COMMITTEE CERTIFICATION OF APPROVED VERSION

The committee for Ning Li certifies that this is the approved version of the following dissertation:

REGULATION OF HEPATOCYTE GROWTH FACTOR RECEPTOR ENDOCYTIC TRAFFICKING

Committee:

Lisa A. Elferink, Ph.D., Supervisor

Stan Watowich, Ph.D.

Cornelis Elferink, Ph.D.

Andrew Bean, Ph.D.

Sarita Sastry, Ph.D.

Dean, Graduate School

REGULATION OF HEPATOCYTE GROWTH FACTOR RECEPTOR ENDOCYTIC TRAFFICKING

by

Ning Li, MB, MMED

Dissertation
Presented to the Faculty of The University of Texas Graduate School of
Biomedical Sciences at Galveston
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Approved by the Supervisory Committee

Lisa A. Elferink, Ph.D.
Stan Watowich, Ph.D.
Cornelis Elferink, Ph.D.
Andrew Bean, Ph.D.
Sarita Sastry, Ph.D.

May 2006
Galveston, Texas

Key words: Endocytosis, Cell signaling

Dedicated to my mother, you are always the wind underneath my wings!

ACKNOWLEDGEMENTS

I first want to give best acknowledgement to my mentor, Dr. Lisa Elferink. She always inspires and challenges me to do my best, giving me advice whenever I needed her help. Without her steadfast support and encouragement, I could not make such accomplishment. Second, I want to acknowledge our collaborator Dr. Keith Ireton in Florida Central University, who generously provided me the reagents, cell lines and protocols for my project. I also want to acknowledge former and current students in our lab, David Strick, Kristen Hill and Jenise Gogan, who make this journey more enjoyable. I would like to acknowledge my committee: Dr. Stan Watowich, Dr. Sarita Sastry, Dr. Cornelis Elferink and Dr. Andrew Bean, for their guidance and encouragement. I also would like to thank my former committee member Dr. Aubrey Thompson. Although he has not been on my committee since he moved to Florida, he still graciously teaches me how to become a good scientist. Our program director Dr. Lillian Chan was always willing to help me when I need her help, and I thank her very much.

I want to acknowledge the former postdoctor Yali Zhao in our lab, Gengming Huang and Li Chen in Dr. Cornelis Elferink's lab for their technical assistance during my early period in this lab. And I also want to thank all my friends in UTMB, Zhifang Zhao, Yafei Huang, Xuan Zhang, Xiaoming Hu, Song Liu, Shijing Liu, Sheng Yan, Zhen Yang, Huolin Tu, and every people not listed here whoever gave me kind help during my days in UTMB. Finally, I would like to thank my family. To my parents and my sister, who love me and support me without condition. And to my dearest husband, Xiaofei Zhang, who is always there, giving me support and understanding.

REGULATION OF HEPATOCYTE GROWTH FACTOR RECEPTOR ENDOCYTIC TRAFFICKING

Publication No._____

Ning Li, Ph.D.
The University of Texas Graduate School of Biomedical Sciences at Galveston, 2006

Supervisor: Dr. Lisa A. Elferink

The Hepatocyte Growth Factor Receptor (HGFR/cMet) is a receptor tyrosine kinase that is essential for multiple cell responses, including cell proliferation, survival, motility and branching morphogenesis. Normal HGFR signaling regulates embryonic development, organ regeneration and wound healing, whereas deregulated HGFR signaling is linked to tumor progression, metastasis and angiogenesis. Ligand activation of HGFR leads to receptor downregulation via endocytosis and lysosomal degradation, the major mechanism for terminating HGFR signaling. Perturbation of HGFR trafficking, either at the level of internalization or during sorting at the early endosome for degradation, leads to oncogenic activation of HGFR. Impaired HGFR trafficking is emerging as a key mechanism for HGFR-induced cancer progression and metastasis.

A major goal of my dissertation was to lay the foundation for future studies examining different mechanisms leading to altered HGFR trafficking in human cancers, by determining and characterizing the mechanisms which normally function to regulate HGFR internalization and degradation. Two ligands have been identified for HGFR, the physiological ligand HGF and the *Listeria* surface protein Internalin B (InlB). I characterized the mechanisms for InlB and HGF induced HGFR trafficking using a combination of imaging, molecular biological and biochemical approaches. First, I demonstrated that InlB and HGF were mechanistically equivalent in triggering HGFR internalization primarily through clathrin-coated pits. Then, I determined that the Y1349 and Y1356 docking sites and tyrosine kinase activity of HGFR were required for receptor internalization. Recruitment of the adaptor protein Grb2, but not Gab1, was essential for ligand induced HGFR internalization. I then showed Cbl, an E3 ubiquitin ligase recruited by Grb2 to HGFR, played an essential role in receptor internalization. Furthermore, the E3 ligase activity of Cbl and ubiquitination machinery was involved in HGFR internalization. Finally, I demonstrated that ligand induced HGFR degradation occurred through the lysosomal pathway, involving the function of Hrs and PI3K. My studies represent the first detailed characterization of the trafficking events that normally function to inactivate HGFR signaling. My findings contribute to better understanding of how HGFR-induced tumorigenesis and tumor metastasis may result from impaired HGFR trafficking, and identify novel mechanisms that may function as therapeutic targets for treatment of human cancers due to impaired HGFR trafficking.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

