-Crk complex is required for Gab1-dependent dorsal ruffle formation.

Having established that Gab1 is required for dorsal ruffle formation downstream from multiple RTKs, we sought to determine the molecular requirements for this Gab1 function. To this end, we employed a structure-function approach in HeLa cells, using Gab1 constructs impaired in their recruitment of different signalling molecules (Lamorte et al., 2002b; Maroun et al., 1999b; Maroun et al., 2000). We show that Gab1 mutants unable to recruit the adaptor molecule Crk, or that lack the pleckstrin homology (PH) domain, fail to induce dorsal ruffle formation in response to HGF stimulation in the presence of serum, whereas mutants lacking recruitment of the p85 subunit of PI3K or Shp2 promote dorsal ruffles to a similar extent as wt Gab1 (Figures 4A and S2C). The Gab1ΔPH mutant is readily phosphorylated in response to HGF, but fails to localize to PIP_3 rich membrane micro-domains in response to HGF (Maroun et al., 1999a), indicating both a requirement for Gab1 subcellular localization as well as recruitment of Crk for its ability to promote dorsal ruffles.

Although multiple Gab1 binding proteins are recruited to dorsal ruffles, generating a unique signalling microenvironment, only the Gab1-Crk interaction is required for the induction of dorsal ruffles. MDCK cells stably over-expressing a Gab1ΔCrk mutant, were impaired by at least 90% in their ability to form dorsal ruffles when compared to cells expressing either Gab1, Gab1Δp85 or Gab1ΔShp2 (Figure 4C), suggesting that the Gab1ΔCrk mutant, which is efficiently recruited and phosphorylated by the Met receptor, interferes with recruitment of endogenous Gab1 to Met (Lamorte et al., 2002b). Over-expression of CrkII alone however, was not sufficient to enhance dorsal ruffle formation in MDCK cells, demonstrating a specific requirement for Gab1-Crk

interaction as well as Gab1-PIP_3 membrane associations, for Met induced dorsal ruffle formation (Figures 4D and S2D).

Increased dorsal ruffles enhance HGF induced Met receptor degradation.

One of the predominant mechanisms of RTK down-regulation is mediated through ligand induced receptor internalization into the endocytic pathway, leading to lysosomal degradation of the receptor (Wiley and Burke, 2001). Concentration of RTKs in dorsal ruffles has been proposed to allow bulk internalization of activated receptors following ligand stimulation (Orth et al., 2006), however this has not been addressed biochemically. To determine if HGF-dependent Met down-regulation is altered by dorsal ruffle formation, we measured the steady state levels of Met post HGF stimulation in MDCK cells, with and without Gab1 over-expression. Notably, in MDCK cells over-expressing Gab1, which form an increased number of dorsal ruffles, degradation of the Met RTK is enhanced significantly (Figure 5A). The half-life of Met was 24.3±2.1 minutes in cells that over-express Gab1, when compared to 71.5±5.8 minutes in MDCK control cells. Enhanced Met degradation requires HGF stimulation, as increasing levels of Gab1 over-expression is not sufficient to induce Met degradation alone (Figure S3A). This implies that Met is efficiently endocytosed from the dorsal ruffle microenvironment to a degradative compartment (Figure 5B). Consistent with this, Met positive puncta are observed on the dorsal ruffle membrane at early time points and at the base of the ruffle at later time points (Figure 1B). In addition, rapid trafficking of Met positive endosomes to a peri-nuclear compartment was observed in response to HGF in Gab1 over-expressing MDCK cells when compared to control cells (Figure 5C). Efficient degradation of the

Met receptor is associated with its ubiquitination (Abella et al., 2005). However, using anti-ubiquitin antibodies, we observe no increase in Met ubiquitination in Gab1 over-expressing cells when compared to vector controls, indicating that the enhanced rate of degradation was not as a result of increased Met ubiquitination (Figure 5D), nor does it reflect targeting Met to a triton insoluble compartment (Figures S3B) (Urbe et al., 2003). Moreover, Gab1 does not traffic with the Met receptor on endosomes, but instead remains at the plasma membrane (Figure S3C), demonstrating that Gab1 does not directly recruit Met through the endocytic pathway, but instead promotes the formation of HGF dependent dorsal ruffles from which Met internalizes.

Disruption of dorsal ruffle formation delays Met receptor degradation and alters HGF induced biological responses.

The localization of Met to the dorsal ruffle micro-domain may be coupled to efficient bulk internalization of the Met receptor. To test if rapid degradation of Met is coupled to the formation of Gab1 dependent dorsal ruffles, we evaluated Met degradation in cells over-expressing the Gab1ΔCrk mutant that fail to promote dorsal ruffles. Using three stable clones of MDCK cells over-expressing Gab1ΔCrk, we show that the initial rate of Met receptor degradation is significantly delayed when compared to MDCK cells over-expressing Gab1. Under steady state conditions, Met levels decrease by 60% within the first 30 minutes of HGF stimulation in cells expressing wt Gab1, whereas, levels of the Met receptor decrease by only 30% in MDCK cells over-expressing Gab1ΔCrk or in MDCK control cells (Figures 6A and S3D). Similarly, Met is degraded more rapidly in HeLa cells over-expressing Gab1 that form dorsal ruffles, when compared to control

HeLa cells (Figures 6B-C), or cells expressing a Gab1ΔPH mutant which fails to induce dorsal ruffles (Figures 6B-C and S2C). Hence, these data demonstrate that recruitment of Met to Gab1-dependent dorsal ruffles facilitates rapid Met receptor degradation.

To establish if enhanced Met degradation is coupled to Gab1 over-expression and/or dorsal ruffle formation, we sought to inhibit dorsal ruffle formation without decreasing Gab1 levels and conversely to promote dorsal ruffle formation by an alternative mechanism utilizing the Pak1 kinase. The stilbene drug SITS (4-acetamido-4'-isothiocyabatostilbene-2'2-disulfonic acid), which inhibits the Na^+ independent Cl^-/HCO^- ion exchanger, inhibits dorsal ruffle formation in MDCK cells (Dowrick et al., 1993). Pre-treatment of Gab1 over-expressing MDCK cells with SITS, significantly reduces HGF induced dorsal ruffle formation (8 fold, Figures 6D and S4B) yet peripheral ruffles and lamellipodia still form under these conditions (Figure 6E). We observe a reduction in scatter of colonies of MDCK cells over-expressing Gab1 treated with SITS (Figure 6F) supporting previous observations that SITS treatment diminished HGF induced cell scatter in MDCK cells (Dowrick et al., 1993). SITS does not interfere with Met phosphorylation in MDCK GFP-Gab1 cells in response to HGF (Figure 6G). Hence, the decreased biological response is not due to decreased Met activation. However, in the presence of SITS, we observe a delay in HGF induced degradation of Met by approximately 2 hours (Figure 6G). This correlates with a delay in the peri-nuclear localization of Met (Figure S4C). In contrast, SITS treatment had no effect on Met degradation and trafficking in HeLa cells, which do not readily form dorsal ruffles in response to HGF (Figures S4D-E). Since SITS is not a general inhibitor of Met phosphorylation or trafficking, we conclude that the ablation of dorsal ruffles in the

presence of SITS, results in an impaired biological response. This is consistent with our previous observations that the Gab1ΔCrk mutant, which is deficient for the induction of dorsal ruffles, also fails to promote cell scatter downstream from Met (Lamorte et al., 2002a).

To establish if enhanced Met degradation was specific only to dorsal ruffles induced following Gab1 over-expression, or was linked to the localization of Met to dorsal ruffles, we examined Met stability in HeLa cells where dorsal ruffles are induced by Pak1. A Pak1 mutant, which is impaired in its auto-inhibition ability (H83-86L) promotes robust dorsal ruffle formation in response to PDGF (Dharmawardhane et al., 1997). When expressed in HeLa cells, Pak1 H83-86L induces dorsal ruffles in response to HGF (Figure 7A). Immuno-staining for endogenous Met revealed that Met was present on the dorsal ruffle membrane (Figure 7A). Importantly, Met was more rapidly degraded in Pak1 H83-86L expressing cells which form dorsal ruffles in response to HGF when compared to vector control cells, which do not form dorsal ruffles (Figure 7B-C). Taken together, these data support that induction of dorsal ruffles per se, in an HGF dependent manner, promotes efficient Met receptor degradation.

Figure 1. The Met receptor and the Gab1 scaffolding protein are recruited to dorsal ruffles. (A) HGF induces peripheral and dorsal ruffle formation in MDCK cells. MDCK cells were stimulated or not with HGF for 5 minutes, fixed and stained with Phalloidin-Alexa Fluor 488 and DAPI. Solid arrows delineate dorsal ruffles and dashed arrows peripheral ruffles. (B) The Met receptor is recruited to dorsal ruffles in MDCK cells. MDCK Met cells were stimulated or not with HGF for 5 minutes, fixed and stained for Met (red) and Phalloidin-Alexa Fluor 488. Bottom panel is a Y-Z section plane through the dorsal ruffle. MDCK Met/HA-Gab1 cells were stimulated or not with HGF for 5 minutes fixed and stained for (C) phospho-Met (pY1234/35) (red) or (D) for phospho-Erk1/2 (red) and HA (green). Confocal images were taken with a 100x objective. Bar represents 10μm.

Figure 1

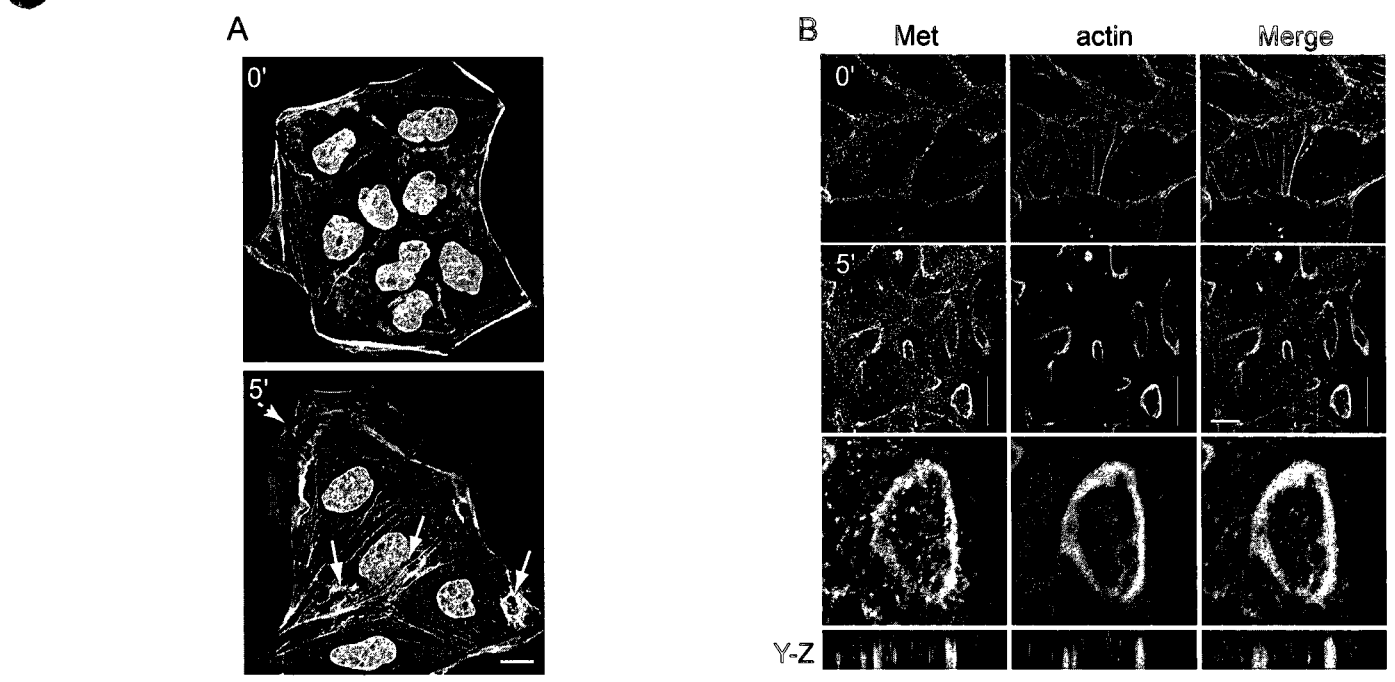
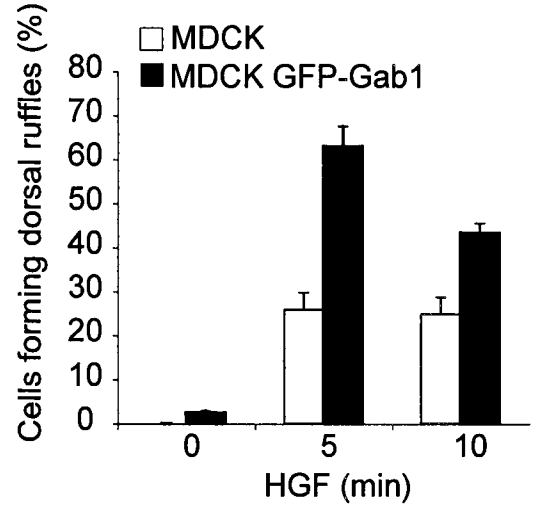

A
0'
5'
B
Met
actin
Merge
0'
5'
Y-Z
C
HA-Gab1
pY1234/35 Met
Merge
0'
5'
D
HA-Gab1
Phospho
Merge
0'
5'

Figure 2. Gab1 regulates dorsal ruffle formation downstream of the Met receptor. (A) MDCK cells, mock, or transfected with scrambled siRNA or three different siRNA duplexes targeting Gab1 were assayed for their ability to make dorsal ruffles. Gab1 protein knock down is shown in inset by western blot analysis 96hrs post transfection. The graph is a representative of three independent experiments. Significant decreases in dorsal ruffle formation are denoted by p-values, where no significant differences were observed in the scramble condition as compared to mock. (B) MDCK and MDCK GFP-Gab1 were stimulated with HGF for 5 minutes, fixed and stained with Phalloidin Alexa-Fluor 546. (C) Protein extracts from MDCK and MDCK GFP-Gab1 cells were separated by SDS PAGE and immuno-blotted for Gab1, GFP and actin to determine the extent of Gab1 expression. (D) Cells forming dorsal ruffles were scored from 10 individual fields in MDCK (□) and MDCK GFP-Gab1 (■) cells at 0, 5 and 10 minutes post HGF stimulation. Values represent the mean + S.E.M. of three separate experiments. (E) HeLa cells transiently transfected with HA-Gab1 were stimulated or not with HGF, fixed and stained for HA (red) to detect transfected cells and Phalloidin-Alexa Fluor 488 to detect the presence of dorsal ruffles (* denotes un-transfected cell).

D


MDCK
MDCK GFP-Gab1
Cells forming dorsal ruffles (%)
80
70
60
50
40
30
20
10
0
0
5
10
HGF (min)

E

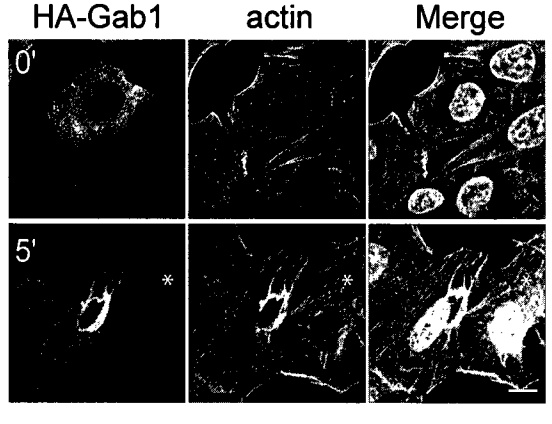

HA-Gab1
actin
Merge
0'
5'
*
*

Figure 2

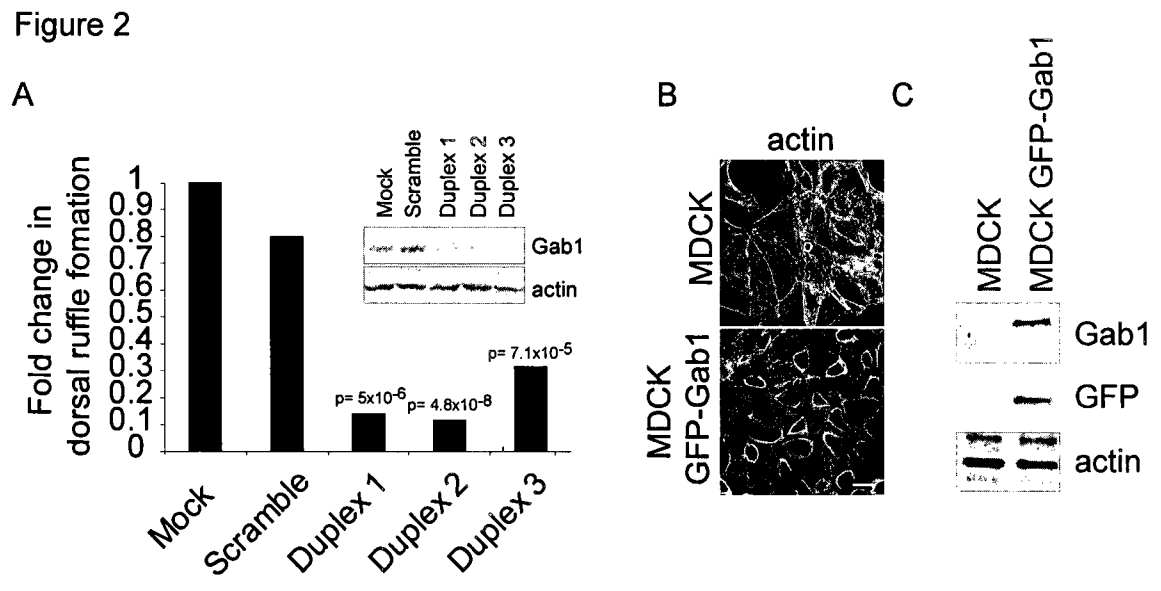

A
B
C
Fold change in dorsal ruffle formation
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Mock
Scramble
Duplex 1
Duplex 2
Duplex 3
p= 5x10-6
p= 4.8x10-8
p= 7.1x10-5
Gab1
actin
actin
MDCK
MDCK GFP-Gab1
MDCK
GFP-Gab1
MDCK
MDCK GFP-Gab1
Gab1
GFP
actin

Figure 3. Gab1 is required for dorsal ruffle formation downstream from the EGF and PDGF receptors.

(A) The ability of wild-type MEF (6B) and two clones of MEF cells from *Gab1*-null animals (7C and 2B) to form dorsal ruffles in response to EGF and PDGF-bb was examined. Cells were serum starved, and stimulated for 5 minutes with indicated ligand. Dorsal ruffle formation is scored based on co-staining with cortactin (red) and Phalloidin-Alexa Fluor 488. (B) The percentage of cells capable of forming dorsal ruffles was scored from 10 individual fields in the un-stimulated condition (□) PDGF (■) or EGF (■). Values represent the mean + S.E.M. of three independent experiments. (C) Re-expression of Gab1 in *Gab1*-null MEF rescues dorsal ruffles formation. Both clones of *Gab1*-null MEF were transfected with GFP-Gab1 for 24 hours, starved and stimulated for 5 minutes with indicated ligand. The actin cytoskeleton is visualized with Phalloidin-Alexa Fluor 546 and representative pictures are shown of MEF 7C. All scale bars represent 10μm.

Figure 3

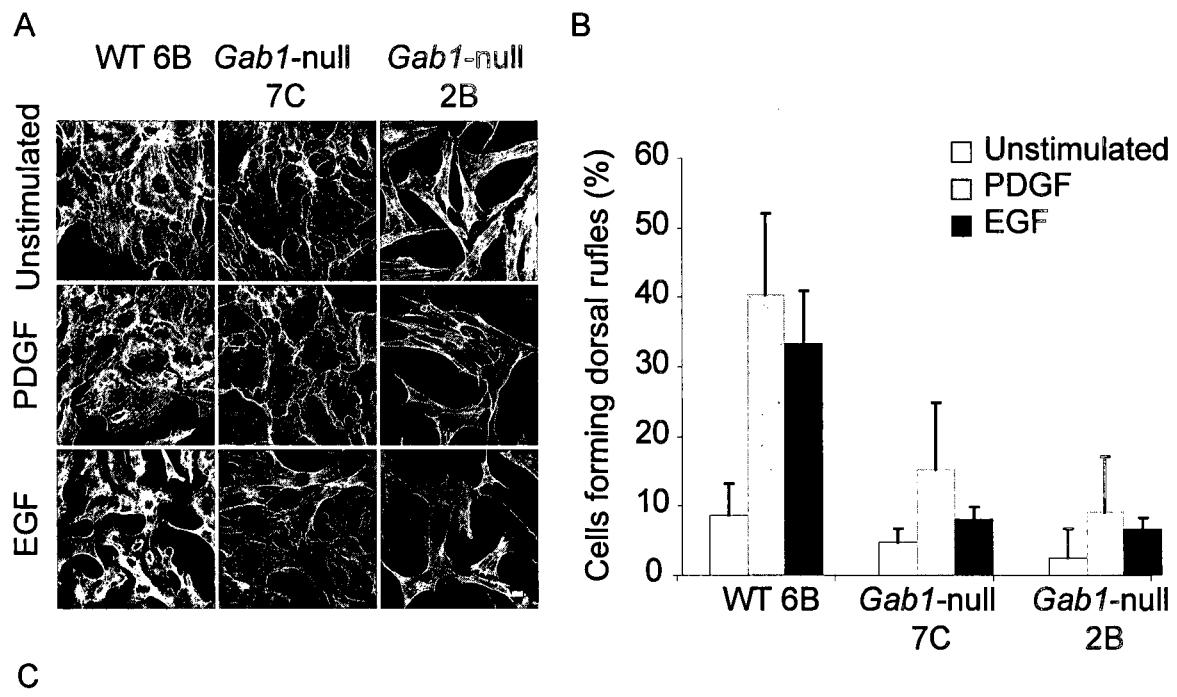

A
WT 6B
Gab1-null 7C
Gab1-null 2B
Unstimulated
PDGF
EGF
B
Cells forming dorsal ruffles (%)
0
10
20
30
40
50
60
Unstimulated
PDGF
EGF
WT 6B
Gab1-null 7C
Gab1-null 2B
C
GFP-Gab1
actin
Merge
Inset
PDGF
EGF

Figure 4. A Gab1-Crk complex is required for dorsal ruffle formation downstream of Met.

(A) HeLa cells transfected with HA-tagged Gab1 or Gab1 mutants (Gab1ΔShp2, Gab1Δp85, or Gab1ΔCrk) were stimulated with HGF for 5 minutes, fixed and stained for HA (red) and Phalloidin-Alexa Fluor 488 to detect dorsal ruffles. Representative images from four separate experiments are shown. Inset in each channel are enlargements of the area denoted in the corresponding merge image. (B) MDCK cells, or MDCK cell lines that stably express HA-tagged Gab1 wt, or Gab1 mutants were left un-stimulated or stimulated for 5 minutes with HGF, fixed stained with Phalloidin-Alexa Fluor 488. Representative confocal images of the most apical z-section from stimulated samples are shown. (C) The percentage of cells which form dorsal ruffles in (B) was scored where cells were left un-stimulated (□) or treated for 5 minutes with HGF (■) and the average response is plotted + S.E.M. (D) Two MDCK cell lines over-expressing CrkII (B1 and C9) were quantified for their ability to form dorsal ruffles without stimulation (□) or following 5 minutes with HGF (■) and the average response plotted + S.E.M. Inset represents protein levels of CrkII in the cell lines used. All scale bars represent 10μm.

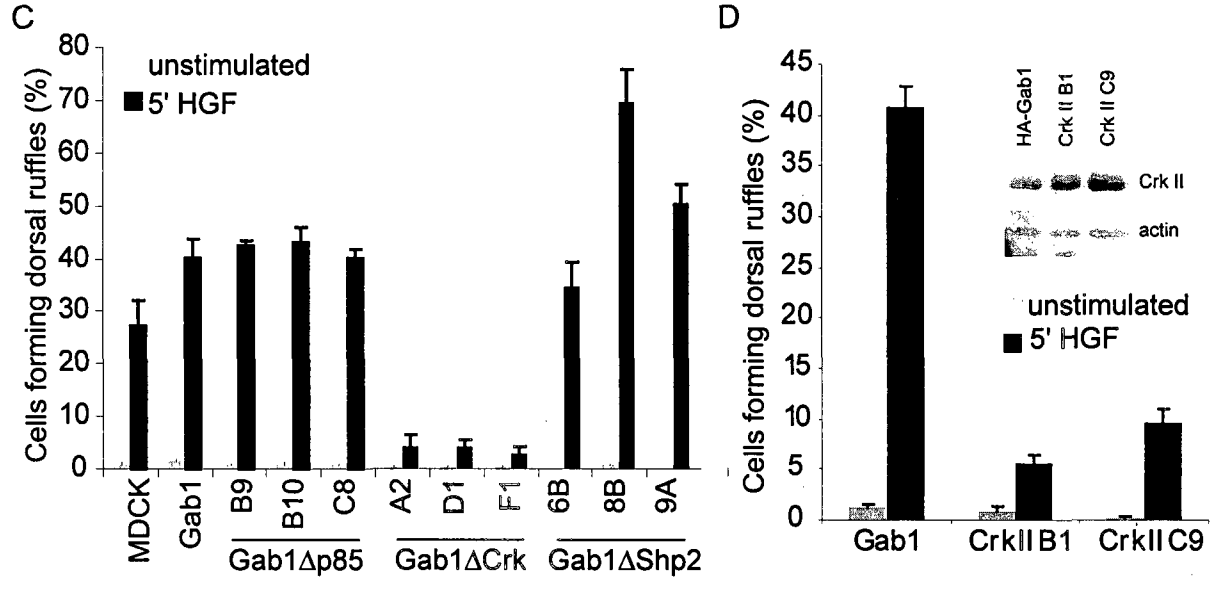

C
unstimulated
5' HGF
Cells forming dorsal ruffles (%)
0
10
20
30
40
50
60
70
80
MDCK
Gab1
B9
B10
C8
A2
D1
F1
6B
8B
9A
Gab1Δp85
Gab1ΔCrk
Gab1ΔShp2
D
Cells forming dorsal ruffles (%)
0
5
10
15
20
25
30
35
40
45
HA-Gab1
Crk II B1
Crk II C9
Crk II
actin
unstimulated
5' HGF
Gab1
CrkII B1
CrkII C9

Figure 4

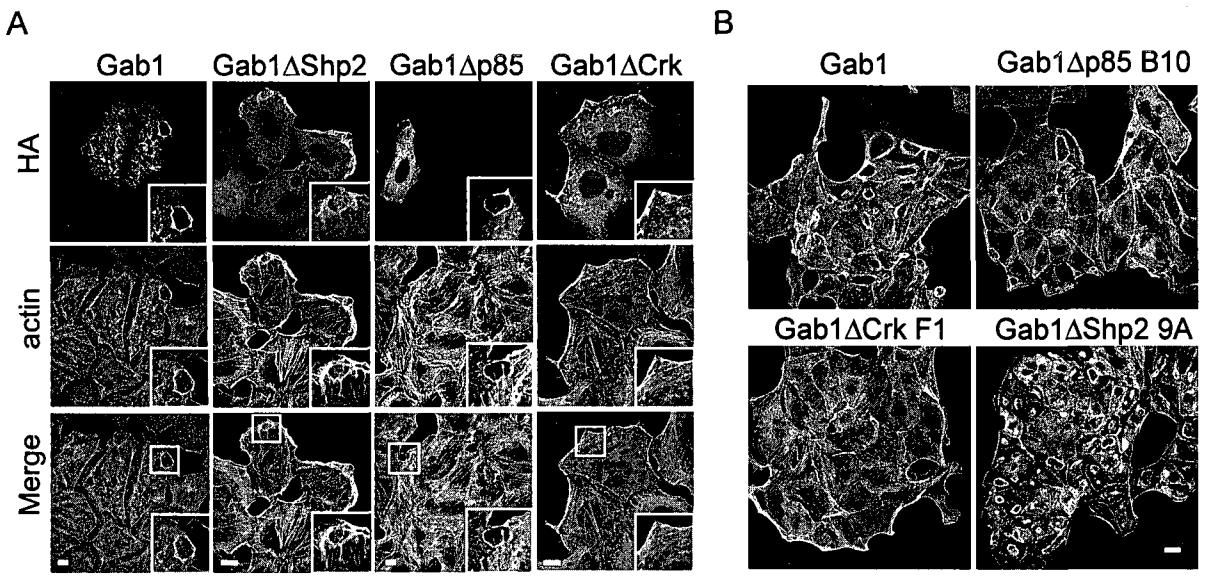

A
Gab1
Gab1ΔShp2
Gab1Δp85
Gab1ΔCrk
HA
actin
Merge
B
Gab1
Gab1Δp85 B10
Gab1ΔCrk F1
Gab1ΔShp2 9A

Figure 5. Dorsal ruffle formation enhances Met receptor degradation.

(A) MDCK and MDCK HA-Gab1 expressing cells were stimulated with HGF for the indicated times. Proteins from cell extracts were separated by SDS PAGE and immuno-blotted for Met, HA-Gab1 and actin. (B) Densitometric analysis of Met degradation as percentage of initial receptor remaining after HGF stimulation +/- S.E.M. as in (A) from three independent experiments was used to generate a best fit one phase decay curve to determine the half life ($t_{1/2}$) of the receptor. (C) MDCK Met and MDCK Met/HA-Gab1 cells were serum starved in the presence of cycloheximide, stimulated with HGF for the indicated time points, fixed and stained for Met (red), EEA1 (green) and DAPI (blue). Images were acquired by CLSM with 100x objective and the outline of the cells in the stimulated conditions, were drawn using the DIC image as a guide. (D) HeLa cells transiently transfected with vector or increasing amounts of HA-Gab1, were stimulated or not with HGF for 5 minutes and lysed in RIPA buffer. Met protein was immuno-precipitated, separated by SDS PAGE and immuno-blotted for ubiquitin, stripped and re-probed for Met (147). Total cell lysates were immuno-blotted for HA-Gab1.

Figure 5

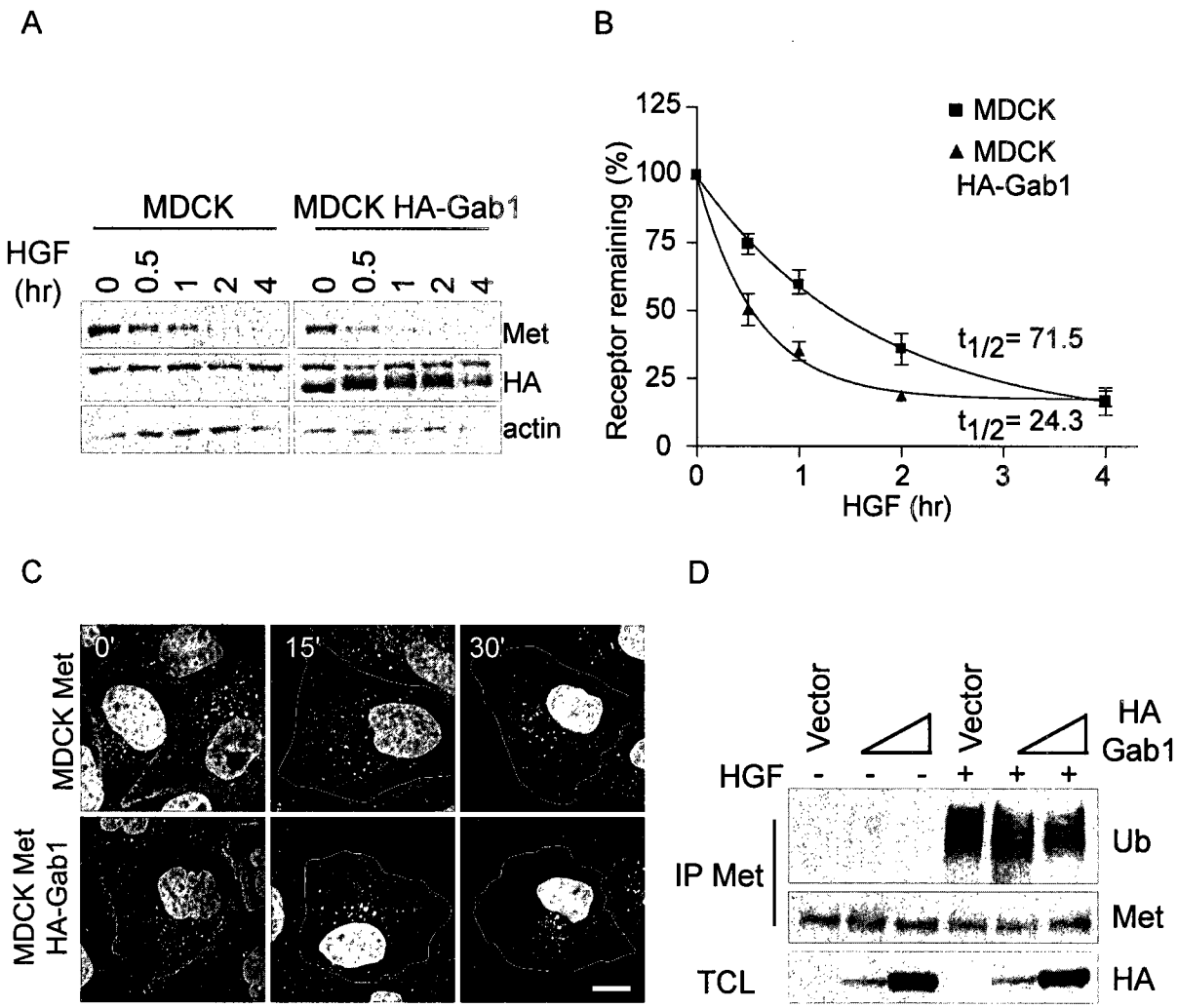

A
MDCK
MDCK HA-Gab1
HGF (hr)
0
0.5
1
2
4
Met
HA
actin
B
MDCK
MDCK HA-Gab1
Receptor remaining (%)
125
100
75
50
25
0
$t_{1/2}$= 71.5
$t_{1/2}$= 24.3
HGF (hr)
C
MDCK Met
MDCK Met HA-Gab1
0'
15'
30'
D
Vector
Vector
HA Gab1
HGF
IP Met
Ub
Met
TCL
HA

Figure 6. Dorsal ruffles are required for Met down-regulation and biological activity. (A) MDCK, MDCK HA-Gab1 cells, and three cell lines expressing HA-Gab1ΔCrk were stimulated with HGF for the indicated times. Densitometric analysis from three independent experiments is presented as percentage of initial receptor remaining after HGF stimulation +/- S.E.M. (B) HeLa cells were transiently transfected with vector, HA-Gab1 or HA-Gab1ΔPH, and 24 hours later stimulated with HGF for indicated times. Proteins from cell lysates were separated by SDS PAGE and immuno-blotted for Met, HA-Gab1 and actin. (C) Densitometric analysis of Met degradation presented as percentage of initial receptor remaining after HGF stimulation, +/- S.E.M. as in (B) from three independent experiments. (D) MDCK GFP-Gab1 cells were pre-treated with DMSO or 0.5mM SITS and stimulated with HGF. The number of cells forming dorsal ruffles was scored from 10 fields of view at 0, 5, 10 and 20 minutes post HGF stimulation, represented as the percentage of cells forming dorsal ruffles over time with DMSO (■) or SITS (□) treatment. (E) MDCK GFP Gab1 cells were pre-treated with 0.5mM SITS then stimulated with HGF and imaged under live conditions. Confocal images of different cells taken with a 100X objective are shown at the indicated time points. The outline of the cells was drawn using the DIC images as a guide. Scale bar represents 10μm. (F) MDCK GFP-Gab1 cells were pre-treated with DMSO or 0.5mM SITS and stimulated or not with HGF. Phase contrast images were taken 24 hours post stimulation. Scale bar represents 100μm. (G) MDCK GFP-Gab1 cells were pre-treated with DMSO or 0.5mM SITS and stimulated with HGF for the indicated times. Cell lysates were immuno-blotted for endogenous Met receptor, pY1234/35 Met, GFP and actin.

Figure 6

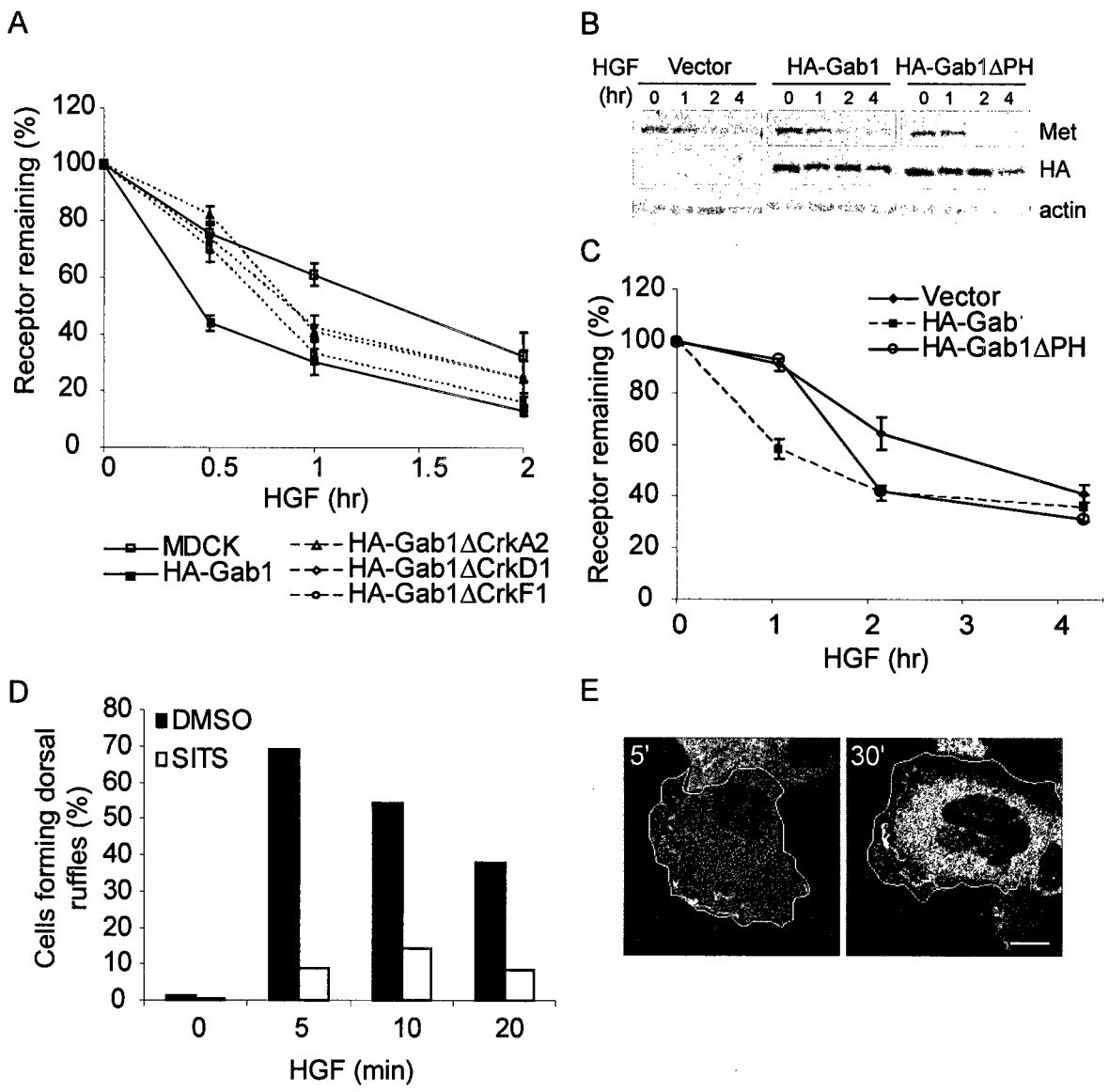

A
Receptor remaining (%)
HGF (hr)
MDCK
HA-Gab1
HA-Gab1ΔCrkA2
HA-Gab1ΔCrkD1
HA-Gab1ΔCrkF1
B
HGF
(hr)
Vector
HA-Gab1
HA-Gab1ΔPH
Met
HA
actin
C
Receptor remaining (%)
HGF (hr)
Vector
HA-Gab1
HA-Gab1ΔPH
D
Cells forming dorsal ruffles (%)
DMSO
SITS
HGF (min)
E
5'
30'
F
DMSO
DMSO +HGF
SITS +HGF
G
DMSO
SITS
HGF (min)
Met
pY1234/35
GFP
actin

Figure 7. Pak1 mediated dorsal ruffles enhance Met receptor degradation.

(A) HeLa cells transiently transfected with myc-Pak1 H83-86L were stimulated or not with HGF for 15 minutes, fixed and stained for endogenous Met receptor (red) and myc (green). (B) HeLa cells transiently transfected with vector or myc-Pak1 H83-86L, were stimulated with HGF for indicated times. Proteins from cell lysates were separated by SDS PAGE and immuno-blotted for endogenous Met receptor, myc-Pak1 and actin. (C) Densitometric analysis of Met degradation presented as a percentage of initial receptor remaining after HGF stimulation as in (B) +/- S.E.M. from three independent experiments.

Figure 7

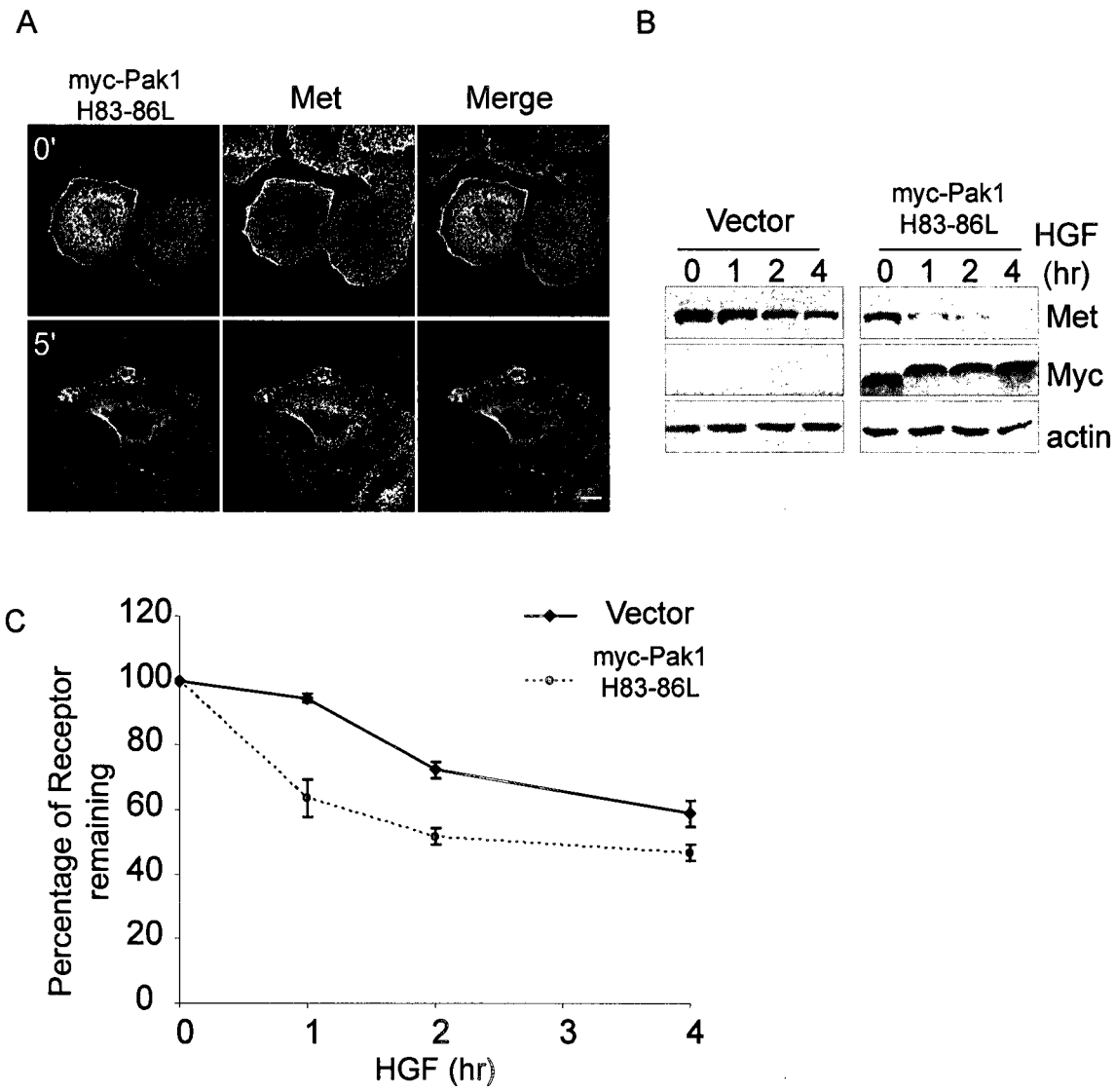
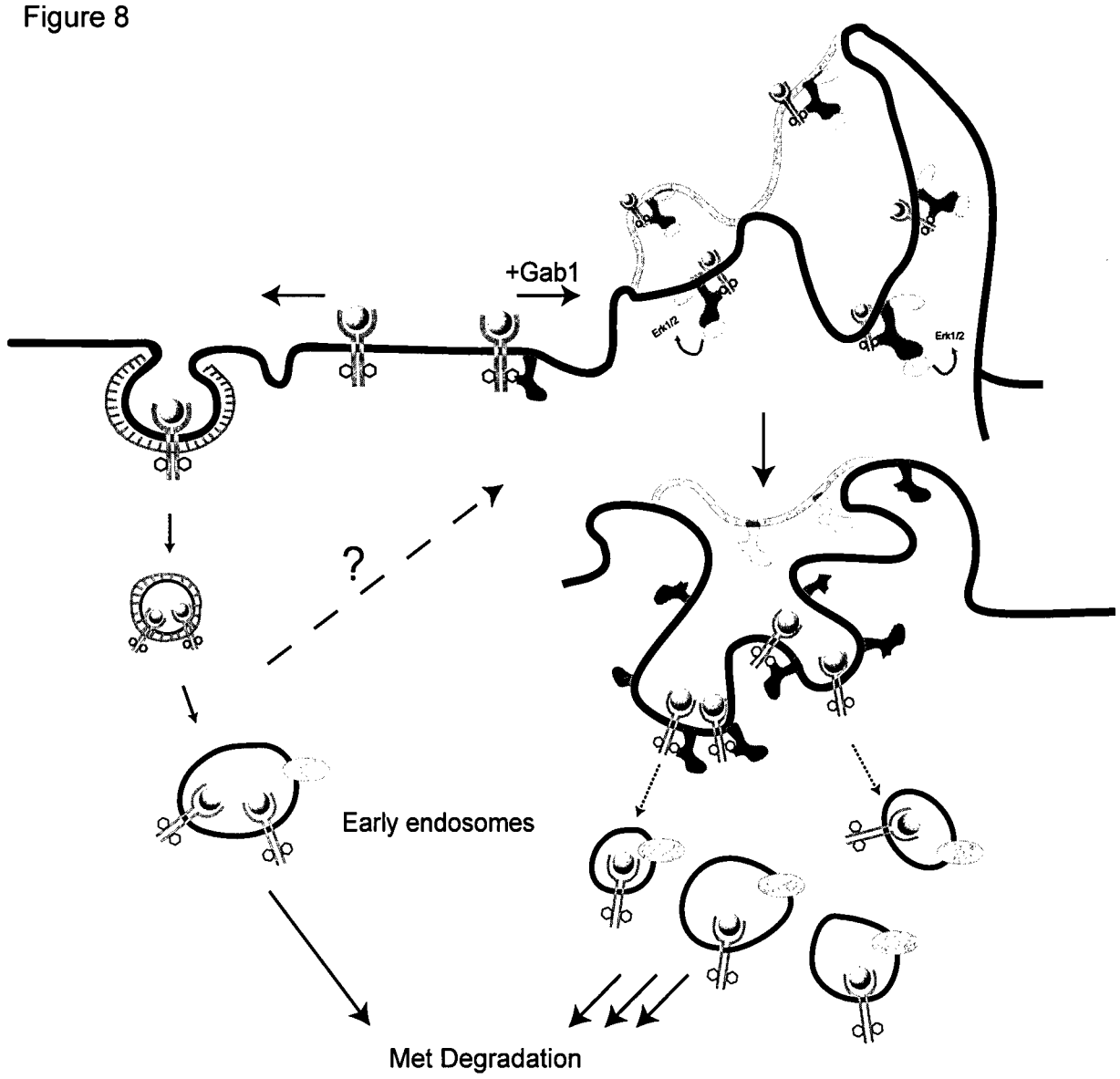

A
myc-Pak1
H83-86L
Met
Merge
0'
5'
B
Vector
myc-Pak1
H83-86L
HGF
0 1 2 4
0 1 2 4
(hr)
Met
Myc
actin
C
Vector
myc-Pak1
H83-86L
Percentage of Receptor remaining
120
100
80
60
40
20
0
0
1
2
3
4
HGF (hr)

Figure 8. Gab1 promotes RTK induced dorsal ruffles which mediate receptor signalling and more efficient down-regulation.