RTK……..Receptor Tyrosine Kinase
HGFR……Hepatocyte Growth Factor
EGFR……Epidermal Growth Factor Receptor
PDGFR……..Platelet Derived Growth Factor Receptor
LDLR…….....Low-density lipoprotein receptor
TGFβ R…….Transforming Growth Factor β Receptor
CSF-1R…….Colony-Stimulating Factor-1 Receptor
TfnR……..Transferrin Receptor
IL-2……Interleukin-2
HGF/SF……..Hepatocyte Growth Factor/Scatter Factor
CTX……....Cholera Toxin
HIP1……...Huntingtin Interacting Protein 1
AP-2……...Assembly Protein 2
Eps15…….EGFR pathway substrate No. 15
CALM……Clathrin-Assembly Lymphoid Myeloid leukemia protein
Hrs……..Hepatocyte-growth-factor-regulated tyrosine-kinase substrate
PI3K……...Phosphatidylinositol 3-kinase
EEA1……..Early Endosomal Antigen 1
CHC……....Clathrin Heavy Chain
PI3P……Phosphatidylinositol 3-Phosphate
PI (4,5)P_2……Phosphatidylinositol 4, 5-Bisphosphate
UIM……Ubiquitin Interacting Motif
MVB……...Multivesicular Body
ESCRT…….....Endosomal Sorting Complex Required for Transport I
UBA……...Ubiquitin-Associated domain
Gab1……...Grb2-Associated Binding protein-1
Grb2……...Growth factor Receptor-Bound protein 2
Sos…… …….Son-of-Sevenless
MEF…….....Mouse Embryonic Fibroblast

CHAPTER 1: GENERAL INTRODUCTION

NEGATIVE REGULATION OF RECEPTOR TYROSINE KINASE IN CANCERS

Cancer is a class of diseases characterized by uncontrolled cell division and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis. During the last few decades, cancer research has revealed that cancer is a disease involving dynamic changes in the genome. This is based on the discovery of mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function. Several lines of evidence indicate that tumorigenesis in humans is a multistep process, that reflect genetic alterations leading to progressive transformation of normal human cells into highly malignant derivatives. Tumor cells acquire several unique capabilities during cancer development (Hanahan and Weinberg 2000), including self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Certain classes of signaling proteins that govern extracellular growth, differentiation and development appear to be targeted much more frequently by oncogenic mutations. Receptor tyrosine kinases (RTKs) are a good example. Of the 58 transmembrane RTKs identified to date, deregulation of 30 has been associated with human cancers during distinct steps leading to cancer progression (Blume-Jensen and Hunter 2001).

Receptor tyrosine kinases (RTKs) are a family of single pass transmembrane proteins that control a wide variety of cellular events in multicellular organisms including cell proliferation, differentiation, survival and migration. Ligand binding to the extracellular domain of the receptor induces receptor dimerization and activation of receptor tyrosine kinase activity. Subsequent autophosphorylation of the receptor at several tyrosine residues within the cytoplasmic domain forms binding sites for proteins that relay downstream biological signals. Ligand induced RTK activation also promotes

receptor internalization. Tyrosine kinase activity and hence receptor signaling is terminated by subsequent lysosomal degradation of the internalized receptor.

Normally, RTK signaling is tightly regulated; however, deregulation of RTK has been linked to a large number of human malignancies. Increased RTK signaling in cancer is caused by several mechanisms, such as gene amplification, chromosomal translocation and point mutations that promote ligand independent RTK autophosphorylation (Bache, Slagsvold et al. 2004). In addition to these mechanisms, escape from negative regulatory mechanisms that normally result in the deregulation of RTK activity leads to neoplastic growth. The major mechanism for terminating RTK signaling is receptor downregulation — desensitization of receptors by removal of activated receptors from the cell surface by endocytosis. The value of this mechanism was first inferred from the finding that a mutant epidermal growth factor receptor (EGFR) that is endocytosis impaired, resulted in enhanced mitogenic signaling and cell transformation (Wells, Welsh et al. 1990). A transformed phenotype and anchorage-independent growth were observed in cells expressing this mutant EGFR at ligand concentrations that failed to elicit these responses in cells expressing wild-type receptors. Therefore, downregulation of EGFR can serve as an attenuation mechanism, without which cell transformation ensues.