Gab1 switches Met internalisation from clathrin mediated endocytosis to dorsal ruffles upon HGF stimulation. Here, activated Met receptors localize with Gab1 and Gab1 recruited signalling molecules (p85, Crk and Shp2). This microenvironment induces local activation of Erk1/2. Upon collapse of dorsal ruffles, Met receptors are internalized and traffic to a peri-nuclear compartment where Met receptor degradation is more efficient downstream from dorsal ruffles. Importantly, the Gab1 scaffold remains at the plasma membrane. It is not clear whether Met receptors internalized through clathrin coated pits can be recycled to membranes where dorsal ruffles form. EEA1 (Early endosomal antigen 1).

Figure 8


+Gab1
Erk1/2
Erk1/2
?
Early endosomes
Met Degradation

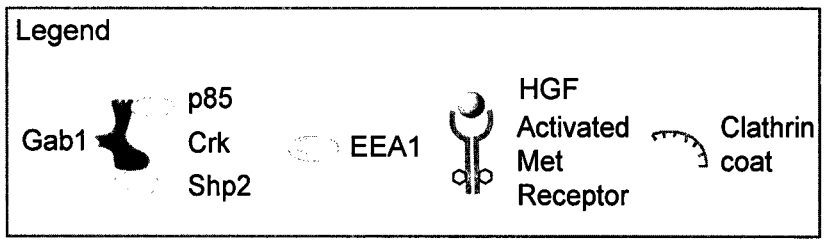

Legend
Gab1
p85
Crk
Shp2
EEA1
HGF
Activated
Met
Receptor
Clathrin
coat

Figure S1. (A) MDCK cells plated on glass coverslips were left untreated (0) or stimulated with indicated amounts of HGF. Cells were fixed and stained with Phalloidin-Alexa Fluor 488 in order to score the percentage of cells that form dorsal ruffles. (B) MDCK GFP-Gab1 cells were stimulated for 5 minutes with HGF, fixed and stained for the actin cytoskeleton and endogenous CrkI/II, (C) endogenous Shp2, or (D) endogenous p85. (E) MDCK cells were transfected with scrambled or three different siRNA duplexes targeting Gab1. Densitometric analysis of Gab1 protein levels from Figure 2A is shown as a percentage of mock condition Gab1 protein levels. (F) Representative images of the actin cytoskeleton (Phalloidin-Alexa Fluor 488 staining) of cells following mock, scrambled or three different siRNA duplexes targeting Gab1 and stimulated with HGF for 5 minutes.

Supplementary Information 1

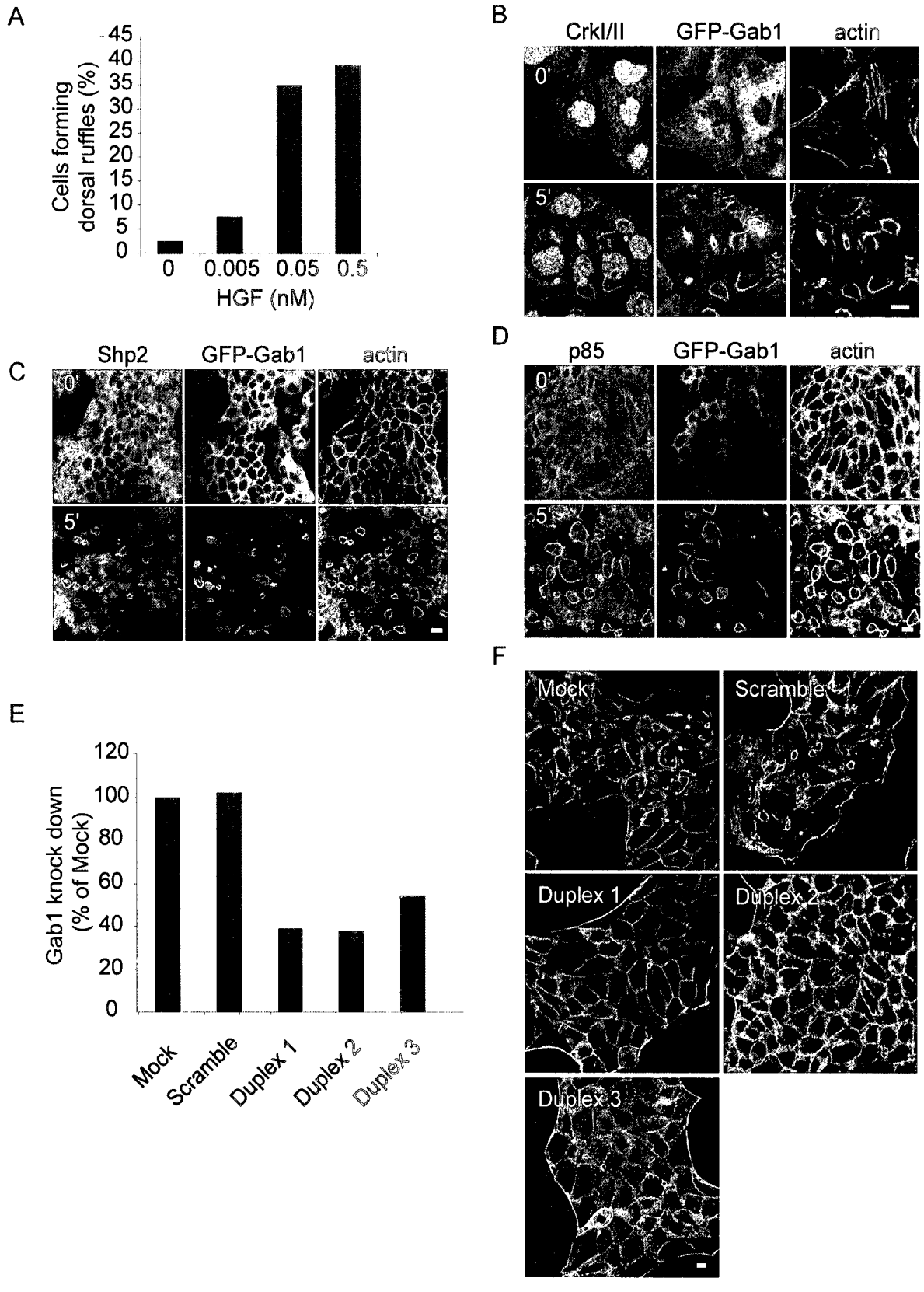
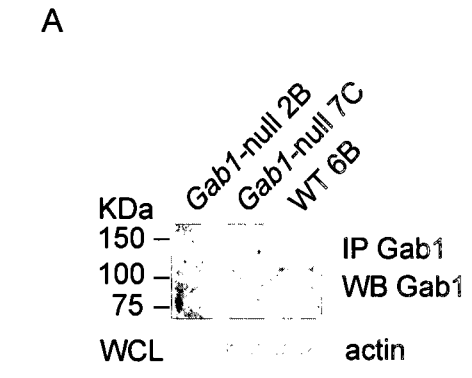
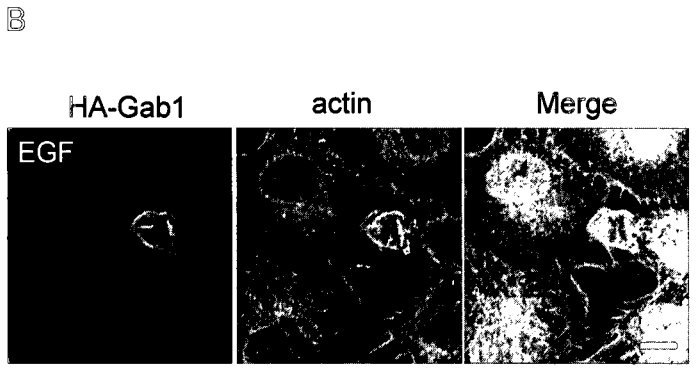
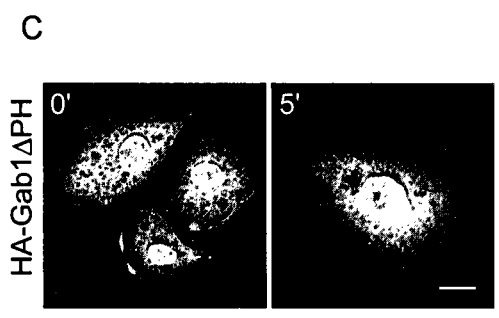


Figure S2. (A) Protein extracts of wt MEF 6B and two clones of *Gab1*-null MEF (2B and 7C) were separated by SDS PAGE and subject to immuno-precipitation with a Gab1 antibody and immuno-blotted for Gab1. Whole cell lysates were blotted for actin as a loading control. (B) HeLa cells plated on glass coverslips and transfected with HA-Gab1 were stimulated 24 hours later with EGF for 5 minutes, fixed and stained for HA-Gab1 (red) and Phalloidin-Alexa Fluor 488. (C) HeLa cells were plated on glass coverslips, transfected with HA-Gab1ΔPH and stimulated or not with HGF for 5 minutes. Cells were fixed and stained for HA (green) and nuclei with DAPI (blue). (D) MDCK GFP-Gab1ΔPH cells were plated on glass coverslips, stimulated or not with HGF for 5 minutes, fixed and stained with Phalloidin Alexa-Fluor 546. All scale bars represent 10μm.

Supplementary Information 2







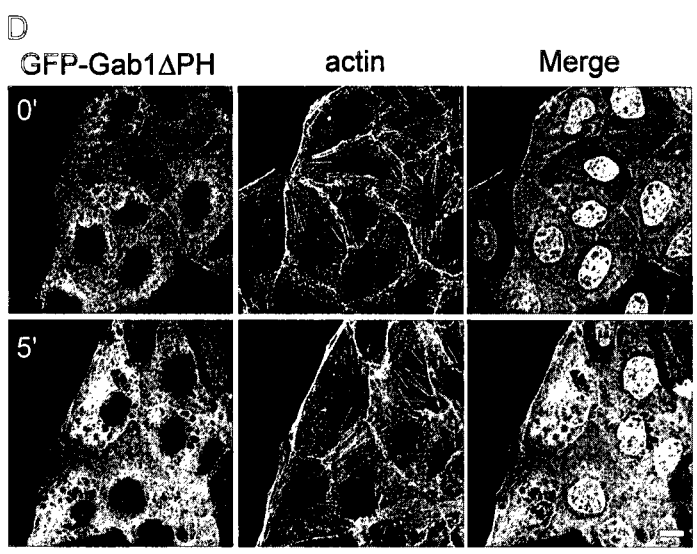
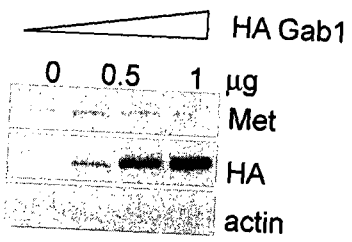


Figure S3. (A) HeLa cells were transiently transfected with increasing amounts of HA-Gab1 cDNA, lysed 24 hours post transfection and immuno-blotted for Met, HA-Gab1 and actin. (B) HeLa cells were transiently transfected with vector or HA-Gab1 and stimulated with HGF for the indicated times. Cells were lysed under boiling lysis conditions, separated by SDS PAGE and immuno-blotted for Met, HA and actin. (C) MDCK Met/HA-Gab1 cells were left un-stimulated or stimulated with HGF for 15 minutes, fixed and stained for HA-Gab1 (red) and EEA1 (green) and confocal images were taken to visualize the localization of Gab1. (D) MDCK, MDCK HA-Gab1, and three cell lines of MDCK cells expressing HA-Gab1ΔCrk were stimulated for indicated times with HGF and proteins from cell extracts were separated by SDS PAGE and immuno-blotted for Met, HA and Actin. Representative blots are shown and densitometric quantification is present in Figure 6A. All scale bars represent 10 μm.

Supplementary Information 3

A



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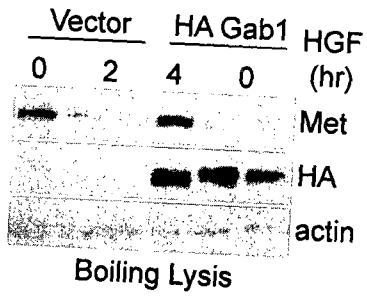

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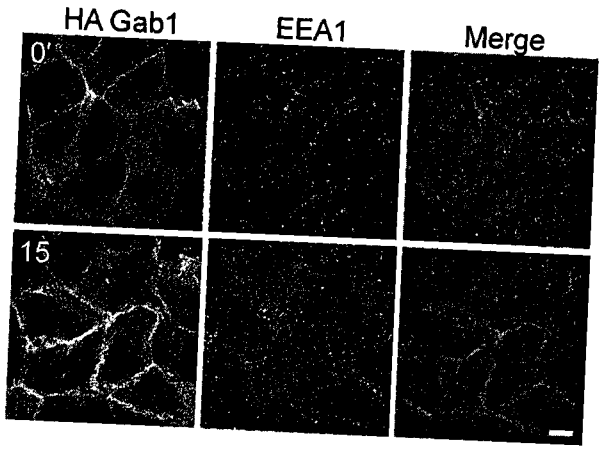


D

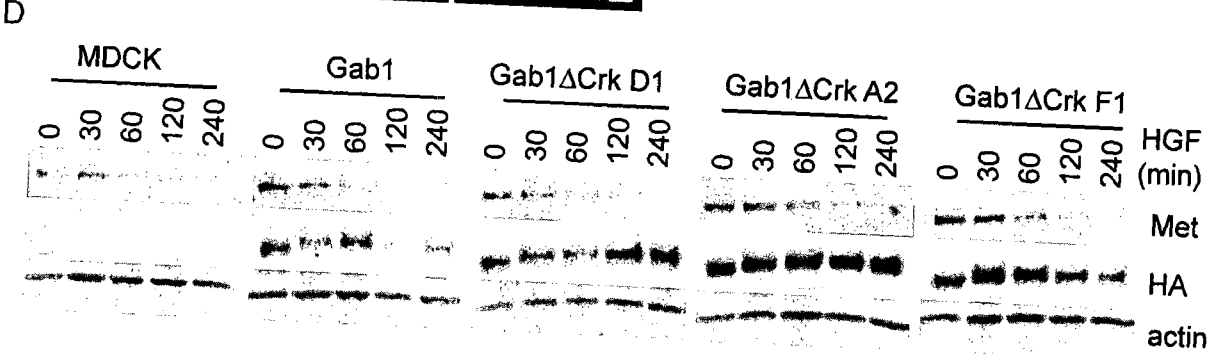
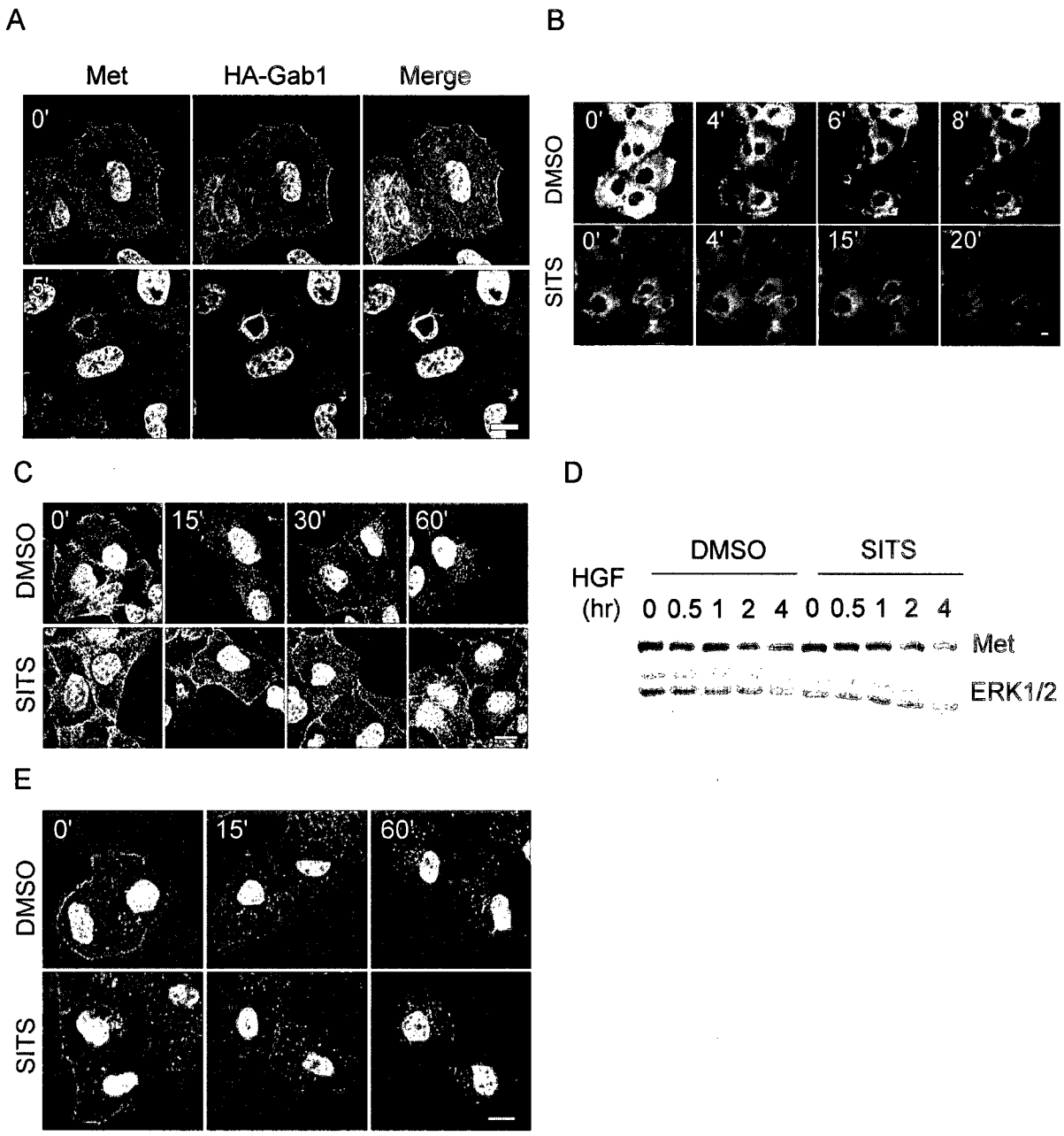


Figure S4. (A) HeLa cells plated on glass coverslips were transiently transfected with HA-Gab1, stimulated with HGF for 5 minutes, fixed and stained for Met (red), HA-Gab1 (green) and DAPI (blue). (B) SDCM with 63x objective was performed on MDCK GFP-Gab1 cells pre-treated with either DMSO or 0.5mM SITS and stimulated with HGF. Five time-lapse images are shown with the indicated times of HGF stimulation. (C) MDCK Met cells were plated on glass coverslips, serum starved in the presence of cycloheximide, pre-treated with DMSO or 0.5 mM SITS and stimulated with HGF for indicated times. Cells were fixed and stained for Met and DAPI (blue). (D) HeLa cells were pre-treated with DMSO or 0.5 mM SITS stimulated or not with HGF in the presence of cycloheximide for the indicated time points. Cells were lysed, separated by SDS PAGE and immuno-blotted for Met and total ERK1/2 as a loading control. (E) HeLa cells plated on glass coverslips were treated as in (C), fixed and stained for Met (red), EEA1 (green) and DAPI (blue). All scale bars represent 10μm.

Supplementary Information 4



Discussion

The physiological significance of dorsal ruffles and their initiation is poorly understood. Our findings demonstrate that the Gab1 scaffold protein is required for dorsal ruffle formation in multiple cell types, including colonies of polarized epithelial cells and downstream from multiple receptor tyrosine kinases (Met, EGFR, PDGFR-β). Gab1 provides a mechanism through which these RTKs couple to signals for dorsal ruffle formation, generating polarized signalling micro-domains that contribute to RTK biological responses and RTK down-regulation.

We provide evidence that Gab1 dependent dorsal ruffles function both as a prolonged signalling micro-environment required for epithelial dispersal (Figures 1 C, D, S1 B-D and 6F), but also as a mechanism for bulk internalization and trafficking of the Met receptor that promotes efficient Met degradation (Figures 5A-C). In the absence of Gab1 over-expression in HeLa cells, Met is internalized by the clathrin dependent pathway, undergoes recycling and is subsequently targeted for degradation (Abella et al., 2005; Hammond et al., 2003). Under these conditions Met is rapidly internalized within 5 minutes post stimulation (Abella et al., 2005; Hammond et al., 2001). Hence, the Met dependent signal at the plasma membrane is transient in nature. In stark contrast, following HGF stimulation of HeLa cells over-expressing Gab1, the activated Met receptor and Gab1 are instead recruited to dorsal ruffles. MDCK cells and HeLa cells transfected with Gab1 continue to form dorsal ruffles up to 20 minutes post-stimulation, providing a prolonged but polarized signalling microenvironment at the plasma membrane, from which Met can be internalized and efficiently degraded (Figures 6 B,D, S4A and 8).

The apparent controversy between maintenance of the Met receptor at the plasma membrane in a dorsal ruffle microenvironment versus the shorter half-life of the Met receptor observed in cells that form dorsal ruffles, may reflect that Met is internalized more efficiently from this site and/or that Met fails to recycle. In support of the former, we observe many Met receptor positive vesicles at the base of the dorsal ruffle as it collapses (Figure 1B), as previously reported for the EGF Receptor (Orth et al., 2006). In addition, Met is more rapidly translocated to a peri-nuclear compartment in MDCK cells over-expressing Gab1 that form dorsal ruffles (Figure 5 C) consistent with a decrease in recycling and the more rapid degradation observed for the Met receptor (Figures 5 A-B). However, it is also possible that Met receptors may first be internalized by a clathrin dependent process and then recycled from an endosomal compartment to the plasma membrane to become incorporated into dorsal ruffles upon HGF stimulation (Figure 8) (Zech and Machesky, 2008).

Multiple mechanisms have been identified for RTK internalization (Mayor and Pagano, 2007; McNiven, 2006). However, the signals that regulate which mechanism is undertaken by RTKs are still unclear. Met internalization from the dorsal ruffle micro-environment results in Met localization onto EEA1 positive endosomes, indicating that dorsal ruffles still deliver receptors to the canonical endocytic pathway (Figure 5C). For the EGFR, ligand concentration can influence the mode of internalization (Sigismund et al., 2005). Importantly, MDCK cells form dorsal ruffles in response to physiological concentrations of HGF (Figure S1), supporting that entry of Met into the endosomal pathway from a dorsal ruffle micro-environment is a physiologically relevant pathway. We did not observe an increase in Met ubiquitination under conditions of Gab1 over-

expression, indicating that enhanced Met ubiquitination was not the signal for the more efficient degradation observed (Figure 5D). In fact, Met ubiquitination was consistently decreased in conditions with Gab1 over-expression, suggesting that Gab1 may compete for binding to Met with Cbl, the E3-ligase for Met, as both are recruited through the adapter protein Grb2 (Peschard et al., 2001). Cbl has been localized to dorsal ruffles (Scaife et al., 2003), but whether it functions both as an ubiquitin ligase and/or a scaffold protein remains to be tested.

A biological function for dorsal ruffles has remained elusive. Our data support a role for the formation of a dorsal ruffle signalling micro-environment in facilitating the dispersal of colonies of epithelial cells. Expression of the Gab1ΔCrk mutant in MDCK cells blocks dorsal ruffle formation and we have previously shown that this mutant impairs cell dispersal (Figure 6A) (Lamorte et al., 2002a). Inhibition of dorsal ruffle formation with the stilbene drug SITS, inhibits scatter of epithelial colonies in response to HGF, independent of Gab1 over-expression (Figure 6F) (Dowrick et al., 1993), whereas SITS treatment did not block peripheral ruffle or lamellipodia formation (Figure 6E). Interestingly, the matrix metallo-proteinease MMP2, has been localized to the tips of dorsal ruffles (Suetsugu et al., 2003), giving rise to the possibility that dorsal ruffles may also promote degradation of the extracellular matrix, to allow for three-dimensional cell migration. This may reflect the inability of the Gab1ΔCrk mutant to promote branching morphogenesis in three-dimensional matrix (Lamorte et al., 2002a).

Gab2, a related protein, regulates Fcγ receptor-mediated phagocytosis in macrophages (Gu et al., 2003), which is required for the internalization of antigen upon engagement with cell surface receptors. Gab2 localizes to nascent phagosomes and by the

subsequent recruitment of p85, functions to amplify PIP_3 production required for phagocytosis (Gu et al., 2003). However, we show that for dorsal ruffle formation, the recruitment of p85 to Gab1 is not essential, although pre-treatment of MDCK cells with inhibitors of PI3K blocks the formation of dorsal ruffles (Chapter 4) consistent with previous studies (Dharmawardhane et al., 1997; Doughman et al., 2003; Orth et al., 2006). Gab1 and Gab2 provide similar functions for bulk receptor internalization, but require distinct signalling complexes from their function since a Gab1 protein uncoupled from recruitment of the Crk adapter fails to promote dorsal ruffles downstream from Met (Figure 4A-C). At the molecular level, the formation of dorsal ruffles is known to be Rac dependent (Krueger et al., 2003; Lanzetti et al., 2004) and at least in part dependent on the activity of WAVE-1, that connects Rac to the actin nucleating complex (Eden et al., 2002; Suetsugu et al., 2003). CrkI/II proteins couple upstream activators to Rac (Feller, 2001) and the Gab1-Crk complex promotes Rac activation in response to HGF (Lamorte et al., 2002a; Lamorte et al., 2002b), hence providing a mechanism for Rac activation in the dorsal ruffle membrane micro-domain.

Since the balance of RTK activation and degradation is critical for normal physiology, a full understanding of the molecular events that control these processes is essential. In this framework, Gab1 had always been considered only as a positive regulator of RTK signalling (Birchmeier et al., 2003; Gu and Neel, 2003). In this context, Gab1 and signalling proteins recruited to Gab1 following RTK activation are present on dorsal ruffles providing a prolonged signalling microenvironment. Paradoxically, positive regulation of dorsal ruffles by Gab1 also induces more efficient degradation of the Met receptor and Gab1 may thus play a key role in recruiting RTKs into dorsal ruffles for

their subsequent ligand dependent down-regulation. This highlights an unsuspected role for Gab1 in RTK homeostasis.

Acknowledgments

We thank members of the Park lab, Dr. Sergio Grinstein and Dr. Stephane Laporte for their helpful comments on the manuscript. We would like to thank Genetech Inc. for HGF, Dr. G Bokoch for Pak1 reagents and Dr. Marina Holgado-Madruga for *Gab1*-null cells. This research was supported by a fellowship to J.V.A from the US Department of Defense Breast Cancer Research Initiative (DAMD17-99-1-9284), to M.M.F. from Fonds de Recherche en Santé du Québec, to C.P. from CIHR Canada Graduate Scholarship (CGS) Doctoral Award and to V.S. from the Terry Fox Foundation and the National Cancer Institute of Canada. This work was supported by an operating grant to M.P. from the National Cancer Institute of Canada with money from the Canadian Cancer Society. M.P. holds the Diane and Sal Guerrera Chair in Cancer Genetics.

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Chapter 4

Gab1 coordinates membrane protrusions by engagement with the actin cytoskeleton, PLCγ and PI3K downstream of the Met Receptor

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Manuscript in preparation

Preface

Dorsal ruffles accumulated Met and Gab1, as well as Gab1 associated molecules, which then function as a signaling compartment. These membrane protrusions must therefore contain other signaling molecules, namely those that regulate the actin cytoskeleton, the lipid composition of the membrane and the regulators of Rac. We sought to determine the other characteristics of dorsal ruffles and further characterize the role of Gab1 in Met mediated dorsal ruffle formation. Furthermore, we inquire as to the role of Gab1 in mediating other types of membrane protrusions. In three-dimensional cultures, the elaboration of a tubulogenic organ requires protrusions of epithelial cells into the extra-cellular matrix, which is in contact with the basal membrane of these cell types. We seek to describe whether or not Gab1 accumulates into basal protrusions in three-dimensional cultures, as well as in basal protrusions known to occur on the ventral surface of invasive breast cancer cells.

Abstract

Upon HGF stimulation, Gab1 is recruited to the Met receptor and functions to diversify the Met signal. This includes the production of membrane protrusions of which Gab1 is required. Gab1 is localized to dorsal ruffles on the apical surface of two-dimensional cell cultures of MDCK cells. HGF induced dorsal ruffles are rich in F-actin and cortactin, as well as the Gab1 associated molecules, PLCγ and the PI3K lipid product PIP_3. Dorsal ruffle formation downstream of Met in MDCK cells requires PI3K and PLCγ enzymatic activity, whereas the MEK pathway is dispensable for dorsal ruffle formation. Gab1 is also localized to the basal surface of MDCK cells as demonstrated by TIRF imaging. Moreover, Gab1 localizes to protrusions induced by HGF in polarized cysts of MDCK cells in a three-dimensional collagen matrix. Gab1 is also required for F-actin rich structures localized on the basal surface of MDA-231 breast cancer cells. Gab1 engages with proteins that remodel the lipid constituent of the plasma membrane, co-localizes with DOCK180 in dorsal ruffles, and can form complexes with actin and cortactin. Therefore, Gab1 forms a multi-purpose complex in the regulation of membrane ruffles downstream of the Met receptor.

Introduction

In response to extra-cellular cues for cell migration, cells must integrate signals to induce changes that will sustain productive cell movement. A cell that suddenly receives cues for movement, begins by establishing a leading membrane edge generating an asymmetric cell shape. This leading edge is a protrusion from the cell body and the force required for the formation of such a structure is provided by the actin cytoskeleton (Stossel et al., 1999). By studying cells in two-dimensional cultures on Petri dishes, overall morphological changes under different conditions have been observed inaugurating the field of cell biology (Abercrombie et al., 1970). There are two types of leading edge membrane protrusions, lamellipodia or filopodia, which are not mutually exclusive as the latter is often a precursor of the former. Filopodia are finger-like projections that extend to serve as sensors for the surrounding extra-cellular environment. These contain long filaments of actin polymers which upon elongation of these filaments of actin function to push on the membrane, forcing the protrusion outward from the cell body (Mattila and Lappalainen, 2008). Lamellipodia are thin sheet-like structures that often arise from multiple filopodia, and are the main site of monomeric actin incorporation into actin filaments (F-actin) (Glacy, 1983). Another species of membrane ruffle, which protrudes from the apical surface of various cell types studied in two-dimensional cultures are dorsal ruffles. It is suggested that cells that are unable to form dorsal ruffles are unable to become motile (Abella and Frigault et al submitted) (Dowrick et al., 1993), however, this correlation has not been widely tested. Dorsal ruffles are protrusions that resemble the phagocytic cup of macrophages, which professionally engulf debris, and have also been described as macro-pinosomes to uptake fluid into the

cell (Araki et al., 2000; Dharmawardhane et al., 2000; Dowrick et al., 1993; Schlunck et al., 2004). More recently, dorsal ruffles have been identified to serve as a mechanism to aggregate signaling proteins into a micro-environment for specialized signaling subsequently followed by bulk RTK internalization and degradation (Abella and Frigault et al. submitted) (Orth et al., 2006). These structures were initially described by Richard Warn in MDCK cells upon HGF stimulation using electron microscopy (Dowrick et al., 1993), and only this year a mechanistic understanding of HGF induced dorsal ruffle formation has been achieved (Abella and Frigault et al. submitted) (Palamidessi et al., 2008). Although the molecular mechanism and function of dorsal ruffles may overlap with those of other membrane ruffle types, numerous studies have identified distinct differences highlighting a unique function for dorsal ruffles.

In order for a cell to generate a large change in cell shape to produce a membrane ruffle, either at the periphery or on its dorsal surface, multiple pathways are activated. One of the most well known drivers of ruffle formation is the activation of the Rho family of small GTPases namely Rac (Ridley and Hall, 1992; Ridley et al., 1992). Rac functions as a molecular switch, which cycles from an activated form when bound to the nucleotide GTP, to an inactive GDP bound state. The cycling between GDP and GTP bound forms of Rac is tightly regulated by various proteins, including exchange factors (GEF) to facilitate reloading of GTP, or activating proteins (GAP), which accelerate hydrolysis of the γ-phosphate (Cote and Vuori, 2007). The cycling from GTP bound to GDP bound states also regulate the membrane association of Rho GTPases where the GTP loaded protein is associated with the membrane and the GDP bound form is cytosolic (Dransart et al., 2005). Integrin activation by engagement with the proteins of

the extra-cellular matrix as well as growth factor stimulation of RTKs leads to Rac activation (Hall, 1998; Price et al., 1998). Stimulation of RTKs also leads to the activation of PI3K and the production of its lipid product PIP_3, which in turn activates GEFs (Cote et al., 2005; Innocenti et al., 2003; Scita et al., 2000; Singleton et al., 2005). Moreover, PIP_2 and PIP_3 accumulate in a polarized manner to sites of active membrane protrusion in chemotactic, migratory, and phagocytic cells (Botelho et al., 2000; Chou et al., 2002; Funamoto et al., 2002; Rickert et al., 2000; Wang et al., 2002; Watt et al., 2002). Downstream of Rac activation, the elaboration of the actin meshwork is regulated and this provides the force for membrane protrusion and is achieved by the activation of the Arp2/3 complex (Machesky and Insall, 1999). Arp2/3 is engaged to form a new F-actin branch on the side of an existing actin filament, and is regulated by nucleating protein factors (NPF). Among these are the WASP and WAVE family of proteins, however, cortactin has been recently described to also function downstream of Rac as a NPF for meshwork elaboration (Lua and Low, 2005; Machesky and Insall, 1998; Mullins, 2000; Takenawa and Suetsugu, 2007; Weaver et al., 2001).

Since it is difficult to isolate membrane ruffles to study them biochemically, much of the elucidation of the molecular mechanisms that regulate them has been performed using microscopy (Danuser and Waterman-Storer, 2003; Hahne et al., 2001; Welch et al., 1997). Indeed, the exploitation of the biophysical properties of imaging technologies and of the innate properties of naturally fluorescent proteins continues to be tailored to the needs of cell biologists. These have produced some techniques that provide insight into mechanisms of actin cytoskeleton rearrangements in response to extra-cellular stimuli and have demonstrated that the modulators of actin dynamics are regulated in a spatial

and temporal manner. Klaus Hahn's group was at the forefront such developments by constructing a sensor composed of fluorescent proteins which when bound to activated Rac would change their behavior (Kraynov et al., 2000). These studies elucidated that although Rac is associated to most membranes, however the site of lamellipodia elaboration occurs only where a large pool of Rac-GTP accumulates (Itoh et al., 2002; Ouyang et al., 2008). Moreover, Rogen Tsien's group has developed a variety of fluorescent proteins that can be fused to various proteins of interest and imaged simultaneously in living cells to observe changes in cellular location (Giepmans et al., 2006; Zhang et al., 2002). It is becoming clear that signaling molecules are not just turned on and off by post-translational modifications, but are regulated also by their subcellular location in time.

The Met RTK is activated by binding to its high affinity ligand, hepatocyte growth factor, HGF. Met activation triggers the activation of signals for cell survival, proliferation, cell scatter and epithelial morphogenesis, and deregulation of the HGF/Met signaling axis results in disease (Peschard and Park, 2007). Met signaling in epithelial cells activates signals for remodeling of the actin cytoskeleton resulting in the formation of membrane protrusions leading to cell scatter (Potempa and Ridley, 1998; Royal et al., 2000; Royal and Park, 1995). Upon Met activation in MDCK epithelial cells, Gab1 is the most highly phosphorylated protein (Nguyen et al., 1997), and most biological signals elicited by HGF/Met are dependent on Gab1. Gab1 is a docking protein that is targeted to the plasma membrane and serves to recruit other signaling molecules to relay signals from the activated Met receptor (Liu and Rohrschneider, 2002).

Indeed, all studies of Gab1 function demonstrate that membrane targeting via a functional PH domain is required. The Gab1ΔPH protein can still form complexes with known signaling proteins downstream of Met, however, this protein is not functional for dorsal ruffle formation (Abella and Frigault et al. submitted), nor can it support cell scatter or epithelial morphogenesis (Maroun et al., 1999a; Maroun et al., 1999b). Downstream of Met, most Gab1 associated signaling molecules are localized with Gab1 to dorsal ruffles (p85 subunit of PI3K, Crk, and Shp2) as well as the activated Met receptor and phosphorylated ERK. Gab1 is required for the formation of HGF induced dorsal ruffles (Abella and Frigault et al. submitted). Therefore we sought to more closely examine the role of Gab1 at sites of membrane protrusions and to determine its association with ruffle machinery.

Materials and Methods

Antibodies and Reagents

Anti-HA (mHA.11) was purchased from Covance (Berkeley, CA), and a rabbit polyclonal HA (H6908) antibody from Sigma (Oakville, ON). Anti-Actin for use in western blot analysis and anti-DOCK180 antibodies monoclonal H-4 and polyclonal N-19 purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). β-Pix, PLCγ, and PY20 that recognizes phosphorylated tyrosine residues were purchased from BD Transduction Laboratories (Mississauga, ON). The pan Ras Ab-3 antibody was purchased from Oncogene Research Products (Boston, MA). The anti-Gab1 antibody used to detect levels of protein in MDA-231 cells, the cortactin antibody 4F11 used in both western blot analysis and for immuno-fluorescence from Upstate Biotechnology Inc. (Lake Placid, NY). F-actin was visualized using Alexa 488, 546, or 635 conjugated phalloidins, secondary antibodies conjugated to Alexa Fluors for indirect immuno-fluorescence, and the lipid dye FM4-64 from Molecular Probes (Burlington, ON). HGF was a gift from Dr. George Van de Woude, Van Andel Research Institute (Grand Rapids, MI). All inhibitors were diluted in DMSO from Sigma and cells were pretreated with inhibitors for 30 minutes at concentrations indicated below prior to 5 minutes HGF stimulation. MEK inhibitor UO126 from Promega (Madison, WI) was used at 20μM, the PLCγ inhibitor U73122 from Calbiochem (Lajolla, CA) was used at 5μM, the PI3K inhibitors LY294002 was used at 20μM, and wortmannin was used at 0.2μM; both are from Calbiochem (Lajolla, CA). Gab1 knock down experiments in MDA-231 cells were done using 50 nM or 100nM Gab1 siGENOME SMARTpool siRNA with SIRNA targeting RISC as a negative control purchased from Dharmacon (Lafayette, CO).

Plasmids and DNA Constructs

DsRed cortactin was a generous gift from Dr. Marc McNiven (Mayo Clinic, Minnesota, USA), GFPPH domain of AKT (GFPPHAKT) was a generous gift from Dr. Sergio Grinstein (Hospital for Sick Children, Toronto, Canada), Met/pXM has been previously described (Rodrigues et al., 1991).

Cell Culture and Transfections

MDCK, MDA-231, and human embryonic kidney (HEK) 293 cells were maintained in DMEM containing 10% fetal bovine serum (Invitrogen Burlington, Ontario, Canada) on Petri dishes from Nunc (Rochester, NY). MDCK cell lines expressing GFP Gab1 or GFP Gab1ΔPH are previously described (Frigault et al., 2008; Maroun et al., 1999a), and stable cell lines expressing DsRed cortactin were generated by co-transfection of DsRed1 cortactin (Dr. McNiven) and PLXSH using the Superfect transfection method (Qiagen) as per manufacturer's directions. Clonal cell lines were generated by selecting with 400μg/ml G418 (Invitrogen) (for GFP/GFP Gab1 selection) and 300μg/ml hygromycin (Roche) (for DsRed cortactin selection). Established cell lines were maintained in half the concentration of the selection pressure, and cells seeded out of drug for experimental set up. MDCK HA Gab1 cells were previously described and characterized (Maroun et al., 1999a) and maintained in 200μg/ml G418, MDCK ras5 cell line was generated by calcium phosphate method (Wigler et al., 1979). HEK 293 cells were plated at 1 X 10^6 cells on a 100 mm dish and the following day transfected by Lipofectamine Plus reagent (Invitrogen) as per manufacturer's directions. 48 hours post-transfection, cells were harvested by lysis in ice-cold 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, 1mM sodium vanadate). MDA-231 cells were placed in a 6 well dish at a density of 1 X 10^5 cells in suspension with DMEM and were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's directions with indicated concentrations of siRNA for 96 hours for optimal knock down of Gab1 protein levels. At 72 hours post-transfection cells treated with mock conditions, RISC siRNA, or Gab1 siRNA were trypsinized, counted and 1.5 X 10^4 cells were plated on glass coverslips in a 24 well dish to observe the actin cytoskeleton, and the remaining cells were plated on a 60mm dish in order to make protein extracts and determine the extent of Gab1 protein expression.

Immuno-precipitation of HA Gab1

MDCK HA Gab1 (7D6) cells were seeded at 1 X 10^6 cells per 100mm dish. Twenty-four hours later, cells were serum starved overnight in DMEM containing 0.02% FBS. For stimulation, 100U HGF/ml warmed DMEM was added to the cells and incubated at 37°C for indicated times. Cells were immediately harvested in ice-cold Triton X-100 lysis buffer (1% Triton X-100, 50mM HEPES pH 8.0, 150mM NaCl, 10% glycerol, 2mM EGTA, 1.5 mM $MgCl_2$, 10μg/ml aprotinin, 10μg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride, 1mM sodium fluoride, 1mM sodium vanadate). HEK293 cells transfected were allowed to express there transfectants for 48 hours and then lysed in ice-cold 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, 1mM sodium vanadate). Following a 10 minute incubation on ice, lysates were

centrifuged at 12000 rpm for 15 minutes at 4°C, and total protein concentration was determined by Bradford Assay using dye reagent concentrate (BIORAD, Hercules, CA). For immuno-precipitation of HA Gab1, 600μg total protein was allowed to mix with mHA.11 antibody at 4°C nutating for 1 h, then 25μl of a 50% slurry of Protein G sepharose beads (Amersham) were added and allowed to nutate for an additional hour. Immuno-precipitates were washed with 0.5ml of their respective lysis buffers three times, loading dye containing DTT was added,samples were boiled for 5 minutes, and proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane, blocked in 3% Bovine Serum Albumin (Roche) for one hour followed by western blot analysis with indicated antibodies.

Collagen Assays

MDCK cells expressing GFP Gab1 or GFP Gab1ΔPH were seeded in collagen as described previously with a few modifications (Zhu et al., 1994). Briefly, $5x10^3$ cells were resuspended in 500μl of Vitrogen 100 collagen solution (Pur-Col by INAMED Biomaterials Fremont, CA) prepared following the manufacturer's directions and layered over 350μl 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. For stimulations of cysts, HGF (10 U/ml) was added to Leibowitz medium supplemented with 2% FBS for 8 hours, washed three times with 1X Phosphate Buffered Saline (PBS), and fixed with fresh 3% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) for 20 minutes, and washed extensively in PBS. Immuno-fluorescence was carried out as described below on slices of the collagen culture, and incubation times are five times as long as for two-dimensional cultures. F-actin staining was done using Alexa 546

phalloidin and a LSCM (Confocal LSM 510, Carl Zeiss) was used to acquire DIC, GFP and red images.

Dorsal ruffle Assay

Cell lines were plated on glass coverslips (Bellco Glass) at a density of $2x10^4$ cells per well in a 24 well dish in duplicate. 24h later, cells were either left un-stimulated or treated with 100 U/ml HGF for anywhere between 4 and 10 minutes and immediately fixed with 2% paraformaldehyde in PBS for 20 minutes at room temperature, stained as described below in *Immuno-fluorescence*. F-actin staining was visualized using Alexa 546 or 488 (1:200 dilution) or Alexa 635(1:50 dilution) conjugated phalloidin. Nuclei were counterstained using a 0.5ng/ml DAPI dilution in water and cover slips were mounted onto microscope slides with immuno-mount (Thermo-Shandon Pittsburgh, PA). LSCM images of the most apical z-section were taken with a 40x oil immersion objective. Images were scored for the total number of dorsal ruffles and represented as a percentage of the total number of cells in each field of view. More than 100 cells were visualized for each condition in each experiment. Multinucleated cells or cells with condensed chromatin were not included.

Immuno-fluorescence

After fixation in paraformaldehyde, cells were washed three times in PBS, and incubated with gentle rocking for 10 min in PBS containing 0.3% Triton X-100. Coverslips were washed three times for 5 minutes in 100mM glycine in PBS, followed by a 30 minute incubation with blocking buffer (2% BSA in IF buffer (0.2% Triton X-100, 0.05% Tween-20 in PBS)) at room temperature. Cover slips were incubated with primary and

secondary antibodies diluted in blocking buffer for 1 h and 40 min respectively at room temperature. Coverslips were washed in IF buffer three times and then once in water before being mounted with immuno-mount onto microscope slides.

Live cell imaging

Cells were seeded on 35mm glass bottom dishes from MatTek Corporation (Ashand, MA) at a 1.5 X 10^5 cell density, and the following day, cells were transported to sites of imaging and allowed to incubate under imaging conditions for 4 hours before the start of the experiment. For optimal live cell imaging conditions see Commentary by Frigault et al. in the Journal of Cell Science (in review). For WFM, cells were imaged with an inverted fluorescent microscope, Axiovert 200 (Zeiss) using a 63X oil immersion objective and Axiovision acquisition software. For TIRF imaging, cells images were acquired with an Olympus illumination arm (Markhan, ON), with AOTF from Prairie Technologies (Middleton, WI) and a Photometrics CoolSNAP EZ camera from MAG Biosystems, (Pleasanton, CA). Image acquisition and processing was done using MetaMorph software from Molecular Devices (Sunnyvale, CA). For SDCM in Movie 5 and Figure 2, images were collected on a WaveFX spinning disk from Quorum Technologies Inc. (Guelph, ON) mounted on a Leica motorized microscope from Wetzlar (Germany) using an EM-CCD camera Hamamastu (Shizuoka, Japan). Movies were not included in the final copy of this thesis since McGill Librabries does not support these.