Impaired RTK Endocytosis is associated with cancer

Recent publications have established that ubiquitination plays a major role in RTK downregulation by targeting receptors to the lysosome. Several RTKs, including EGFR, Platelet Derived Growth Factor Receptor (PDGFR), Colony-Stimulating Factor-1 Receptor (CSF-1R) and Hepatocyte Growth Factor receptor (HGFR or cMet) are ubiquitinated following recruitment of the E3 ubiquitin ligase, Cbl. Many RTK-derived oncoproteins avoid downregulation due to mutations that result in the loss of Cbl binding sites, inefficient Cbl recruitment or increased Cbl degradation. For example, the mutant EGFRvIII resulting from the deletion of exons 2-7 located in the extracellular domain of EGFR is the most common genetic alteration detected in glioblastomas. EGFRvIII is constitutively active due to a low level of tyrosine phosphorylation. Moreover, EGFRvIII has a reduced ability to recruit Cbl and CIN85, an adaptor protein for clathrin-mediated

endocytosis. Accordingly, EGFRvIII is not ubiquitinated or internalized, resulting in the increased receptor signaling from the cell surface (Schmidt, Furnari et al. 2003). An oncogenic form of HGFR, called Tpr-Met, was caused by a chromosomal rearrangement, resulting in the fusion of the dimerization domain of the Tpr (translocated promoter region) to the kinase domain of the HGFR that lacks the Cbl binding site. Accordingly, Tpr-Met is constitutively active and not degraded. A germ line HGFR mutation (P991S) in a primary gastric cancer contains a mutation within the juxtamembrane domain of the receptor, close to the direct binding site for Cbl. Once stimulated with HGF, the HGFR-P991S shows increased and prolonged phosphorylation compared to wild-type receptors (Lee, Han et al. 2000). It remains unclear whether this mutation alters the endocytic properties of the mutant HGFR. However, this might be another example of the link between endocytic pathway and cancer.

Oncogenic endocytic machinery components

In addition to mutant RTKs that are impaired in endocytic trafficking, some endocytic machinery components were also found to be disrupted in certain types of cancers. There are two types of mechanisms that could disrupt the endocytic machinery in cancer cells. One mechanism involves the overexpression of endocytic accessory proteins, while the other involves chromosomal translocation events that generate fusion proteins between an endocytic component and a proto-oncogene. The resulting chimeric protein has oncogenic activity. A discussion of examples for each of these mechanisms follows.

Overexpression of endocytic machinery components

HIP1 (huntingtin interacting protein 1) was first cloned as a binding partner for Huntingtin, a protein whose gene is mutated in Huntington's disease (Kalchman, Koide et al. 1997; Wanker, Rovira et al. 1997). HIP1 is a cofactor in clathrin-mediated endocytosis, which associates with AP-2, clathrin and phosphoinositide. HIP1 overexpression was detected in several primary epithelial tumors including breast, ovarian, prostate, lung and colon cancer cells (Rao, Hyun et al. 2002). The expression of HIP1 in normal breast tissue is very low, whereas elevated HIP1 expression is frequently

found in invasive breast cancers. Increased HIP1 expression correlates closely with the aggressiveness of prostate cancer, and increased HIP1 expression is an independent predictor of relapse in patients with prostate cancer (Rao, Hyun et al. 2002). Overexpression of HIP1 has been reported to transform NIH/3T3 fibroblasts and upregulate multiple growth factor receptors including EGFR (Rao, Bradley et al. 2003), Fibroblast Growth Factor Receptor (FGFR) and PDGF-β receptor (Hyun and Ross 2004).

In addition to HIP1, other endocytic proteins that transform cells and hence could play a role in cancer progression include intersectin, Eps15 (EGFR pathway substrate No. 15), and Hrs (Hepatocyte-growth-factor-regulated tyrosine-kinase substrate). Overexpression of intersectin regulated mitogenic signaling pathways and caused transformation of rodent fibroblasts (Adams, Thorn et al. 2000). Similarly, overexpression of Eps15 in NIH/3T3 cells also resulted in cell transformation (Fazioli, Minichiello et al. 1993). The transformation of cells in response to overexpression of intersectin or Eps15 could result from the general disruption of growth factor receptor endocytosis, and increased signaling from multiple receptors.

Oncogenic endocytic fusion proteins

The genes encoding some of the endocytic accessory proteins in clathrin-coated pits formation have been identified as targets of chromosomal translocations in human haematopoietic malignancies (Floyd and De Camilli 1998). For example, the N-terminal domain of ALL1/HRX (a transcription regulatory factor gene) is fused in frame with C-terminal domain of Eps15. The ALL1/HRX gene is involved in a large number of chromosomal translocation events resulting in a range of ALL1/HRX fusion proteins. These different types of translocations tend to be associated with acute myeloid leukaemia. Similarly, the N-terminal domain of ALL1/HRX was also detected fused with C-terminal domain of endophilin-2, another endocytic protein in some types of human leukaemias. A third endocytic protein CALM (clathrin-assembly lymphoid myeloid leukemia protein) is detected fused to a transcription factor AF-10 (ALL1 fused gene from chromosome 10) in human U937 cell line derived from a patient with diffuse histocytic lymphoma (Floyd and De Camilli 1998). PDGFR was detected expressed in

frame fusion with Rabaptin-5, a protein important for early endosomal fusion (Magnusson, Meade et al. 2001) and HIP1 (Ross, Bernard et al. 1998). Since both Rabaptin-5 and HIP1 have coiled-coil dimerization domains, the oncogenic transformation resulting from expression of the fusion proteins could be caused by the constitutive dimerization of PDGFR, leading to ligand-independent signaling receptor. Alternatively, these fusion proteins may interfere with normal receptor downregulation.

RECEPTOR MEDIATED ENDOCYTOSIS PATHWAYS

Endocytosis occurs by multiple mechanisms that can be divided into two major categories, "phagocytosis" or cell eating and "pinocytosis" or cell drinking. Phagocytosis in mammals is restricted to specialized cells such as macrophages and neutrophils for the clearance of large pathogens or cell debris. Pinocytosis occurs in all cells and can be divided into five subtypes: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, lipid raft-mediated endocytosis and non clathrin and non caveolae/lipid raft-mediated endocytosis. In this section, I will briefly summarize the mechanisms underlying these endocytic routes, with a particular emphasis on clathrin mediated endocytosis, the focus of this thesis.

Macropinocytosis

Macropinocytosis occurs by the fusion of membrane ruffles that is induced upon stimulation by growth factors or other signals, leading to the formation of macropinosomes. Activation of the GTPase Arf6 by GTP binding leads to membrane ruffling and accumulation of macropinosomes mediated through the production of PIP_2 (Brown, Rozelle et al. 2001). Inhibition of PI-3Kinase or phospholipase C prevents the formation of macropinosomes (Amyere, Payrastre et al. 2000). Macropinocytosis has been shown to mediate antigen uptake, and pathogen entry. For example, *Legionella* and HIV have been shown to be localized in macropinosomes after entry into macrophages (Marechal, Prevost et al. 2001; Watarai, Derre et al. 2001).

Caveolae/lipid rafts

Lipid rafts are detergent insoluble, low-density membrane fractions that are rich in cholesterol and glycosphingolipid. Caveolae are flask-shaped invagination of the plasma membrane enriched in cholesterol and sphingolipid, in which many diverse signaling molecules are concentrated. The other characteristic feature of caveolae is the presence of the cholesterol binding protein caveolin 1. Therefore caveolae are considered a subdomain of biochemically defined lipid rafts (Anderson 1998). Both caveolae and lipid rafts can be disrupted by drugs that deplete (cyclodextrins) or sequester cholesterol (filipin or nystatin), by overexpression of dominant-negative caveolin 1 mutants and through genetic knock out of the caveolin 1 gene (Razani and Lisanti 2001). Caveolae have been involved in the internalization of sphingolipid binding toxin cholera toxin (CTX) (Wolf, Fujinaga et al. 2002), GPI-anchored proteins endocytosis (Kurzchalia and Parton 1999) and SV40 virus entry (Pelkmans, Kartenbeck et al. 2001). The GTPase activity of dynamin is required for the subsequent fission of caveolae or lipid raft enriched vesicles from the plasma membrane (Henley, Krueger et al. 1998; Lamaze, Dujeancourt et al. 2001). Caveolae ($t_{1/2}$ >20min) mediated endocytosis is slow relative to clathrin mediated endocytosis ($t_{1/2}$ ~5min), and the resulting caveosome does not contain classic clathrin derived endosomal markers such as transferrin receptors. Conversely, lipid rafts mediated endocytosis functions independently of caveolin 1. For example, clathrin independent endocytosis of Interleukin-2 (IL-2) receptor was detected in lymphocytes that are devoid of caveolae, implicating a lipid raft dependent mechanism in this process. However, it is important to note that lipid raft-mediated endocytosis also occurs in the cells with abundant caveolae (Kirkham, Fujita et al. 2005). The functional relationship between these distinct endocytic routes currently remains unclear.

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is accompanied by the temporally and spatially regulated interaction of multiple factors, consisting of several steps. First, clathrin triskelions, composed of three clathrin heavy chains (CHC) and three light chains (CLC), assemble into polygonal lattices on the plasma membrane. Binding of clathrin to the

plasma membrane is mediated through the major adaptor protein, AP-2 complex, which interacts with clathrin, PtdIns(4,5)P_2 and transmembrane proteins targeted for internalization. The initial stage of clathrin-mediated endocytosis requires membrane curvature in a process dependent on accessory proteins such as epsin. The late stage of clathrin-coated pits invagination requires other accessory proteins such as endophilin and amphiphysin. After formation of deeply invaginated pits, the GTPase dynamin functions as a GTP dependent "pinchase" for scission of the nascent endocytic vesicles. Finally, removal of the clathrin coat in a process called uncoating is accomplished with the participation of the synaptojanin 1 and a chaperone complex consisting of auxilin and Hsc70 (Conner and Schmid 2003).