Results

Gab1 is localized to the plasma membrane and in membrane protrusions of three-dimensional organoid cultures.

The inherent capacity of Gab1 to be associated with the plasma membrane is directly linked to its ability to functionally relay Met mediated biological processes. Gab1 mutants that lack a PH domain, or are unable to mediate PIP_3 binding, cannot mediate signals for epithelial morphogenesis (Maroun et al., 1999a; Maroun et al., 1999b). Moreover, lipidation of a Gab1 PH domain deletion mutant, or of a Gab2 molecule with the Met binding motif (MBM) re-introduced, rescues the defect of these to promote morphogenesis (Frigault et al., 2008; Maroun et al., 2003). The signaling capacity of Gab1 has been well characterized and elements of membrane targeting are required for Gab1 function, however, the dynamics of Gab1 localization to membranes during these processes remains to be demonstrated.

In order to determine the subcellular localization of Gab1 during epithelial morphogenesis, MDCK cells which express a fusion protein of eGFP and Gab1 (termed GFP Gab1) were seeded into a collagen matrix and allowed to form three-dimensional cysts consisting of a spheroid of single cells (Figure 1 A-top panel). Fixed samples were stained with Alexa 546 conjugated phalloidin to visualize F-actin and analyzed by laser scanning confocal microscopy (LSCM). Images of the most basal surface of the cyst determine that Gab1 is predominantly localized to sites of cell-cell contacts and co-localizes with F-actin cables, which demarcate the perimeter of the cell. While Gab1 accumulates mostly at sites of cell-cell contacts, a Gab1 mutant lacking the PH domain is localized to the cytosol, and is excluded from the nucleus without accumulation at F-actin

Three-dimensional surface rendering

In order to visualize a three-dimensional protrusion, LSCM with a Zeiss LSM 510 was used to acquire many z-planes at optimal 0.3μM intervals. Data was used to generate a pseudo surface using the intensities from the raw data using Imaris software from Bitplane Inc. (Saint Paul, MN). These were used to determine the fluorescence intensity along the length of multiple protrusions.

demarcated cell contacts. Cells expressing an eGFP fusion with Gab1 lacking the PH domain were treated as wild-type, and the localization of GFP Gab1ΔPH was visualized by LSCM (Figure 1 A-bottom panel). These results are in agreement with two-dimensional cultures of MDCK cells that express an HA epitope tagged Gab1 or Gab1ΔPH protein, where indirect immuno-fluorescence demonstrates that Gab1 is localized to sites of cell-cell contacts where Gab1ΔPH is diffuse, but extra-nuclear (Maroun et al., 1999a; Maroun et al., 2003).

Gab1 localization following activation of the Met receptor by HGF stimulation in two-dimensional cultures has been well described and involves the accumulation of Gab1 into sites of active membrane ruffling (Frigault et al., 2008; Maroun et al., 2003) (Abella and Frigault et al. submitted). Therefore, we wanted to determine whether Gab1 also accumulated into membrane protrusions in a three-dimensional model for branching morphogenesis. For the elaboration of the formation of an epithelial organ from a cyst, the effects of external morphogenic factors such as HGF induce a rearrangement of the actin cytoskeleton as well as reorganization of cell-cell contacts. HGF treatment of a cyst leads to the initiation of a protrusion from the basal surface of a single cell into the extra-cellular matrix (Figure 1 E). MDCK GFP Gab1 cells were allowed to form cysts in a collagen matrix for 7 days and then either left untreated (no HGF) or stimulated with HGF for 8 hours (8h HGF). Digital interference contrast (DIC) images acquired by LSCM demonstrate the formation of protrusions from cells in the polarized spherical monolayer of the cyst (Figure 1B). These structures were stained for F-actin and serial z-planes were acquired. Thirty-seven z-planes were acquired and the fluorescence data was de-convolved and subsequently the fluorescence intensity from GFP Gab1 and F-actin

(Red) were used to generate a rendered surface (Figure 1 C). A 16μm line was drawn along one of the protrusions and the intensity of GFP Gab1 and F-actin were determined from the rendered surface and plotted using Imaris software (Figure 1 D). The plot demonstrates that Gab1 is localized along the membrane protrusion until the tip. These data demonstrate that Gab1 is co-localized with F-actin at sites of cell-cell contact and in protrusions stimulated by HGF (Figure 1 E – boxed cartoon).

In order to confirm the localization of Gab1 at the basal membrane of epithelial cells, MDCK GFP Gab1 cells were imaged using total internal reflective fluorescence microscopy (TIRF) where only those molecules within 100 nm of the basal cell surface can be visualized. Cells were plated on glass bottom dishes in a two-dimensional culture and sequential images acquired by widefield microscopy (WFM) and TIRF demonstrating that Gab1 is localized throughout the cell as well as near the basal surface (Figure 1F and data not shown). Moreover, upon HGF stimulation, GFP Gab1 concentrates at the edges of the cell prior to membrane protrusion as seen in live cell imaging.

Gab1 accumulates into F-actin rich membrane ruffles and associates with actin.

In two-dimensional cell culture, MDCK epithelial cells grow and divide forming colonies of cells. When colonies of MDCK cells are treated with HGF, changes in cell shape become immediately noticeable. Membrane ruffling begins as early as two minutes post-stimulation and can persist for 30 minutes. Cells in the center of a colony as well as those on the periphery form dorsal ruffles on their apical surface (Dowrick et

al., 1993). Moreover, Gab1 is localized to these structures and is required for their induction (Abella and Frigault et al submitted).

Cells use actin polymerization to change cell shape by elaboration of the actin network which provides a physical force for the generation of membrane protrusions (Stossel et al., 1999). In order to determine whether or not Gab1 is linked to the actin cytoskeleton, MDCK cells expressing HA Gab1 were plated on glass coverslips and allowed to form colonies overnight. These were left un-stimulated or stimulated for 8.5 minutes with HGF to induce dorsal ruffle formation. Gab1 co-localizes with F-actin following immuno-fluorescence and LSCM (Figure 2 A). In the un-stimulated (0') condition, Gab1 is localized to both the cytosol as well as at sites of cell-cell contact where it co-localizes with F-actin. Upon stimulation (8.5'), Gab1 accumulates into F-actin rich dorsal ruffles. The inset panel further demonstrates the extent to which Gab1 and F-actin co-localize. In order to examine with greater detail the localization of Gab1 and F-actin in dorsal ruffles, GFP Gab1 MDCK cells were stimulated for 5 minutes with HGF to activate the formation of dorsal ruffles, fixed, stained for F-actin and imaged by spinning disk confocal microscopy (SDCM). Shown is one representative field of view where Gab1 is localized to most all of the apical cell surface whereas F-actin accumulates specifically into the dorsal ruffle structures. The X-Y plane is a maximum projection of all 37 z-planes acquired, whereas the Z-Y image on the left is an image of all Z planes at the X position denoted by the blue line and the Z-X plane is taken from the Y position demarcated by the green line (Figure 2 B). Indeed, Gab1 and F-actin accumulate into dorsal ruffles occurring above the rest of the cell at the apical surface.

Since Gab1 and F-actin are closely linked as determined by LSCM and SDCM, we determined whether or not Gab1 could be found in a complex with actin. MDCK cells expressing HA Gab1 were stimulated for indicated times with HGF and equal amounts of protein from whole cell extracts were subject to anti-HA immuno-precipitation. Western blot analysis demonstrates that Gab1 is tyrosine phosphorylated in response to HGF stimulation and that Gab1 coimmuno-precipitates with actin over a prolonged time-course of HGF stimulation but not in un-stimulated conditions (Figure 2 C). These results demonstrate that Gab1 is found in a complex with actin downstream of Met, which correlates with these co-localized in ruffles upon stimulation with HGF.

Gab1 associates with and co-localizes with cortactin.

Gab1 over-expression drives the formation of Met mediated dorsal ruffles in both HeLa and MDCK cells (Abella and Frigault et al submitted). HeLa cells transiently transfected with HA Gab1 (outlined with a white line), and stimulated with HGF, form dorsal ruffles (Figure 3 A). LSCM images were acquired and demonstrate that these ruffles are F-actin rich and also contain cortactin. Cortactin is well characterized to be a nucleating factor which functions to provide branch points by binding to F-actin in order to elaborate the actin meshwork for membrane ruffles (Uruno et al., 2001; Weed et al., 2000).

In order to further characterize the association of Gab1 with the actin cytoskeleton, we determined the extent to which cortactin was associated with Gab1. MDCK GFP Gab1 cells were made to over-express a DsRed fusion of cortactin in order to observe the changes in the subcellular localization of both Gab1 and cortactin

simultaneously downstream of Met. By WFM, time lapse microscopy demonstrate that both Gab1 and cortactin localize to active sites of membrane ruffling, both peripheral and dorsal ruffles, upon HGF stimulation. A single frame from time lapse WFM of both the GFP Gab1 and DsRed cortactin channels 18 minutes 20 seconds post-HGF stimulation are shown with a section of the field of view enlarged to see the dorsal ruffle (Figure 3 B).

Since Gab1 and cortactin co-localize in dorsal ruffles in a Met dependent manner, we sought to determine whether or not Gab1 could be associated with cortactin. By transient transfection of HEK 293 cells with HA Gab1, Met and increasing concentrations of DsRed cortactin. Equal amounts of protein from whole cell extracts were subject to immuno-precipitation with an HA antibody, and cortactin was found in a complex with Gab1 by western blot analysis (Figure 3 C). MDCK cells expressing GFP, GFP Gab1, GFP and DsRed cortactin, or GFP Gab1 and DsRed cortactin were assayed for their ability to make dorsal ruffles as quantified by F-actin staining as a percentage of total cell number without (no stim) or with HGF treatment (HGF) (Figure 3 D). Gab1 can induce the formation of dorsal ruffles (Abella and Frigault et al submitted), and although Gab1 and cortactin are found in a complex together and co-localize at sites of ruffling, only Gab1 and not cortactin can induce the formation of dorsal ruffles.

Gab1 is required for F-actin structures in breast cancer epithelial cells.

We have demonstrated that Gab1 is localized with F-actin and cortactin into dorsal ruffles on the apical surface of MDCK cells cultured in a two-dimensional manner. Moreover, we demonstrate that Gab1 accumulates into the basal protrusions, which

extend into the matrix in a three-dimensional collagen matrix. Therefore we sought to determine if Gab1 also plays a role in other types of membrane protrusions that have been well characterized, namely those that occur on the basal membrane and are rich in F-actin and cortactin. Transformed fibroblasts and some breast cancer cells lines make invadopodia. These have been characterized to be degradative protrusions which occur on the basal surface and may be contributive to the invasive and metastatic potential of breast cancer cells (Chen et al., 1994). The MDA-231 breast cancer cell line has been extensively studied to have invadopodia at their basal surface, which are F-actin and cortactin rich (Bowden et al., 2006). We therefore determined whether or not Gab1 was localized to invadopodia. MDA-231 cells plated on glass coverslips were transiently transfected with GFP Gab1 and cells were fixed and stained for F-actin. Images were acquired by CLSM looking at only the most basal z-plane. Indeed, Gab1 can be found to be in the vicinity of F-actin structures on the basal surface of MDA-231 cells (Figure 4 A).

Since Gab1 is localized to these F-actin structures, and Gab1 is known to be required for dorsal ruffle formation downstream of Met (Abella and Frigault et al. submitted), we sought out to determine the effect of Gab1 knock-down in MDA-231 cells on the formation of F-actin structures suggestive of invadopodia. Using siRNA silencing technologies, MDA-231 cells treated with either a pool of siRNA duplexes targeting the Gab1 gene or duplexes which are not targeted by the RISC complex, the expression levels of Gab1 protein was determined (Figure 4 B). Under conditions where Gab1 protein level was optimally reduced (50nM), and those equivalent RISC and mock conditions, the actin cytoskeleton was examined by acquiring the most basal z-plane

images by CLSM (Figure 4 C). The F-actin rich structures at the basal surface of MDA-231 cells treated with 50nM RISC siRNA did not result in drastic changes of F-actin staining as compared to mock treated cells. The F-actin structures remain intense and appear to exist in as an organized structure as previously described (Figure 4 C –inset) (Artym et al., 2006). However, knock-down of Gab1 protein levels results in the apparent dissemination of these F-actin punctate structures, suggesting that Gab1 may play a coordinate role in these types of protrusions (Figure 4 B and C).

Gab1 potentiation of Met mediated dorsal ruffles is dependent on lipid metabolism.

Although the actin cytoskeleton is an integral part of membrane ruffling, the membrane itself is dynamically regulated during this process. In order to confirm that Gab1 is tightly associated with membranes, live cell microscopy of MDCK cells expressing GFP Gab1 plated on glass bottom dishes was preformed with SDCM using a membrane dye FM-464. Time lapse SDCM reveals that Gab1 is co-localized with the membrane extensively upon HGF stimulation. A series of z-planes were acquired in the far-red channel to capture FM-464 data followed by the green channel to collect localization data from GFP Gab1 in time and three-dimensional space. The maximum projection of all z-planes for each time point is shown with time. Three frames extracted from time lapse experiments at indicated time points post HGF stimulation demonstrate that Gab1 and the membrane are dynamically related since GFP Gab1 and FM-464 are co-localized over the duration of these experiments (Figure 5 A).

Since Gab1 is a multi-protein scaffold, which coordinates and activates many signaling pathways, we set out to determine which pathways are required for dorsal

ruffles. Upon HGF stimulation, Gab1 becomes associated with PLCγ (Gual et al., 2000), the p85 subunit of PI3K (Maroun et al., 1999a), as well as Shp2 leading to ERK activation (Maroun et al., 2000) and ERK itself binds to Gab1 (Osawa et al., 2004; Yu et al., 2001). In order to determine which Gab1 associated enzymes were required for dorsal ruffles formation, we treated cells with pharmacological inhibitors of either MEK (UO126), PI3K (LY294002 or wortmannin), or PLCγ (U73122). MDCK GFP Gab1 cells were plated on glass coverslips and the following day, were pretreated with indicated inhibitors or DMSO alone (the vehicle in which the inhibitors were dissolved). Cells were stimulated for 5 minutes with HGF, fixed and stained for F-actin (Figure 5 B). By collecting the apical z-plane by LSCM of five fields of view, the response of cells treated with indicated conditions was determined in two independent experiments and is represented as compared to the control DMSO. Inhibition of the MEK-ERK pathway has no appreciable effect on the formation of dorsal ruffles, however, both inhibitors of PI3K and PLCγ drastically reduce the ability of cells to make dorsal ruffles in response to HGF (Figure 5 C).

In order to further characterize the requirement for these enzymes in dorsal ruffle formation, we sought to identify their subcellular localization. MDCK GFP Gab1 cells were plated on glass coverslips and the following day were stimulated for 5 minutes with HGF, fixed and stained with antibodies against PLCγ. Indeed Gab1 co-localizes with endogenous PLCγ in dorsal ruffles as determined by imaging the most apical z-plane with LSCM (Figure 5 D). We have previously shown that the p85 subunit of PI3K also co-localizes with Gab1 in dorsal ruffles in the same manner (Abella and Frigault et al. submitted). Furthermore, we demonstrate that HGF induced dorsal ruffles are PIP_3 rich,

which supports the fact that PI3K activity is required for dorsal ruffle formation. We transiently transfected MDCK cells with a GFP fusion with the PH domain of AKT which is widely used as a PIP_3 fluorescent reporter (Haugh et al., 2000), to visualize the localization of PIP_3 post HGF stimulation. F-actin dorsal ruffles also localize PIP_3 as determined by LSCM of the apical surface of cells stimulated with HGF for 8 minutes (Figure 5 E). Interestingly, although phosphorylated ERK is localized to sites of HGF induced membrane ruffling in MDCK cells (Frigault et al., 2008) (Abella and Frigault et al. submitted), inhibition of the ERK kinase MEK, does not significantly result impede dorsal ruffle formation. In agreement with these results, MDCK cells which over-express Ras, the upstream activator of MEK and ERK, do not enhance dorsal ruffle formation as assessed in comparison with MDCK cells by stimulating for 6 minutes with HGF, and looking at F-actin staining by LSCM to quantify the dorsal ruffle response.

Gab1 co-localizes with Rac1 GEFs in dorsal ruffles.

Cell movement begins with membrane ruffling in order to generate a leading edge. Ruffle formation requires the coordination of actin and membrane remodeling. This regulated by the Rho GTPase family of proteins, including Rac. Activation of Rac is regulated by a family of proteins known as Guanine Exchange Factors (GEFs) some of which have been characterized as being associated with Gab1 binding proteins. Namely, the adaptor molecule Crk has been identified in complexes with both DOCK180 and β-Pix (Akakura et al., 2005; Lamorte et al., 2003). Moreover, PLCγ function is dependant on association with β-Pix and regulates the downstream activation of Rac (Jones and Katan, 2007). We have also shown that the Gab1-Crk association is required for dorsal

ruffle formation (Abella and Frigault et al submitted to EMBO) and this may provide a link to Rac activation. In order to test this, MDCK GFP Gab1 cells were plated on glass coverslips and the following day stimulated for 5 minutes which HGF, fixed and stained with Alexa 635 conjugated phalloidin (F-actin) and antibodies against DOCK180 or β-Pix and the most apical z-plane was imaged using CLSM. Representative images of experiments performed using two different antibodies against DOCK180 demonstrated that this GEF co-localizes with Gab1 and F-actin in dorsal ruffles (Figure 6A). Moreover, although β-Pix is localized in the proximity of the dorsal ruffle, this GEF does not co-localize to the same extent as DOCK180 with Gab1. Representative LSCM images of immuno-fluorescence studies using two different antibodies on two different clonal MDCK GFP Gab1 cell lines are shown (Figure 6 B).

Although Gab1 is localized to and is required for the formation of dorsal ruffles, not all Gab1 associated enzymes and adaptor molecules that are localized to dorsal ruffles are required for their formation. Those molecules in Figure 6C that are draw in shades of blue, both localize and shown to be required for HGF stimulated Gab1 enhanced dorsal ruffles. Those in red have been localized to these dorsal ruffles but are not required. Green molecules are localized to dorsal ruffles but remain to be tested as to their requirement in this system. Interestingly, although many molecules that are localized to dorsal ruffles are also required for their formation, we have demonstrated that this is not always the case as in the example of MEK-ERK. Thus the elucidation of the differences between location and function can begin to provide the details of how these elaborate membrane protrusions are generated.

Figure 1. Gab1 is localized to sites of cell-cell contacts in three-dimensional MDCK cell culture and accumulates into basal membrane protrusions. (A) MDCK cells expressing either GFP Gab1 or GFP Gab1ΔPH were allowed to form cysts in a three-dimensional collagen matrix for 7 days. Immuno-fluorescence with Alexa conjugated 546 phalloidin demonstrate the localization of Gab1 and Gab1ΔPH in an organoid structure. Inset of the area indicated with a white box are a 2 fold enlargement. (B) MDCK GFP Gab1 cells were treated as in (A) and a DIC image with CLSM was acquired using a 63X objective, left un-stimulated or treated with 10U/ml HGF for 8 hours. All scale bars represent 10μm. (C) Bottom panel in (B) was stained for F-actin as in (A) and imaged in three-dimensions with LSCM where 37 z-planes at 1.26μm intervals and a 63X objective, followed by surface rendering using Imaris software. A 16μm line is drawn along a protrusion and annotated by points A and B. (D) Fluorescence intensity of F-actin (red) and GFP Gab1 (green) over the distance of the line from point A to B drawn in (C). (E) Pictorial representation of polarized epithelial cells with cell-cell contacts, apical membrane, and basal surface where a protrusion as in (B and C) would occur with enlargement of protrusion demonstrating Gab1 and F-actin localization. (F) Sequential images of a single MDCK GFP Gab1 cell acquired with either TIRF or WFM using 100X objective.

Figure 1

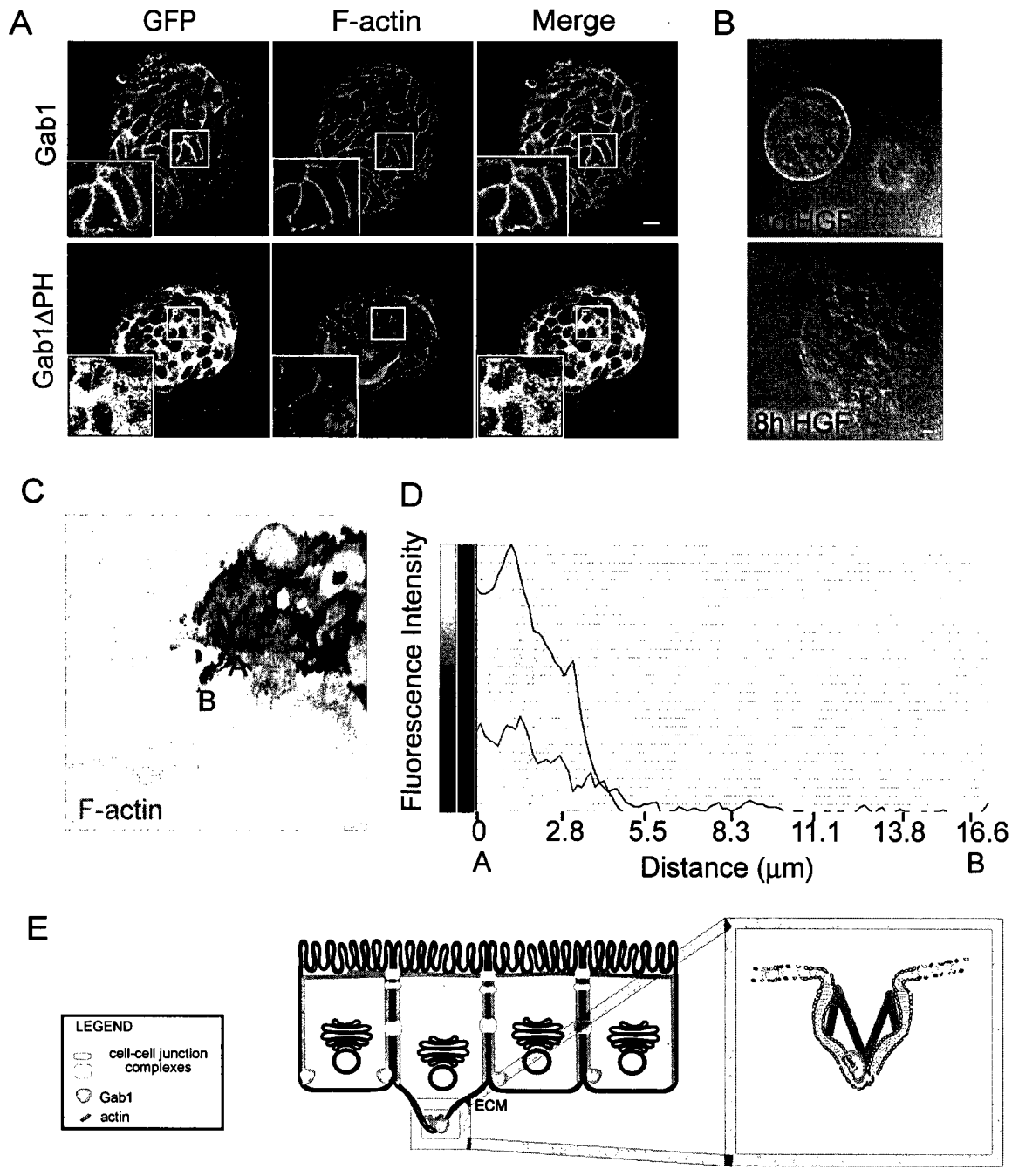

A
GFP
F-actin
Merge
Gab1
Gab1ΔPH
B
8h HGF
C
A
B
F-actin
D
Fluorescence Intensity
0
2.8
5.5
8.3
11.1
13.8
16.6
A
Distance (μm)
B
E
LEGEND
cell-cell junction complexes
Gab1
actin
ECM
F
TIRF
WFM

Figure 2. Gab1 co-localizes with F-actin in dorsal ruffles MDCK cells cultured in two-dimensions. (A) MDCK HA Gab1 cells were allowed to form colonies overnight and left un-stimulated or stimulated with 100U/ml HGF for 8.5 minutes. Indirect immuno-fluorescence with anti HA antibody and F-actin staining is shown. The most apical z-plane is acquired with CLSM and 100X objective and the inset is a 3 fold enlargement of the denoted area. Scale bars represent 10μm. (B) MDCK GFP Gab1 colonies of cells were stimulated for 5 minutes with 100U/ml HGF, fixed and stained for F-actin. SDCM acquired 61 z-planes with 0.2 μn intervals and these were deconvolved using the blind re-itterative method. The X-Y plane shown is a maximum projection of the deconvolved data where GFP Gab1 is in green and F-actin in red. The florescence data alone the blue and green lines are shown for the Y-Z and X-Z planes respectively. Scale bar represents 7 μm. (C) MDCK HA Gab1 cells were stimulated at indicated times with 100U/ml HGF and protein extracts were subject to anti HA immuno-precipitation and western blot analysis with indicated antibodies. Whole Cell Lysates (WCL) were probed for actin and serve as a loading control.