Clathrin-dependent endocytosis can be divided into two distinct categories, constitutive versus regulated endocytosis. Constitutive internalization is dependent on a short linear internalization motif within the cargo protein, such as the TfnR (transferrin receptor), the LDLR (Low-density lipoprotein receptor) and catalytically inactive RTK. In contrast to constitutive internalization, regulated endocytosis depends on ligand binding which leads to phosphorylation and ubiquitin-mediated interactions, essential for the internalization process. In the absence of ligand, TfnR and LDLR are internalized and recycled back to the plasma membrane constitutively. The unoccupied RTKs are internalized constitutively at a low rate, however, binding of EGF to its receptor increases the internalization rate about 10 fold. Both constitutive endocytosis and regulated endocytosis utilize common endocytic components, but via different molecular interactions. For example, Eps15 is involved in both TfnR and EGFR internalization via clathrin-coated pits. However, the tyrosine phosphorylation and monoubiquitination of Eps15 is only required for clathrin-mediated EGFR internalization. Endocytosis of different cargoes, through clathrin-coated pits (CCP), is differently controlled. EGFR, TfnR and LDLR are all internalized through independent and saturable pathways, that are not competitive (Wiley 1988; Warren, Green et al. 1997; Warren, Green et al. 1998). These studies suggest that different limiting steps exist for receptors endocytosis through

CCP, including differences in the endocytic accessory components, posttranslational modification and their protein-protein interaction.

AP-2 adaptor dependent endocytosis

AP-2 (Assembly Protein 2) was the first adaptor protein identified in clathrin dependent endocytosis. AP-2 is a heterodimer consisting of four subunits: α, β2, μ2 and σ2 adaptins. The N-terminus of the α subunit binds to phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P_2) (Gaidarov and Keen 1999), while the c-terminus of α subunit binds endocytic accessory proteins such as amphiphysin 1 (Slepnev, Ochoa et al. 2000). The β2 subunit is responsible for binding the clathrin heavy chain (Owen, Vallis et al. 2000) (Brodsky, Chen et al. 2001). The interaction between the AP-2 complex and the cargo protein is mediated by two interactions: the cargo binding domain of μ2 and the tyrosine-based motif Yxxθ present in the cytoplasmic tail of cargo proteins (x is a variable residue while θ is a bulky hydrophobic residue) (Owen and Evans 1998); a second tyrosine-based binding motif for the μ2 subunit of AP-2 is FxNPxY, and was first identified in the cytoplasmic tail of LDLR (Boll, Rapoport et al. 2002). The Leucine-based motif (DE)xxxLL(I) has also been shown to bind the β2 subunit of the AP-2 complex (Rapoport, Chen et al. 1998) and may bind to the μ2 subunit, as suggested by an *in vitro* binding assay (Rodionov and Bakke 1998). The protein kinases AAK1 (adaptor-associated kinase 1) and GAK (cyclin-G-associated protein kinase/auxilin 2) have been proposed to phosphorylate μ2 *in vivo* (Korolchuk and Banting 2002). AP-2 phosphorylation significantly increases the affinity of AP-2 for receptor internalization signals *in vitro* (Ricotta, Conner et al. 2002).

Alternate clathrin adaptors for signaling receptor endocytosis

Tyrosine-based and di-leucine motifs have been identified in several signaling receptors such as the EGFR (Sorkin, Mazzotti et al. 1996), PDGF-β receptor (Yao, Ehrlich et al. 2002; Wu, Windmiller et al. 2003) and the TGF-β receptor (Yao, Ehrlich et al. 2002). However, mutational analysis revealed that neither of these motifs nor AP-2

interactions plays an essential role in clathrin mediated endocytosis of these receptors. For example, AP-2 knockdown abolished clathrin localization to the plasma membrane. However, EGFR endocytosis proceeded normally in cells depleted of endogenous AP-2 (Hinrichsen, Harborth et al. 2003). This implied that additional adaptor proteins are involved in signaling receptor internalization through clathrin-coated pits. β-arrestins have been shown to be required for linking some G-protein coupled receptor (GPCR) to coated pits (Goodman, Krupnick et al. 1996; Zhang, Ferguson et al. 1996). β-arrestins interact with clathrin heavy chain and the AP-2 complex and are implicated in the recruitment of agonist-activated GPCR into clathrin-coated pits (Claing, Laporte et al. 2002). A role for β-arrestin in mediating coated-pit recruitment of other membrane cargo proteins such as type-III TGFβ receptors (Chen, Kirkbride et al. 2003), has also been suggested. Additional clathrin adaptors, including AP180, epsin, Dab2 and HIP, have been described for receptor endocytosis. These adaptor proteins bind PI (4,5)P2, AP-2 and clathrin. It remains unclear whether these alternate adaptors function independently or with AP-2 to optimize clathrin recruitment and assembly for receptor internalization (Ford, Pearse et al. 2001; Mishra, Agostinelli et al. 2001; Mishra, Keyel et al. 2002). Clearly, multiple adaptors provide the necessary plasticity to allow the precise temporal control in the face of high traffic volumes.