Figure 2

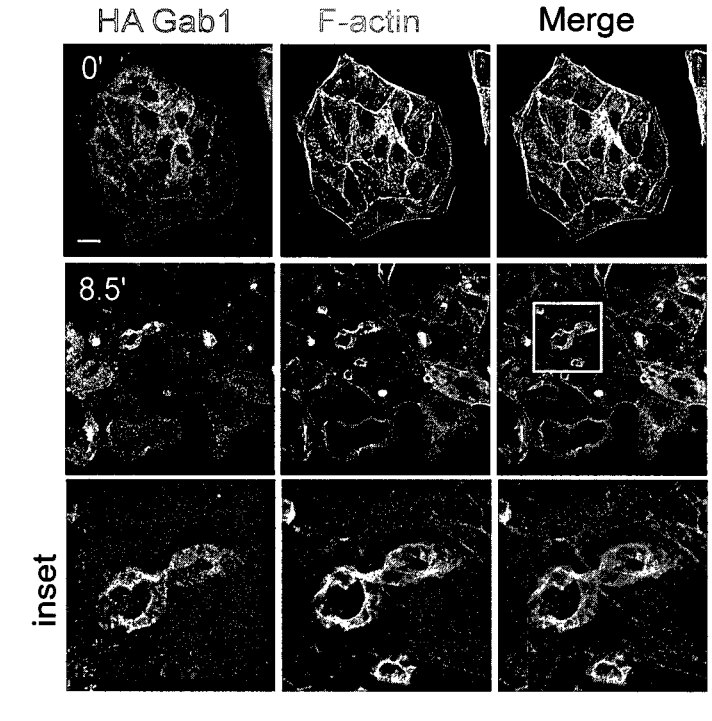
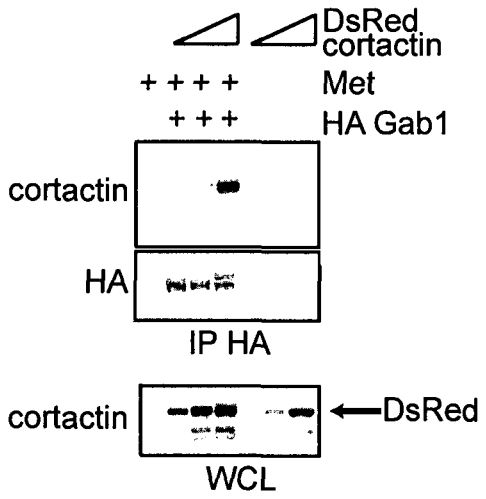

A
HA Gab1
F-actin
Merge
0'
8.5'
inset
B
z
z
GFP Gab1
F-actin
C
HGF (minutes): 0 15 30 60 180
phospho-Tyr
HA
actin
IP HA
actin
WCL

Figure 3. Gab1 engages with cortactin and are both localized to HGF stimulated dorsal ruffles. (A) HeLa cells are transiently transfected with HA Gab1 cDNA and the next day, cells are stimulated with 100U/ml HGF for 8 minutes, fixed, and indirect immuno-fluorescence to identify transfected cells (outlined) and co-stained with cortactin and F-actin. Area marked with a white box is enlarged 5 fold. Scale bar is 10μm. (B) Living MDCK cells expressing both GFP Gab1 and DsRed cortactin were imaged using WFM, and a frame is shown from time-lapse microscopy 18 minutes 20 seconds post 100U/ml HGF stimulation. Enlargement of indicated area is 3 fold. (C) HEK 293 cells are transfected with vectors expressing indicated cDNA, and protein extracts are immuno-precipitated with HA antibody and western blot analysis with indicated antibodies. WCL demonstrate increasing amounts of DsRed transfected. (D) Dorsal ruffle assays of MDCK cell lines expressing either fluorescently tagged Gab1 or cortactin, or both were done by visualizing F-actin and determining the percentage of cells that make dorsal ruffles from three independent experiments.

C


DsRed
cortactin
+ + + +
+ + +
Met
HA Gab1
cortactin
HA
IP HA
cortactin
DsRed
WCL

D

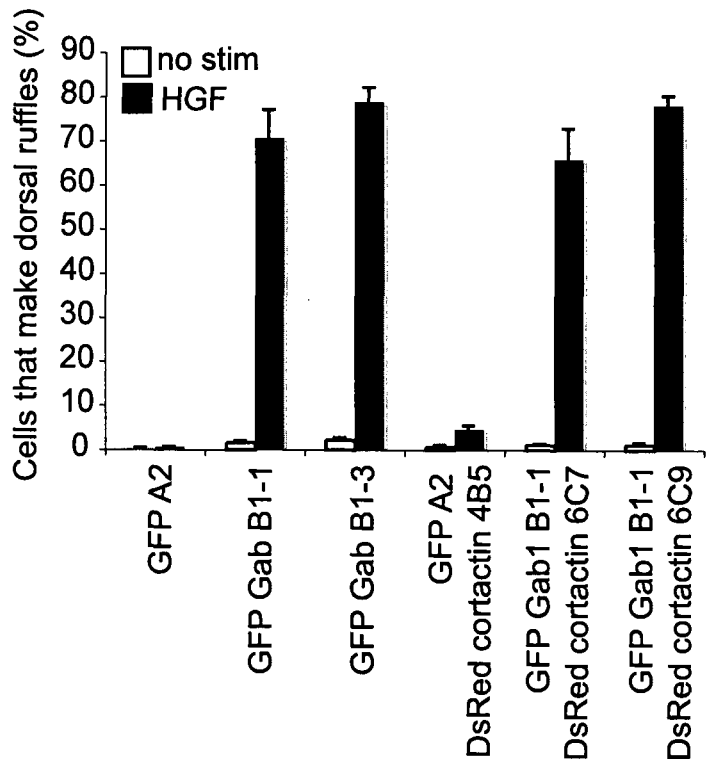

Cells that make dorsal ruffles (%)
90
80
70
60
50
40
30
20
10
0
no stim
HGF
GFP A2
GFP Gab B1-1
GFP Gab B1-3
GFP A2
DsRed cortactin 4B5
GFP Gab1 B1-1
DsRed cortactin 6C7
GFP Gab1 B1-1
DsRed cortactin 6C9

Figure 3

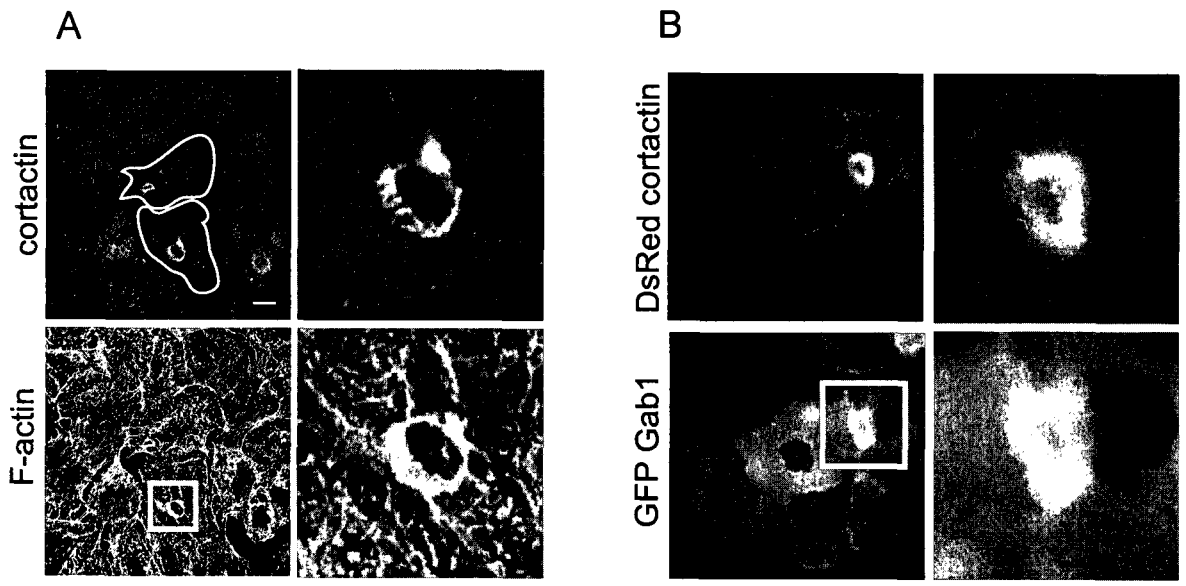
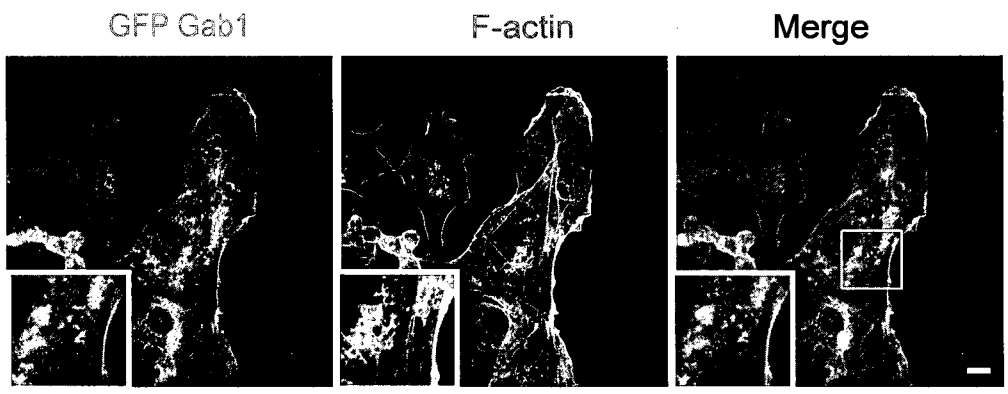


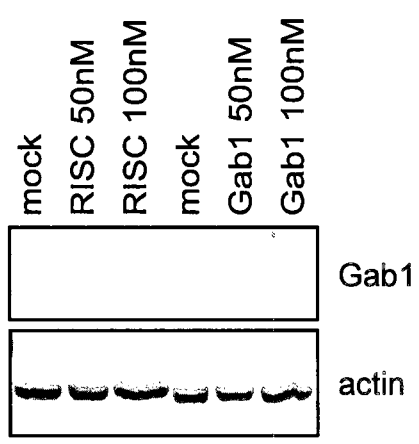
Figure 4. Gab1 is required for the integrity of F-actin rich structures in MDA-231 cells. (A) The MDA-231 breast cancer cell line was transfected with GFP Gab1, fixed, stained for F-actin and visualized with LSCM. The boxed area in the merge image is enlarged 2 fold in the inset. (B) MDA-231 cells were treated either under mock conditions (no siRNA), with RISC targeting siRNA as a negative control, or with Gab1 targeted siRNA at indicated concentrations. Western blot analysis with Gab1 antibody was used to determine extent of Gab1 knock-down and actin was used as a loading control. (C) The actin cytoskeleton is visualized of MDA-231 cells treated by using LSCM and acquiring the most basal z-plane. Insets are a 2 fold enlargement of the area of the cell where F-actin structures are found. All scale bars represent 10μm.

Figure 4

A


GFP Gab1
F-actin
Merge

B


mock
RISC 50nM
RISC 100nM
mock
Gab1 50nM
Gab1 100nM
Gab1
actin

C

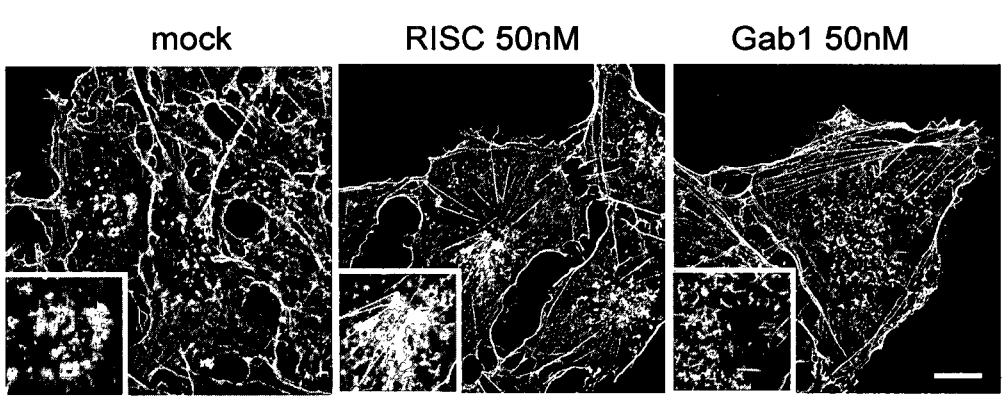
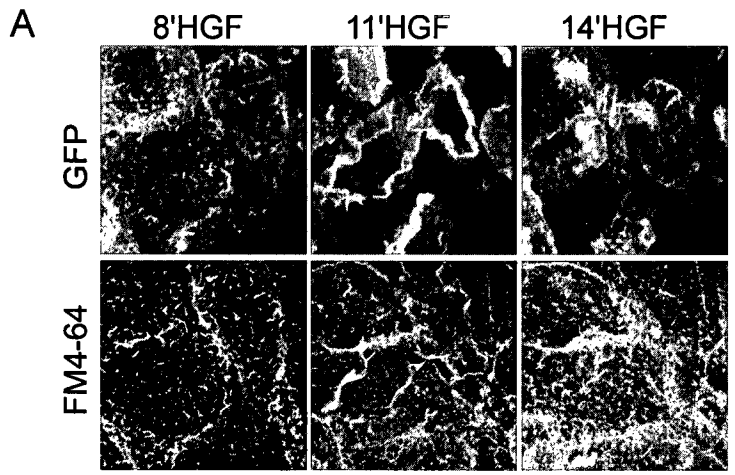
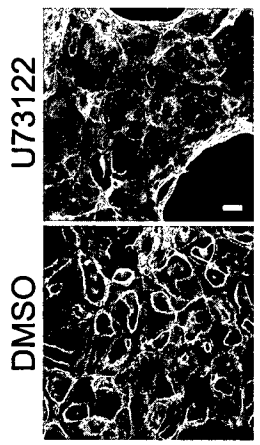
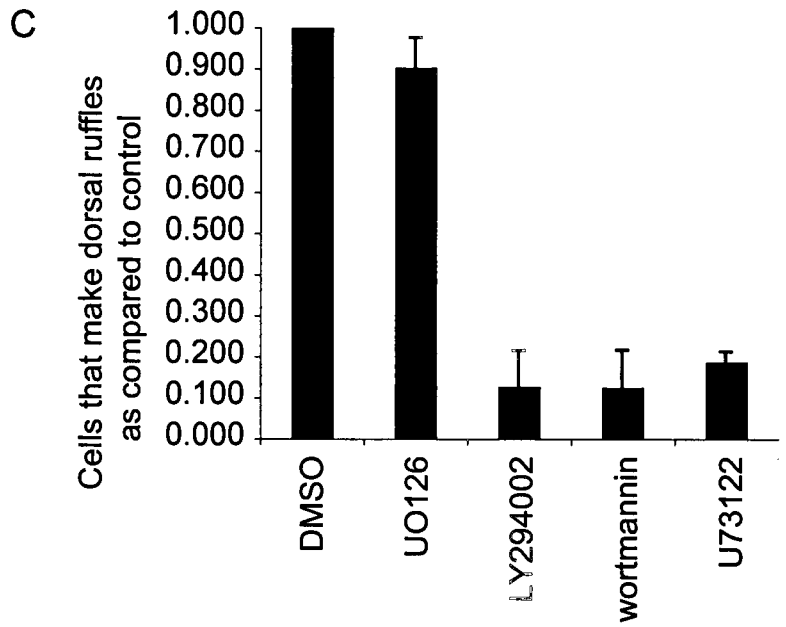

mock
RISC 50nM
Gab1 50nM

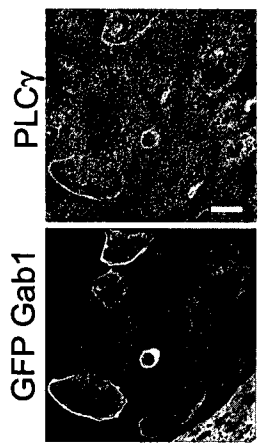
Figure 5. Gab1 dorsal ruffles are sensitive to inhibitors of PI3K and PLCγ, but not MEK. (A) MDCK GFP Gab1 cells imaged live by SDCM. Cells were stimulated with 100U/ml HGF and a membrane dye FM4-64. The images are a maximum intensity projection from 25 z-planes acquired with 0.5μm intervals and a 63X objective. These are images from time-lapse experiments with a 4 fold enlargement at indicated time points post-HGF stimulation. There is a 19 second delay between the two fluorophore acquisition times due to the time it takes to capturing the z-stack for first FM4-64 then GFP Gab1. (B) Dorsal ruffle assays of MDCK GFP Gab1 cells were done where cells were either pretreated with DMSO or the PLCγ inhibitor U73122 for 30 minutes before stimulation for 5 minutes with 100U/ml HGF. A representative image of an apical z-plane using CLSM and the effect of U73122 on dorsal ruffle formation by visualization of the actin cytoskeleton. (C) Dorsal ruffle assays from two independent experiments of MDCK GFP Gab1 cells pretreated with indicated conditions as in (B) and the response represented as compared to DMSO control. (D) MDCK GFP Gab1 cells plated on glass coverslips and the following day stimulated with 100U/ml HGF for 5 minutes, fixed and indirect immuno-fluorescence using anti-PLCγ antibodies to determine the subcellular localization of PLCγ with respect to GFP Gab1. (E) MDCK cells transfected with a fluorescence reporter of PIP_3 localization (GFPPHAKT) and stimulated for 8 minutes with 100U/ml HGF. Images were acquired of the most apical z-plane using LSCM. Area indicated by a white box in merge image is enlarged 3.5 fold. All scale bars represent 10 μm. (F) MDCK cells and those that stably express Ras were subject to dorsal ruffle assays. The extent of Ras over-expression over parental cells is demonstrated by the inset western blot analysis.

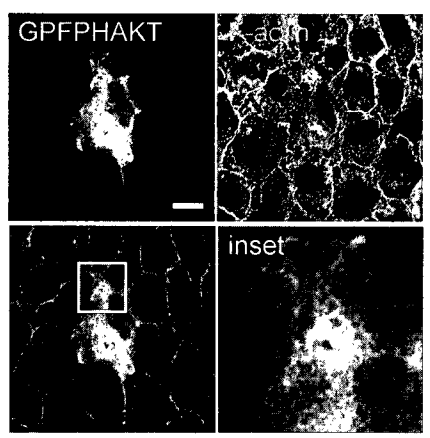
Figure 5


A
8'HGF
11'HGF
14'HGF
GFP
FM4-64


B
U73122
DMSO


C
Cells that make dorsal ruffles as compared to control
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
DMSO
UO126
LY294002
wortmannin
U73122


D
PLCγ
GFP Gab1


E
GPFPHAKT
actin
inset

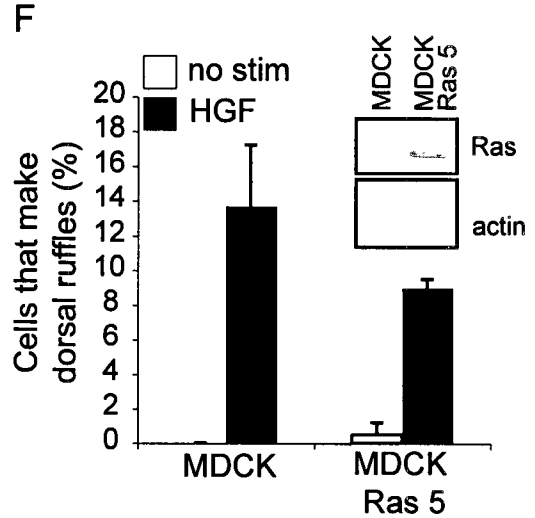
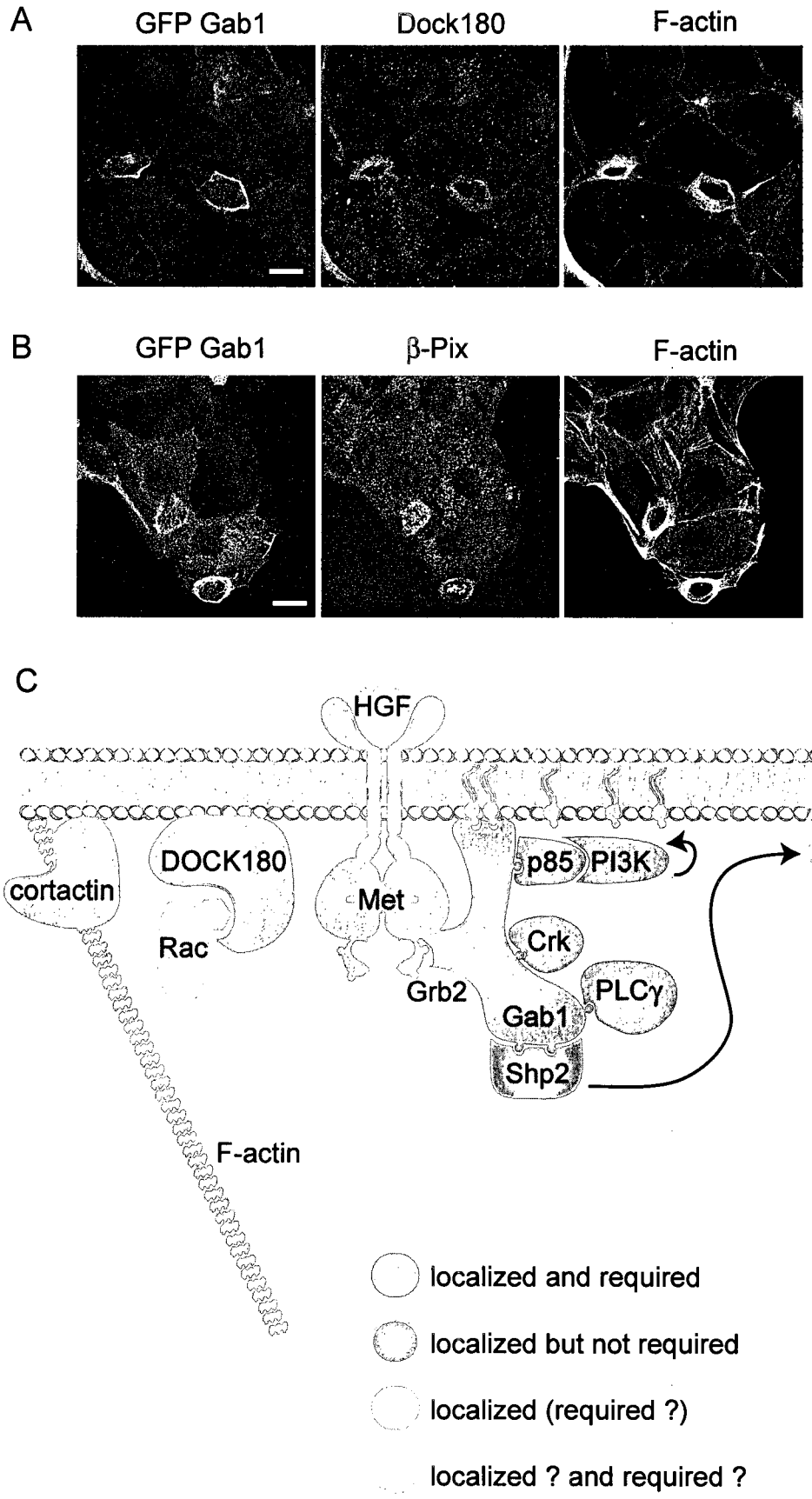

F
no stim
HGF
Cells that make dorsal ruffles (%)
20
18
16
14
12
10
8
6
4
2
0
MDCK
MDCK Ras 5
MDCK
MDCK Ras 5
Ras
actin

Figure 6. Gab1 co-localizes with DOCK180 in HGF stimulated dorsal ruffles. MDCK GFP Gab1 cells were stimulated for 10 minutes with 100U/ml HGF to activate dorsal ruffles and cells were fixed, and stained for DOCK180 (A) or β-Pix (B) and F-actin. LSCM images using 100X objective of the most apical z-plane are shown here. Scale bar represents 10μm. (C) Model drawing of the proteins localized to and required for HGF induced, Gab1 enhanced dorsal ruffle formation in MDCK cells.

Figure 6


A
GFP Gab1
Dock180
F-actin
B
GFP Gab1
β-Pix
F-actin
C
HGF
DOCK180
cortactin
Rac
Met
Grb2
p85
PI3K
Crk
PLCγ
Gab1
Shp2
Ras
Raf
MEK
ERK
F-actin
localized and required
localized but not required
localized (required ?)
localized ? and required ?

Discussion

Gab1 expression is required for dorsal ruffle formation in MDCK cells (Abella and Frigault et al. submitted). Also, Gab1 is required for the integrity of F-actin structures at the basal surface of a breast cancer cell line MDA-231 (Figure 4). We propose that the requirement of Gab1 in these processes is not solely for the relay of signals to activate other molecules, which in turn regulate the productive formation of these protrusions. Gab1 may function directly at the site of membrane protrusion to coordinate the local activation of required signals, such as providing a complex of proteins that dynamically remodels the constituents of the membrane and provides a physical link to the actin cytoskeleton. Evidence for this is provided herein, where Gab1 is actively localized to sites of F-actin rich membrane ruffling both at apical (MDCK two-dimensional cultures Figure 2) and basal surfaces (MDCK three dimensional cultures and MDA-231 two-dimensional cultures Figures 1 and 4). Using various different methods of imaging, Gab1 is co-localized in a dynamic fashion with both F-actin as well as the actin associated protein cortactin (Figures 1, 2 and 3, and live cell imaging movies not shown). Upon HGF stimulation, Gab1 engages with actin (Figure 2) and Gab1 can also be coimmuno-precipitated with cortactin in the presence of Met (Figure 3). These data demonstrate that Gab1 can be physically linked to the actin cytoskeleton.

Cortactin is a nucleating factor which functions downstream of Rac to bridge F-actin cables and activate the elaboration of new branches via the Arp2/3 complex. The creation of branched actin networks occurs at sites of active membrane ruffling and provides the force to push the membrane in order to create an extension. Taken together with the co-localization of Gab1 into cortactin and F-actin rich protrusions, Gab1 may be

involved in physically linking the actin meshwork with the membrane at the leading edge.

Indeed Gab1 is tightly associated with the plasma membrane (Movies 5 and 6). The membrane of HGF induced dorsal ruffles is PIP_3 rich and PI3K enzymatic activity is required for their formation (Figure 5). Studies of phagocytosis have demonstrated that there is dynamic turnover of both PIP_2 and PIP_3 during engulfment via the formation of large phagocytic ruffles on the apical surface of macrophages (Botelho et al., 2000; Marshall et al., 2001). The dynamics of PIP_2 disappearance and PIP_3 appearance at sites of dorsal ruffling in response to EGF is highly dynamic (Araki et al., 2007). Moreover, PIP_2 is concentrated at sites of actin remodeling and ruffling and not localized diffusely throughout the plasma membrane (Tall et al., 2000). In MDCK cells, Gab1 is co-localized with enzymes of lipid metabolism, PLCγ, and the p85 subunit of PI3K in dorsal ruffles (Figure 5 and Abella and Frigault et al. submitted). It therefore is attractive to speculate that Gab1 may recruit these molecules to active sites of membrane remodeling to provide the temporal fine-tuning of phospho-lipids required to sustain dorsal ruffle formation.

However, previous data demonstrates that the recruitment of the p85 subunit of PI3K to Gab1 is dispensable for the activation of signals to induce dorsal ruffles (Abella and Frigault et al. submitted). Since the enzymatic activity of PI3K is absolutely required for dorsal ruffle formation downstream of Met we suggest that Gab1 mediated PI3K signaling is dispensable and that PI3K activity is an upstream signal for dorsal ruffle formation. The PI3K signal may thus function to transduce signals that will activate the physical machinery for ruffle formation. PI3K is required for the complex coordination

of signals that dictate ruffling dynamics and motility (Innocenti et al., 2003; Zhou et al., 1998). Moreover, the Gab1 PH domain specifically binds PIP_3, the PI3K product, and Gab1 must be membrane targeted to be functional. Therefore, it is possible that PI3K activity generates higher concentrations of PIP_3 in the membrane generating a binding site for Gab1 to the plasma membrane (Rodrigues et al., 2000). The onset of PI3K activation and therefore the production of PIP_3 may serve as an initiating event in HGF induced dorsal ruffle formation.

PLCγ is localized to dorsal ruffles with Gab1 upon HGF stimulation (Figure 5). This enzyme has been reported to bind Gab1 downstream of Met (Gual et al., 2000), suggesting that this complex exists in dorsal ruffles. Moreover we have previously shown that a Gab1 mutant uncoupled from both Crk and PLCγ is unable to activate the robust induction of dorsal ruffle formation upon HGF stimulation (Abella and Frigault et al. submitted). In accordance with this, inhibition of PLCγ abrogates dorsal ruffle formation (Figure 5). Taken together, PLCγ signaling in dorsal ruffle formation downstream of Met is essential.

PLCγ hydrolyses PIP_2 into two lipid second messengers IP_3 and DAG. These subsequently serve to activate calcium and PKC signaling pathways respectively, both of which have yet to be determined as to whether or not they play a role in dorsal ruffle formation or maintenance. Although we show that enzymatic activity of PLCγ is required for dorsal ruffle formation, it is also likely that non-enzymatic functions of this protein may regulate dorsal ruffle formation. Indeed, PLCγ recruitment to the plasma membrane is dependent on its SH2 and SH3 domains (Bar-Sagi et al., 1993). Additionally, the SH3 domain of PLCγ has been reported to function as a GEF for PIKE

proteins which are enhancers of PI3K signaling thus amplifying the signal (Wang and Moran, 2002). The role of PLCγ in chemotaxing *Dictyostelium discoideum* is to function as a regulator of the PI3K signal (Kolsch et al., 2008), and *plc*-null cannot activate the production of PIP_3 (Korthol et al., 2007). It is therefore likely that PLCγ regulates PI3K activity, alternatively PLCγ may control the concentration of PIP_2 substrate available to PI3K via a competitive mechanism, although this has not been demonstrated in the literature. Another role for PLCγ in dorsal ruffles might be related to its functions as a GEF for the GTPase dynamin (Choi et al., 2004), which is localized to and regulates dorsal ruffles in fibroblasts stimulated with EGF and PGDF (Krueger et al., 2003; Orth et al., 2006).

The activation of Rac is a hallmark mechanism for remodeling of the actin cytoskeleton. Rac activation results in the formation of branched F-actin networks. Moreover, cortactin is required for dorsal ruffle formation (Weed et al., 1998). However, the question remains, how is Rac1 activated downstream of Met? We observe that DOCK180 is co-localized with Gab1 in HGF stimulated dorsal ruffles in MDCK cells (Figure 6) and we propose that this GEF might function locally to activate Rac and hence localized actin cytoskeletal meshwork elaboration and membrane protrusion. DOCK180 binds PIP_3 in membrane ruffles via its DHR domain, an unsuspected modular protein domain for lipid binding (Cote et al., 2005). Moreover, the DOCK180 exchange function is regulated by its association with Crk in a mutli-molecular complex (Akakura et al., 2005; Gumienny et al., 2001; Valles et al., 2004). Crk is co-localized with Gab1 in dorsal ruffles and the Gab1-Crk interaction is required for the induction of HGF induced dorsal ruffles (Abella and Frigault et al. submitted). We suggest that the Gab1-Crk

complex recruits a functional DOCK180 that binds to the same lipid ligand as Gab1, the PI3K product PIP_3. These data provide evidence to suggest that Gab1 coordinates the activation of signals for remodeling the cytoskeleton as well as the membrane in order to make dorsal ruffles downstream of the Met receptor. The elucidation of the requirement as well as dynamic localization of signaling molecules downstream of Met in dorsal ruffles, will provide mechanistic insight as to how these dynamic structures are formed.

Acknowledgements

We would like to thank Dr. Marc McNiven for the DsRed1cortactin cDNA, and Dr. Sergio Grinstein for the GFP tagged PH domain of AKT used in these studies. Thank you to Dr. Claire Brown for her help with the TIRF imaging and her function as a microscopy reference as well as to Dr. Judith Lacoste for her help on with SDCM and velocity. Thank you to Dr. Robin Battye from Quorum Technologies, for demonstrating his SDCM and helping us acquire detailed images of Gab1 in a dorsal ruffle.

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Chapter 5

General Discussion

1. Conclusions

The work presented in this thesis demonstrates the function of Gab1 as a multi-purpose Met specific modulator, which regulates the ensuing signal via multi-protein complex formation for signal relay. Moreover, the data presented herein delineates specific elements of Gab1 as essential for formation of various types of membrane protrusions in epithelial cells downstream of Met. The studies presented here also demonstrate that Gab1 is consistently recruited to active sites of membrane ruffling, no matter the type of ruffle, and suggest that the requirement for Gab1 is not just in the initiation of the signal, but for the capacity of Gab1 to function as a docking protein at the site of ruffle formation.

2. Microenvironments of the plasma membrane

Signal transduction initiated by Met, via Gab1, from the plasma membrane leading to the activation of signals in the cytoplasm which will eventually be relayed to the activation of changes in transcription of genes in the nucleus, is a simplistic view. In fact, a lot of data has suggested that the subcellular localization of Gab1, namely targeting to the plasma membrane, dictates the capacity of Gab1 to signal. However, the cell surface is not all the same, there exist a variety of membrane micro-environments which are defined by certain characteristics.

2.1 Lipid Rafts

Lipid rafts are assemblies of specialized lipids and proteins in distinct regions of the plasma membrane. These are detergent insoluble glycoprotein rich membrane

pockets, which are cholesterol rich and thus are more compact ordered membrane platforms and not as fluid as non-raft plasma membranes (Brown and London, 2000). In T and B lymphocytes, lipid rafts organize signaling complexes by clustering the multi-component T cell receptor (TCR) and B cell receptor (BCR) for productive signaling (Sedwick and Altman, 2002). These receptors do not have intrinsic kinase activity and rely on the recruitment and activation of Src family kinases, which are tightly associated with the membrane by lipidation and constitutively localized to lipid rafts (Filipp et al., 2004; Melkonian et al., 1999; Shenoy-Scaria et al., 1993). Lipid rafts are essential for axon guidance, which is a chemotactic response that requires active remodeling of the actin cytoskeleton (Guirland et al., 2004; Herincs et al., 2005).

Lipid rafts have been identified in epithelial cells, including MDCK cells, and are found to traffic as an entity from the synthetic pathway to the apical surface. Moreover, lipid rafts undergo endocytosis by a caveolin-associated mechanisms in MDCK cells (Schuck and Simons, 2004). Since dorsal ruffles arise on the apical surface, are dorsal ruffles composed of lipid rafts? Studies downstream of PDGF stimulation in fibroblasts demonstrate that there are two distinct pools of PDGFR-Src family kinases (SFK) complexes. Those in caveolin, cholesterol rich lipid rafts are required for mitogenic signals such as DNA synthesis, whereas those complexes in the non-raft pool activate signals for dorsal ruffle formation (Veracini et al., 2006). This therefore suggests that dorsal ruffles are not lipid rafts, however, we have seen endogenous caveolin co-localized with Gab1 in HGF stimulated dorsal ruffles in MDCK cell (data not shown). Caveolin is required for the transport of apical surface receptors by transcytosis in MDCK cells and may accumulate in dorsal ruffles passively (Shmuel et al., 2007).

2.2 Phagocytic membrane protrusions

We have described the extensive localization of the p85 subunit of PI3K in dorsal ruffles (Chapter 3 Supplemental Figure 1), as well as its lipid product PIP_3 and have demonstrated the requirement of PI3K enzymatic activity for HGF induced dorsal ruffle formation (Chapter 4 Figure 5). Therefore, to confirm or deny dorsal ruffles as lipid rafts, a survey of RTKs signaling to PI3K from lipid rafts is discussed. FGFR2 in osteoclasts associates with the ubiquitin ligase Cbl in a raft and this leads to the formation of a PI3K-Cbl complex, PI3K protein degradation and induction of cell death (Dufour et al., 2008). These data suggest that non-raft associated PI3K is a positive signal and thus would support dorsal ruffles. Contradicting evidence in a small cell lung cancer cell line demonstrates that c-Kit signaling to PI3K requires the integrity of lipid rafts (Arcaro et al., 2007), however, these are cells with oncogenic potential, and PI3K signaling from rafts may be an acquired mechanism for cell survival of tumorigenic cells. Phagocytic cells generate large membrane protrusions for the engulfment of pathogens. PI3K activity is necessary for this process and some reports demonstrate that pathogen entry occurs via lipid rafts. *Group B streptococci* use lipid rafts and PI3K signaling to invade endometrial cells (Goluszko et al., 2008), and infection of alveolar macrophages by *Pseudomonas aeruginosa* occurs via cholesterol rich membranes and activates PI3K signaling (Kannan et al., 2008). Although phagocytosis generates membrane protrusions for engulfment, this is clearly a distinct cellular process from dorsal ruffle formation as described in this thesis.

The elucidation of much of the elements involved in actin cytoskeleton dynamics was done by studying pathogens, which sequester the actin machinery to drive entry into cells (Frischknecht and Way, 2001). Viruses, bacteria, and parasites utilize tyrosine kinase and Rho GTPase signaling pathways of the host cell to achieve efficient entry, which requires remodeling and recruitment of the actin cytoskeleton as well as the recruitment of molecules associated with actin (Munter et al., 2006). A protein on the bacterial cell surface of *Listeria monocytogenes*, InlB binds to and activates the Met receptor and is sufficient to activate MDCK cell scatter (Shen et al., 2000). This bacterial protein is a PI3K agonist, leads to Gab1 phosphorylation and complex formation with p85, and activates the localized accumulation of PIP_3 in large membrane ruffles (Ireton et al., 1999; Shen et al., 2000). The ensuing large membrane ruffles produced by InlB engagement with Met are sensitive to cholesterol depletion, but not at the level of Met activation, rather only for the subsequent signals to remodel actin. These data suggest that lipid rafts may also play a role in this process (Seveau et al., 2004). However, InlB binds a second receptor, gC1q-R, which also leads to Gab1 phosphorylation and PI3K activation (Braun et al., 2000) and since Met activation is not sensitive to cholesterol depletion, perhaps gC1q-R requires the integrity of lipid rafts for signaling to the actin cytoskeleton. We have shown that dorsal ruffles require the Gab1-Crk complex but that the Gab1-p85 complex is dispensable (Chapter 3 Figure 4). Similarly, Gab1 over-expression facilitates *Listeria* entry whereas Gab1ΔCrk and Gab1Δp85 mutants are unable to do so (Sun et al., 2005), demonstrating similar requirements for Gab1-Crk but also distinct complexes for bacterial entry (Gab1-p85). *Listeria* has therefore evolved to selectively activate the Met receptor, activating the signals required for dorsal ruffle

formation, and likely to provide a method of entry into its host since Met is itself internalized via dorsal ruffles (Chapter 3). While some phagocytic ruffles involve cholesterol rich membranes, dorsal ruffles may not, however, this possibility should be tested in light of all the evidence discussed herein. It is controversial whether or not lipid rafts are present in all cell types, and that these are a uniform mechanism of compartmentalization of signaling pathways, since much of the studies of these structures relies on detergent insolubility, and they remain to be definitively imaged and widely accepted (Munro, 2003).

2.3 Membrane ruffles as a signaling compartment

Signaling pathways are regulated by the composition of complexes, the magnitude of the output, the duration, and the location of the signal. For actin-based cellular protrusions, it has been established that numerous regulators of this process are localized specifically to sites of membrane ruffles. As discussed in other sections of this thesis, but briefly summarized here, molecules that are essential for membrane ruffle formation are localized to sites of active membrane ruffling. The membrane constituent PIP_3 is specifically localized at the leading edge of chemotaxing unicellular and multicellular eukaryotic cells and in migrating cells (Kolsch et al., 2008; Wang et al., 2002), GTP loaded Rac accumulates at sites of membrane protrusions (Del Pozo et al., 2002; Gardiner et al., 2002), and the subcellular localization of cortactin is regulated such that in migrating cells, it is protected from cleavage only at the leading edge of the cell (Perrin et al., 2006).

Gab1 is also dynamically localized to membrane ruffles. By observing the changes in localization of GFP tagged Gab1 upon HGF stimulation in MDCK cells, we have shown that nearly all Gab1 accumulates into active sites of dorsal ruffles (Chapter 4 and live cell imaging, data not shown). Moreover, in MDCK cells stimulated for 15 minutes with HGF and fixed, Gab1 localizes to the leading edge of lamellipodia (Chapter 2 Figure 5). Also, in cells where the mutant CSF-MetΔGrb2 receptor is stimulated for the same amount of time, Gab1 accumulates into lamellipodia (Chapter 2 Figure 5). Gab1 accumulates into dorsal ruffles as does the activated Met receptor (Chapter 3 Figure 1), and Met also is co-localized into lamellipodia with Gab1 in HeLa cells stimulated with HGF for 15 minutes (Figure 1).

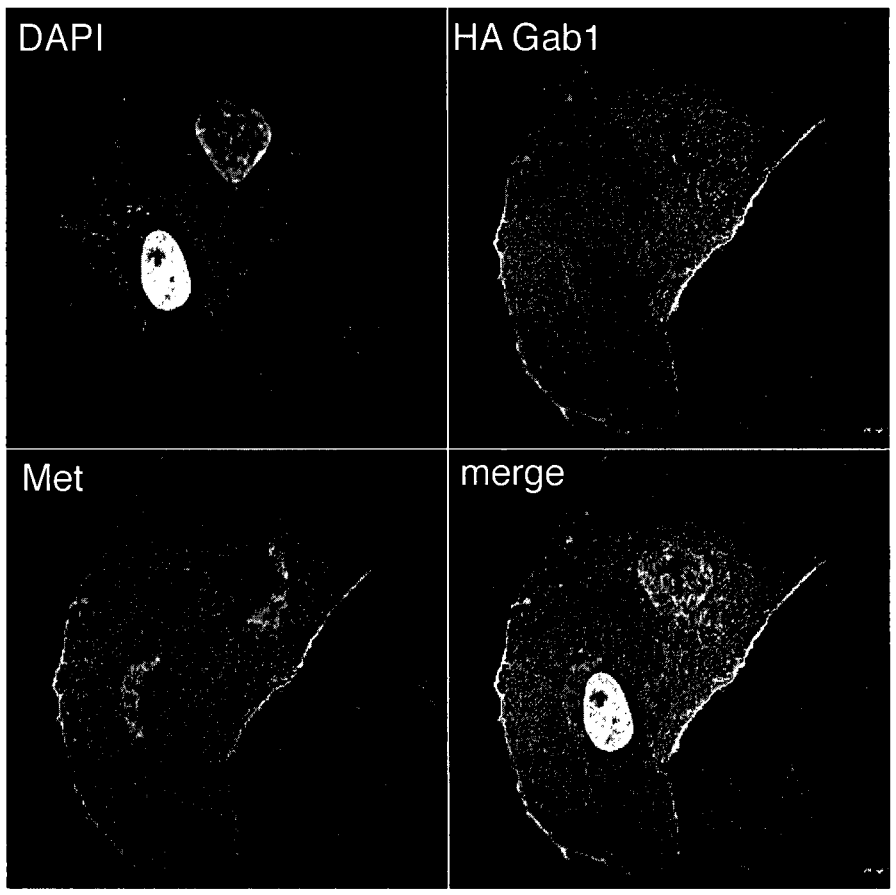

Figure 1. Met and Gab1 co-localize in lamellipodia induced by HGF stimulation.

HeLa cells were plated on glass coverslips and transfected the following day with HA tagged Gab1. Cells were stimulated 48hours later for 15 minutes with 100U/ml HGF, fixed and stained using anti HA (green), anti-Met (red) antibodies as well as nuclei by dapi (blue). SDCM with 100X objective were used to acquire images of two cells, which have elaborated lamellipodia in opposing directions. The predominate localization of Gab1 occurs at the edge of the lamellipodia, whereas Met is also localized to lamellipodia and also to the synthetic pathway in the golgi.

Membrane ruffles are an actin-based compartment, which contain all the regulators necessary to remodel and regulate protrusions. Since Gab1 binds PIP_3, is involved in complex formation which regulates Rac activation, and can form a complex with actin and cortactin, we propose that Gab1 is localized to HGF induced ruffles to

coordinate their formation and to form a signaling complex in order to transduce information from this location.

3. Gab1 associated signals downstream of Met for membrane ruffle formation.

Gab1 is a docking adaptor protein, which recruits several other signaling molecules, both adaptors and enzymes. By structure function studies described in this thesis, and work from previous members of the lab, the function of the Gab1-associated protein interactions can be inferred. The work presented throughout this thesis has elucidated that specific Gab1 complexes may not only regulate different signals, but also different types of membrane ruffles downstream of Met.

3.1 Sustained Recruitment of Gab to RTKs for lamellipodia formation.

Downstream of the Met receptor, Gab1 and Gab2 differ in that Gab1 is able to promote epithelial morphogenesis and cell scatter, but not Gab2 (Frigault et al., 2008; Lock et al., 2002). Similarly, Gab1 promotes robust lamellipodia formation whereas Gab2 is unable to do so (Chapter 2 Figure 4). One of the most observable differences between Gab1 and Gab2 is their different mechanism of recruitment to the Met receptor. Gab1 is recruited indirectly by the adaptor Grb2 as well as via the Met binding Motif (MBM), whereas Gab2 is recruited to Met only via its interaction with Grb2 (Chapter 1 Figure 7) (Lock et al., 2002; Schaeper et al., 2000). Indeed, Gab1 recruitment via its MBM is required for its ability to promote epithelial morphogenesis since substitution of residues in the MBM for alanine abrogates Gab1 function (Lock et al., 2002). Another essential element of Gab1 for biological function downstream of Met, is the PH domain.

Gab1 must be membrane targeted via a functional PH domain or by myristoylation (myr) in order to rescue Met mediated epithelial morphogenesis (Maroun et al., 1999a; Maroun et al., 1999b). Both of these elements of Gab1, MBM and membrane targeting, are required for function in epithelial morphogenesis since myristoylation of Gab2, or MBM insertion into Gab2 are not sufficient to promote epithelial morphogenesis (Frigault et al., 2008; Lock et al., 2002). However, the morphogenic defect of Gab2 is switched by inserting both of these Gab1 required elements into Gab2, myrGab2MBM (Chapter 2 Figure 2).