RECEPTOR ENDOCYTOSIS CONTROLS SIGNALING OUTPUT

Cells use various internalization pathways to control the number of cell surface receptors. This is crucial for the regulation of cell signaling, receptor turnover, and the magnitude and duration of signaling endpoints. For example, EGFRs have been shown to localize in caveolae and lipid rafts. The relative amounts of caveolae and lipid raft associated EGFRs varies between 60% and just a few percent, and seems cell type dependent (Pike and Casey 1996; Mineo, Gill et al. 1999; Roepstorff, Thomsen et al. 2002). The localization of EGFR within or outside of lipid rafts is important for ligand activation of the receptor and downstream signaling. Cholesterol depletion increases EGF binding, whereas cholesterol loading lowers EGF binding. This is because sequestration

of the EGFR in lipid rafts inhibits EGF binding to the receptor (Roepstorff, Thomsen et al. 2002). The localization of EGFR to lipid rafts is mediated by an extracellular sequence in the receptor (Yamabhai and Anderson 2002). EGF binding and receptor autophosphorylation initiates EGFR migration from lipid raft into clathrin coated pits. (Mineo, Gill et al. 1999).

It has been shown that EGFR can be internalized through both clathrin-mediated and nonclathrin-mediated pathways, depending on the ligand concentration and receptor expression level. At a low EGF concentration (<10ng/ml), EGFR is internalized through clathrin-coated pits. Treatment using higher concentrations of EGF saturated the clathrin-mediated endocytosis of the EGFR, rerouting the receptor to a clathrin-independent pathway for internalization. For example, in a tumor cell line A431 which expresses high level of EGFR, EGFR was internalized through a clathrin-independent pathway with a slower rate (compared to the EGFR internalization rate through the clathrin-mediated pathway). Moreover, the EGFRs in these cells do not undergo efficient downregulation. (Wiley 1988). This mechanism may contribute to the increased EGFR signaling observed in this tumor cell line.

In a separate study, the TGF-β receptor was shown to be internalized through clathrin-coated pits as well as caveolae (Di Guglielmo, Le Roy et al. 2003). TGF-β binding to the receptor initiates the recruitment of SMAD2, an important downstream signaling molecule for the TGF-β receptor. Phosphatidylinositol-3 Phosphate (PI3P) containing early endosomes are enriched for the SMAD2-anchor SARA, which contains a PI3P binding FYVE domain (Hayes, Chawla et al. 2002; Itoh, Divecha et al. 2002). The clathrin-mediated endocytic pathway was shown to mediate the colocalization of TGF-β receptor with downstream signaling molecules such as SARA and SMAD2 in early endosomes. However, internalization of TGF-β R through caveolae causes association with the Smad7-Smurf2 ubiquitin ligase complex, subsequent receptor ubiquitination and lysosomal degradation of the receptor (Di Guglielmo, Le Roy et al. 2003). Thus, the fate of the activated TGF-β R is different depending on different entry route.

UBIQUITINATION, RTK TRAFFICKING AND CANCER

It has become evident that modification of ligand binding RTKs and endocytic accessory proteins with ubiquitin, plays an important role in receptor trafficking (EGFR trafficking model shown in Figure 1) In this section, I will summarize the types of ubiquitination detected *in vivo*, the enzymes involved in this process and the different families of E3 enzymes. Then I will describe the role of ubiquitination in RTK internalization and degradation and how this is related to cancer. Finally, I will discuss the role of an E3 ubiquitin ligase Cbl in RTK endocytosis and in the development and progression of human cancers.