Epithelial morphogenesis requires cellular processes such as actin cytoskeleton rearrangement and the formation of a membrane protrusion. In agreement with this, myrGab2MBM expressing cells make lamellipodia in response to stimulation to the same extent as Gab1 expressing cells, demonstrating that both the Gab1-Met interaction in concert with targeting to the membrane is required to produce lamellipodia (Chapter 2 Figure 4). Insertion of MBM into Gab2 may provide a more efficient recruitment of Gab2 to Met, thus permitting Met to continue to phosphorylate the Gab protein, which subsequently activates sustained signals. This suggests that Gab1 function requires both, direct recruitment to Met as well as specific membrane targeting, to result in lamellipodia formation. Since myrGab2MBM was sufficient to activate prolonged ERK and AKT signals (Chapter 2 Figure 6 and Supplemental Figure 1) and promote lamellipodia formation (Chapter 2 Figure 4), it is attractive to speculate that if a Gab scaffold is recruited to an RTK complex in a sustained manner where it is normally transient, this might change the fate of signaling.

Similarly, Gab1 recruitment to different RTKs also occurs via different mechanisms. For example, recruitment of Gab1 to Met occurs by a dual mechanism, however, recruitment of Gab1 to the EGFR occurs only in an indirect manner via Grb2. EGF signaling in MDCK cells does not promote the morphogenic program (Maroun et al., 2003), robust lamellipodia formation (Chapter 2 Figure 5), or dorsal ruffle formation (data not shown). Perhaps, by inducing a prolonged recruitment of Gab1 to the EGFR and subsequent prolonged activation of downstream signaling pathways this may switch the biological function of EGFR. Myristoylation of Gab1 indeed switches EGF to activate epithelial morphogenesis in MDCK cells as well as the activation of sustained tyrosine phosphorylation of Gab1 and downstream signaling pathways (Maroun et al., 2003). This protein is localized to the membrane constitutively, and membrane targeting is a requirement for recruitment of Gab1 to EGFR (Rodrigues et al., 2000). Perhaps membrane targeting provides a second mechanism of recruitment for a more stable association with the receptor, thus inducing a more prolonged phosphorylation of Gab1 as well as the formation of prolonged Gab1-associated protein complexes.

We suggest that if a Gab docking protein is recruited in a sustained manner to a RTK, it has conferred the ability to activate sustained signaling pathways and make a lamellipodia, which then can function to confer the morphogenic program. The ability of cells that over-express myrGab2MBM or myrGab2 to switch the EGF signal from non-morphogenic to one that produces cells to activate sustained signaling, lamellipodia and morphogenesis remains to be tested. These data would delineate the specificity of which Gab scaffold is required for the assembly of a complex that is lamellipodia and morphogenesis competent downstream of EGF, thus comparing these complex

requirements to those for Met induced epithelial morphogenesis. These experiments would also confirm or deny the hypothesis that sustained recruitment of a Gab protein to a receptor is the basic requirement for lamellipodia formation.

3.2 Gab1-Shp2 signals lead to lamellipodia formation.

Once the Gab scaffold is recruited to the cell surface receptor in a sustained manner, the composition of the complex then provides signal specificity. Namely, the Gab1-Shp2 complex is required for 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 (Chapter 2 Figure 7). Moreover, sustained activation of ERK as well as the morphogenic program, are impaired in cells expressing Gab1ΔShp2 (Chapter 2 Figure 7) (Maroun et al., 2000). These data delineate the requirement for a Gab1-Shp2 complex to make lamellipodia. Indeed, phosphorylated ERK is localized to lamellipodia in a sustained manner (Chapter 2 Figure 6) and MEK inhibition diminishes lamellipodia formation (Chapter 2 Figure 7), further demonstrating that the Gab1-Shp2-MEK-ERK pathway regulate lamellipodia formation.

The Gab1-Shp2 complex will activate a prolonged ERK signal downstream of the Met receptor, however, the mechanism by which Shp2 functions as a positive regulator of ERK activation remains to be comprehended. Gab1 itself may be a substrate of Shp2 resulting in de-phosphorylation of Gab1, thereby regulating other phosphorylated tyrosine mediated interactions. Indeed, in *SHP2* null fibroblasts or in cells that over-express a dominant negative Shp2 protein, Gab1 is hyper-phosphorylated (Agazie and

Hayman, 2003; Zhang et al., 2002). A Gab1 protein uncoupled from Shp2 has increased phosphorylation of tyrosine residues that lie in p85 recruitment sites, leading to a more robust activation of the PI3K pathway (Zhang et al., 2002). These data suggest Gab1 is a substrate of Shp2 but does not explain the mechanism by which Shp2 leads to ERK activation. One such molecular mechanism has been proposed where Shp2 regulates the phosphorylation of the CSK regulator PAG, which in turn controls the ability of CSK to activate Src, which functions to activate Ras, the first kinase in the cascade for ERK activation (Zhang et al., 2004). However, the phosphorylation site which regulates this mechanism characterized in mouse cells is not conserved in dog or human and thus cannot provide a mechanism for Met mediated Gab1-Shp2 activation of the Ras-ERK pathway in MDCK cells. A more plausible mechanism of ERK activation is that Gab1 may function to target Shp2 to the proper sub-cellular localization to reach its substrates. It has been demonstrated that PIP_3 concentrations are required for Gab1-Shp2 mediated ERK activation (Yart et al., 2001). Moreover, studies of fusion protein chimeras where the Gab1 PH domain fused to Shp2 without the auto-inhibition SH2 domain could activate a sustained MEK (ERK kinase) signal (Cunnick et al., 2002). The work presented in Chapter 2 further supports the concept that Gab1, or myrGab2MBM, functions to mediate epithelial morphogenesis and the activation of a sustained ERK signal by being localized specifically to lamellipodia downstream of Met, where Shp2 and activated ERK are also co-localized (Chapter 2 Figures 6 and 7). Thus Gab1 serves to localize Shp2 to a specific membrane domain where substrates of Shp2 are presumably readily accessible and thereby facilitate ERK activation due to the local accumulation of protein complexes required to propagate the signal.

Gab1, Shp2 and phosphorylated ERK are also co-localized in HGF stimulated dorsal ruffles in MDCK cells (Chapter 3 Figure 1 and Supplemental Figure 1). In contrast to the requirement for lamellipodia formation, the Gab1-Shp2 complex is not required for the induction of dorsal ruffles. Where the Gab1-Shp2 complex is instructive for the formation of lamellipodia, it is not necessary in order to activate signals to make dorsal ruffles. There may be temporal differences at work here, a dorsal ruffle has been described to be a precursor of peripheral ruffles in fibroblast cells where cells that makes a dorsal ruffle are 2.5 fold more likely to produce lamellipodia than those that do not (Krueger et al., 2003). In MDCK cells, Gab1 accumulates into dorsal ruffles as early as 2 minutes post-HGF stimulation, whereas lamellipodia are only visible at earliest 10 minutes post-HGF stimulation. However, Gab1 has been detected to localize in dorsal ruffles and lamellipodia simultaneously. The accumulation of Gab1 and Shp2 into a dorsal ruffle could be a precursor to determine the elaboration of a lamellipodia, and thus the dorsal ruffle in itself may serve to bring these two signaling molecules together to produce a signal for the commitment to making lamellipodia.

3.3 The Gab1-Crk complex for dorsal ruffles.

We have demonstrated that over-expression of Gab1, but not Crk, drives the formation of dorsal ruffles in response to HGF in MDCK cells (Chapter 3 Figures 2 and 4). The Gab1-Crk complex is required to activate the formation of Met dependant dorsal ruffles, but the Gab1-Shp2 or Gab1-p85 complexes are dispensable for dorsal ruffle formation (Chapter 3 Figure 4). This demonstrates that although Gab1-Shp2 is necessary for lamellipodia, it is dispensable for dorsal ruffle formation. Although Gab1 is co-

localized with p85, Shp2 and Crk in dorsal ruffles, only the Gab1-Crk complex is instructive for dorsal ruffle formation (Chapter 3 Supplemental Figure 1). Crk is a known activator of Rac (Abassi and Vuori, 2002) and we suppose that Crk functions in this manner via the GEF DOCK180, which is also localized to dorsal ruffles with Gab1 (Chapter 4 Figure 6). Crk is an integral adaptor for activation of DOCK180 function (Akakura et al., 2005; Gumienny et al., 2001; Valles et al., 2004), and Crk has been found by proteomic analyses of pseudopod preparations to be localized in these protrusions with Rac (Cho and Klemke, 2002). Gab1 and Crk are both found to be drivers of *Listeria* entry (Sun et al., 2005), which occurs via phagocytic cups reminiscent of dorsal ruffles (see above).

There may also be a role for Crk in other Gab1 related functions for membrane ruffling. In the absence of Crk protein by using siRNA silencing technologies, Gab1 has reduced tyrosine phosphorylation downstream of Met activation (Watanabe et al., 2006). This may suggest that a Gab1 protein is impeded to reach its upstream kinase to become phosphorylated in the absence of Crk. Moreover, Crk complex formation with Gab1 drive a Tpr-Met induced cellular transformation (Lamorte et al., 2000) and perhaps the enhancement of this complex is required for Gab1 mediated signals. Evidence that Crk regulates the localization of other proteins is supported by the inability of Cbl mutants which are uncoupled from Crk to localize to lamellipodia in fibroblasts (Scaife and Langdon, 2000). Perhaps the Gab1–Crk interaction is required for proper targeting of Gab1 and Gab1-associated molecules to lamellipodia or other membrane ruffles as well. The cellular extensions that engulf the bacterium *Shigella flexneri* contain cortactin-Crk complexes and cortactin is required for actin polymerization activated by *Shigella*

(Bougneres et al., 2004). These data suggest that perhaps Crk may target Gab1 to cortactin in membrane ruffles. Indeed, the requirement for Crk in the basal protrusions of MDA-231 breast cancer cell lines such as those in Chapter 4 Figure 4, are cortactin rich and the role of Crk in these is currently under investigation in the lab.

3.4.1 The role of PLCγ in HGF induced ruffles.

Gab1 recruits the lipid lipase PLCγ downstream of Met activation in MDCK cells (Gual et al., 2000). The Gab1-PLCγ complex also forms upon osmotic shock of adipocytes, which triggers the translocation of the glucose transporter GLUT4 to the cell surface in order to uptake glucose (Ueno et al., 2001). Insulin stimulation of muscle cells also results in the translocation of GLUT4 to the membrane and into sites of active membrane ruffling on the dorsal surface of the cells (Khayat et al., 2000; Tong et al., 2001). This suggests that perhaps the Gab1-PLCγ complex serves a role in dorsal ruffle formation in other cell types and downstream of other RTKs. PLCγ enzymatic activity is required for HGF induced dorsal ruffle formation in MDCK cells and this enzyme co-localizes with Gab1 in dorsal ruffles (Chapter 4 Figure 5). A precedent has been set for the role of PLCγ in dorsal ruffle formation downstream of other RTKs. For example, the PDGFR signals via PLCγ for actin cytoskeleton remodeling and PLCγ is localized to actin rich protrusions (Yu et al., 1998).

What remains intriguing, however, is that although the Gab1-PLCγ complex is required for Met mediated morphogenesis, it is dispensable for cell scatter. In agreement with this, the enzymatic activity of PLCγ does not have an effect on cell scatter but is

essential for the morphogenic program (Gual et al., 2000; Royal et al., 2000). These data uncouple cell migration in two-dimensional cell cultures from three-dimensional cultures. Perhaps the requirements of the enzymatic activity of PLCγ are required for signal integration between signals downstream of Met and those from the extra-cellular matrix present in a three dimensional system. However, the results presented herein, demonstrate that HGF mediated dorsal ruffle formation is dependent on the enzymatic activity of PLCγ (Chapter 4 Figure 5), which suggests that the formation of dorsal ruffles may not be required for HGF mediated cell scatter, but may be involved in the morphogenic program.

3.5 Gab1 engages with the actin cytoskeleton.

Work presented in Chapter 4 of this thesis demonstrates that Gab1 engages with actin and cortactin downstream of Met and these also co-localize with Gab1 in dorsal and peripheral ruffles (Chapter 4 Figures 2 and 3 and Movies 3 and 4). Remodeling of the cytoskeleton occurs via Rac activation, which results in the engagement of NPFs such as cortactin with the actin branching machinery. Downstream of Met activation, DOCK180 co-localizes with Gab1 and F-actin to dorsal ruffles (Chapter 4 Figure 6), and Tiam1 localizes to endosomes with Rac (Palamidessi et al., 2008). These two GEFs may be functioning to activate two distinct pools of Rac, one at the site of dorsal ruffle formation and another at an intracellular vesicular compartment thought to deliver membrane and associated molecules to the active sites of HGF induced membrane ruffling. These data demonstrate the regulators of the actin cytoskeleton downstream of Met can be differentially compartmentalized.

4. Gab1 is required for the formation of membrane protrusions.

Gab1 is required for the formation of dorsal ruffles, since siRNA knock-down of Gab1 in MDCK cells abrogates, whereas over-expression of Gab1 induces HGF dorsal ruffle formation (Chapter 3 Figure 2). Knock-down studies of Gab1 in endothelial cells demonstrate that it is also required for migration of individual cells downstream of VEGFR2 (Laramee et al., 2007), which implies that in the absence of Gab1, endothelial cells are unable to produce a leading edge lamellipodia. We have demonstrated that lamellipodia formation downstream of the mutant CSF-MetΔGrb2 receptor is only achievable by over-expression of Gab1 and not the closely related Gab2 protein (Chapter 2 Figure 4). Also, Gab1 knock-down abrogates the formation of F-actin rich punctae on the basal surface of MDA-231 cells (Chapter 4 Figure 4) which are suggestive of a basal membrane protrusion called invadopodia (Bowden et al., 2006).

It is likely that there exist different pools of Gab1, perhaps a signaling pool, and a localized effector pool. Purification of the various Gab1 complexes that occur and determining their constituents would provide insight into the mechanisms by which Gab1 mediates membrane ruffle formation.

5. Gab1 is an initiator or required for maintenance of ruffles.

The signaling capacity of Gab proteins studied in Chapter 2 of this thesis correlates with their localization to lamellipodia and their ability to induce cell scatter and epithelial morphogenesis. myrGab2MBM can switch Gab2 to behaving identically to Gab1. myrGab2MBM and Gab1 activate sustained signaling pathways ERK, AKT, and JNK (Chapter 2 Figure 6 and Supplemental Figure 1), myrGab2MBM and Gab1 are

localized to lamellipodia (Chapter 2 Figure 5) and in cells expressing myrGab2MBM or Gab1, phosphorylated ERK is localized to lamellipodia in a sustained manner (Chapter 2 Figure 6). myrGab2MBM and Gab1 but not Gab2, Gab2MBM, or myrGab2 can induce cell scatter and epithelial morphogenesis (Chapter 2 Figures 2 and 3).

Is the switch of function due to the fact that myrGab2MBM is recruited to the receptor in a more enhanced fashion, and therefore activates signals required to activate membrane ruffles downstream? Or does myrGab2MBM switch the capacity of Gab2 to function in that it becomes a ruffle-localized regulator providing maintenance of the ruffle/signal? Moreover, the work presented in Chapter 3 describe Gab1 as being required for dorsal ruffle formation and this also correlates with Gab1 being localized to these ruffles.

The unanswered question that arises from this work is, does Gab1 function as an initiator for membrane ruffles or is it required for maintenance? The work presented herein provides evidence for both possibilities, and it is likely that both initiation and maintenance are interconnected. In cases where the Gab1 signal is permissive for ruffle formation, Gab1 is consistently localized to these. These two functions of Gab1 as a possible instructive and maintenance signal for membrane ruffles may not have to be uncoupled. Perhaps more elements of seemingly unrelated signaling pathways will begin to emerge as integrated modulators of transmission of the signal and effectors of their signals. Indeed, SNX9 is a protein bound to phosphoinositides, is required for membrane remodeling during endocytosis and directly binds and activates N-Wasp leading to Arp2/3 remodeling the cytoskeleton (Yarar et al., 2007).

6. Future directions

Often, function can be inferred from location. In order to further characterize the role of Gab1 in membrane ruffles, Gab1 localization may be further characterized. Co-localization studies of Gab1 with cortactin presented in Chapter 4 suggests that Gab1 may be involved with the elaboration of the meshwork of the actin cytoskeleton. Interestingly, Gab1 binds to PIP_3 in membranes as well as to elements of the actin cytoskeleton, which suggests that Gab1 may be a link between the membrane and the actin cytoskeleton. Evaluating whether or not Gab1 is at the leading tip of a protrusion providing contact between the cytoskeleton and the membrane, such as the Abl interacting protein (Abi), would be exciting (Stradal et al., 2001). Also, the role of Gab1 as a regulator of the actin cytoskeleton has been inferred by studies from another student in the lab who has characterized a Rac/cdc42 effector, Pak4, to be a *bona fide* binding protein of Gab1 and characterized this interaction demonstrating that these two synergize to make more aggressive protrusive structures in three-dimensional MDCK cell cultures.

In order to fully comprehend the mechanisms regulated by Gab1 interaction with the actin cytoskeleton, structure-function studies to uncouple Gab1 from actin would definitively show the requirement for this complex in Met mediated cellular responses. Moreover, emerging data implicates the microtubule cytoskeleton to regulate cell polarity and protrusions (Etienne-Manneville and Hall, 2003), and the mechanisms by which Met activates changes in microtubules, and whether or not Gab1 is involved should be addressed. In addition, cutting edge imaging technologies will be able to reveal the temporal dynamics of Gab1 and will continue to provide clues to its function.

7. Implications

Although much of the work presented in this thesis aim to understand the mechanisms of Gab1 action in Met mediated biological processes, there are surely general mechanisms for membrane protrusions such as the requirement of Gab1 for dorsal ruffle formation downstream of EGF, PDGF and HGF receptors (Chapter 3 Figure 3). Conversely, there are clearly numerous minute details that vary between proteins of the same family (Gab1 and Gab2, and EGFR and Met), as well as the variety of signaling complexes for different membrane ruffles (Gab1-Shp2 for lamellipodia and Gab1-Crk from dorsal ruffles). However, most of these studies are carried out on cells that produce membrane ruffles cultured in a two-dimensional system. Cells have been shown *in vivo* to in fact migrate not as single entities, but collectively (Wolf et al., 2007). Cues from the third dimension must be considered when studying cell migration.

The importance of studying cell migration is highlighted by the fact that in humans disease, cells use this process as a mechanism to survive and become invasive. Human tumors have been characterized as being hyperactive in their ability to make filopodial protrusions (Mattila and Lappalainen, 2008). Moreover, cells extracted from a Met induced murine breast cancer make many protrusions, and thus must suggest that the active sites of protrusion may be part of the pathological mechanism for tumor progression.

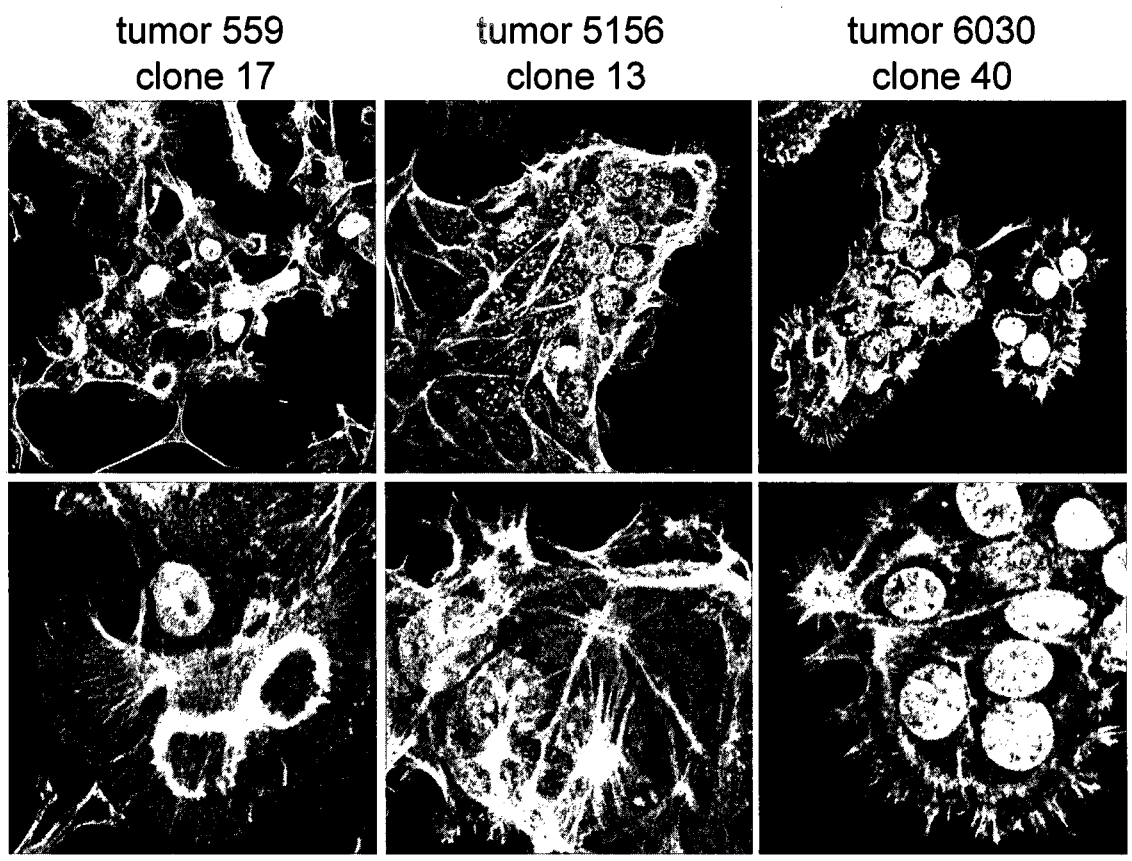

Figure 2. Met induced mammary tumor cell lines form ruffles constitutively.

Mice expressing MMTV driven activated Met transgene (Y1003F/M1250T) develop mammary tumors. Cell lines were generated from these tumors (Marisa Ponzo) and their actin cytoskeleton examined. Cells cultured in serum were plated on glass coverslips and the following day, were fixed and their actin cytoskeleton (green) and nuclei (blue) were stained. CLSM was performed with 40x objective and representative images are shown from three clonal cell lines from three different tumors. The cell line clone 17 from tumor 559 forms many dorsal ruffles. The cell line clone 13 from tumor 5156 also makes some dorsal ruffles and lamellipodia. The cell line clone 40 from tumor 6030 makes mostly filopodia.

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Chapter 6

Contributions to original research

1. Identified that both the Met Binding Motif as well as membrane targeting via myristoylation is required to confer the morphogenic phenotype to Gab2 downstream of Met.
2. Demonstrated that Gab1, not Gab2, is able to promote lamellipodia formation.
3. Identified that Gab1 is localized to the leading edge of lamellipodia as is Shp2 as well as phosphorylated ERK.
4. Demonstrated that HGF stimulation promotes the formation of lamellipodia in contrast to EGF stimulation.
5. Demonstrated that the Gab1-Shp2 complex is required for the formation of lamellipodia, and that MEK activity is also required.
6. Identified that Gab1, Crk, Shp2, PLCγ, the p85 subunit of PI3K, PIP_3 and DOCK180 are all localized to dorsal ruffles downstream of Met activation.
7. Demonstrated that Gab1 over-expression leads to enhanced dorsal ruffle formation.
8. Identified that Gab1 is required for dorsal ruffle formation downstream of PDGF and EGF RTKs.
9. Identified the Gab1-Crk complex as required for Met mediated dorsal ruffle formation.
10. Demonstrated that Gab1 can engage with the actin cytoskeleton downstream of Met by coimmuno-precipitation with actin and cortactin.
11. Identified a role for Gab1 in F-actin rich invadopdia structures in MDA-231 cells by siRNA silencing.