Types of Ubiquitination and E3 ubiquitin ligases

RTKs such as EGFR and HGFR are monoubiquitinated at multiple sites following ligand stimulation. Ubiquitin has been shown to act as a signal for routing internalized receptor to the lysosome for degradation and downregulation. However, the role of receptor ubiquitination in controlling receptor internalization remains unclear. Ubiquitin is a highly conserved 76 amino acid polypeptide that is covalently attached to target proteins via an isopeptide bond between the c-terminal glycine of ubiquitin and a lysine in substrate proteins. Substrate ubiquitination is mediated by a series of enzymatic reactions: ATP-dependent conjugation of ubiquitin to an active site cysteine residue in an E1 ubiquitin-activating enzyme; followed by transfer to an E2 ubiquitin-conjugating enzyme through a thiol-ester linkage; and E3 ubiquitin ligases mediate substrate recognition and are responsible for ubiquitin conjugation to substrates. First, monoubiquitination is the addition of a single ubiquitin to a substrate. Second, multiple monoubiquitination is the attachment of single ubiquitin molecules to different lysine residues within a substrate protein. Third, polyubiquitination is obtained by the formation of a polymeric chain of ubiquitin molecules by the subsequent addition of ubiquitin molecules to the lysine residue of the preceding ubiquitin. Monoubiquitination and multiple monoubiquitination are involved in endocytosis, membrane trafficking, histone modification, transcription and DNA repair, while polyubiquitination functions as a targeting signal for proteasomal degradation (Haglund, Di Fiore et al. 2003). There are

several types of E3 ligases (Weissman 2001). One group includes the catalytic HECT-domain containing E3 ligase, such as Nedd4 family ligases, which have been shown to ubiquitinate Eps15 and Hrs (Katz, Shtiegman et al. 2002; Polo, Sigismund et al. 2002). This family of E3 ligase proceeds through a thiolester:ubiquitin intermediate to transfer ubiquitin. The other group are RING finger domain containing E3 ligases, such as Cbl, and U-box-containing proteins, such as CHIP (Marmor and Yarden 2004). This group of E3 ligases bind both the substrate and E2 enzymes to enable the ubiquitination. The same substrate can undergo all three modifications by distinct ligases. Similarly, the same E3 ligase can mono, multi or polyubiquitinate different substrates. For example, Cbl, an E3 ubiquitin ligase, polyubiqutinates Sprouty, Src and Abl proteins, degraded by the proteasome pathway (Hall, Jura et al. 2003; Soubeyran, Barac et al. 2003; Kim, Tezuka et al. 2004). Cbl can also multi/monoubiquitinate RTK targeted to the endocytic/lysosomal pathway including EGFR, PDGFR and HGFR (Marmor and Yarden 2004).

Receptor ubiquitination and internalization

Ubiquitination of cargo protein is required for internalization in yeast, but in mammalian cells the role of receptor ubiquitination in receptor internalization is still controversial. Fusion of monoubiquitin to a truncated EGFR mutant lacking the cytoplasmic domain is sufficient to initiate receptor internalization and lysosomal degradation (Haglund, Sigismund et al. 2003). Unlike the full-length EGFR the chimeric receptor is internalized constitutively by the lipid raft-mediated pathway. Clathrin independent internalization of ubiquitinated EGFR depends on its interaction with proteins harboring a Ub-interacting motif, such as Eps15, Eps15R and Epsin (Sigismund, Woelk et al. 2005). However, the interaction between multiple monoubiquitinated full-length EGFR and UIM (Ubiquitin Interacting Motif) of Eps15 has also been reported to be involved in the recruitment of EGFR into the clathrin-coated pits (de Melker, van der Horst et al. 2004; Stang, Blystad et al. 2004). Ubiquitination of the beta2-adrenergic receptor has also been shown not to be required for receptor internalization, whereas ubiquitination of the adaptor protein β-arrestin was essential for receptor internalization (Shenoy, McDonald et al. 2001). Similarly, a mutant growth hormone receptor (GHR)

lacking all of the cytosolic lysine residues, is internalized at the same rate as the wild type receptor (Govers, ten Broeke et al. 1999). These studies suggest that receptor ubiquitination may function in a receptor specific and cell specific context to regulate receptor uptake. Several endocytic accessory proteins that are monoubiquitinated in response to receptor activation contain one or more UIMs, including Eps15, Eps15R, epsin and CIN85. These ubiquitin and UIM containing molecules are proposed to form protein scaffolding complexes via multiple ubiquitin-UIM interactions, which may contribute to receptor clustering into clathrin-coated pits. The role of ubiquitin in receptor internalization remains controversial because multiple and redundant mechanisms appear to mediate the receptor internalization from the cell surface, as compared with an essential role of ubiquitin in the sorting of internalized receptor to lysosomes for degradation.

Ubiquitination and RTK degradation

The "multivesicular body" (MVB) is an organelle that consists of a limiting membrane enclosing many internal vesicles of 40-90 nm. Ubiquitin has been shown to serve as a signal for receptor sorting within the MVB (Longva, Blystad et al. 2002). The endocytic protein Hrs plays a pivotal role in this process. Hrs is a 115 kDa protein localized to the early endosomes by its FYVE and coiled-coil domains (Komada, Masaki et al. 1997; Raiborg, Bremnes et al. 2001). Hrs can recruit clathrin to early endosomes through a PI3P dependent manner (Raiborg, Bache et al. 2001). Monoubiquitinated proteins are detected in the clathrin enriched microdomain in early endosomes along with Hrs, while EEA1 (Early Endosomal Antigen 1) is found in different microdomains in early endosomal membranes. Hrs contains UIMs that recognize ubiquitinated proteins, which could partly explain the accumulation of ubiquitinated proteins in Hrs/clathrin enriched microdomains. Hrs is complexed to other UIM-containing proteins, such as Eps15 and STAM (signal-transducing adaptor molecule) (Bilodeau, Urbanowski et al. 2002; Polo, Sigismund et al. 2002; Shih, Katzmann et al. 2002). The UIM-containing endocytic proteins Epsin, Eps15, Eps15R, Hrs and STAM have been shown to be monoubiquitinated upon receptor activation (Katz, Shtiegman et al. 2002; Polo,

Sigismund et al. 2002). Monoubiquitination may serve to regulate the sorting functions of UIM-containing proteins by controlling interactions with cargo or sorting components, or by facilitating the formation of large complexes through multiple UIM-ubiquitin interactions. Overexpression of Hrs leads to the intracellular accumulation of the ubiquitinated EGFR, whereas a UIM point mutant that is unable to bind ubiquitin can not, implicating a role for Hrs in the recognition of ubiquitinated receptors (Raiborg, Bache et al. 2002). Hrs also recruits the Ub-binding complex ESCRT-I (endosomal sorting complex required for transport I), which is composed of three other Vps proteins, Vps23p, Vps28 and Vps37. ESCRT-I binds to ubiquitinated proteins and directs sorting into the MVB pathway (Katzmann, Babst et al. 2001). This is followed by the recruitment of two ESCRT complexes (II and III) that sequentially interact with the ubiquitinated cargoes and deliver them to the sorting process (Babst, Katzmann et al. 2002; Babst, Katzmann et al. 2002).

The E3 ligase Cbl and RTK internalization

Cbl is an E3 ubiquitin-protein ligase that also functions as an important adaptor protein. v-Cbl, the first member of the Cbl family of proteins, was cloned from the Cas NS-1 retrovirus, the causative agent for pre-B-cell lymphomas and myelogenous leukaemia in mice (Langdon, Hartley et al. 1989). It was subsequently shown that v-Cbl corresponded to a truncated form of a larger cellular homologue called c-Cbl. The full-length form of c-Cbl consists of an N-terminal tyrosine-kinase-binding domain (TKB domain), a ring finger motif and a short linker sequence, a proline-rich region and a C-terminal ubiquitin-associated (UBA) domain that overlaps with a leucine zipper motif (LZ).

Three mammalian Cbl homologues have been characterized, c-Cbl, Cbl-b and Cbl-c ((Keane, Rivero-Lezcano et al. 1995; Keane, Ettenberg et al. 1999). c-Cbl and Cbl-b are ubiquitously expressed proteins. Invertebrate orthologues have been identified in *Drosophila melanogaster* (D-Cbl) and *C. elegans* (SLI-1) (Yoon, Lee et al. 1995; Hime, Dhungat et al. 1997). Cbl-c is a shorter Cbl isoform without the c-terminal UBA/LZ domain. The TKB domain of cCbl consists of a four-helix bundle, a calcium-binding EF

hand and a variant Src homology 2 (SH2) domain. Together these domains have been shown to recognize the receptor tyrosine kinases EGFR, PDGFR and HGFR and non-receptor tyrosine kinases (ZAP-70 and Syk). The Ring finger motif is important for the transfer of ubiquitin moieties from E2 enzymes to the substrate molecules. The linker region is often deleted or mutated in oncogenic Cbl variants (Thien, Walker et al. 2001). Cbl functions as an E3 ligase by ubiquitination of associated proteins. Proteins that are polyubiquitinated by Cbl are targeted for proteosomal degradation, whereas monoubiquitination of the Cbl substrates is important for recruitment of Ubiquitin-binding proteins to form a large macromolecular complex (Polo, Sigismund et al. 2002). The Proline-rich region is important for the interaction with the SH3 domain containing protein such as Grb2 (growth factor receptor-bound protein 2) and CIN85. c-Cbl is a prominent substrate of tyrosine kinases and is phosphorylated after the stimulation of many cell-surface receptors (Peschard, Fournier et al. 2001; Kassenbrock, Hunter et al. 2002; Thien and Langdon 2005). C-Cbl is efficiently phosphorylated by Syk and Src family kinases Fyn, Yes and Lyn (Feshchenko, Langdon et al. 1998). These tyrosine phosphorylation sites provide docking sites for the SH2 domain containing proteins such as Vav, CrkL and p85 regulatory subunit of Phosphatidylinositol-3 Kinase (PI3K) (Marengere, Mirtsos et al. 1997; Hunter, Burton et al. 1999). UBA and LZ domain is important of the oligomerization of Cbl and for ubiquitin binding.

Overexpression of Cbl promotes EGF, PDGF and CSF-1 receptor multiple monoubiquitination and degradation (Levkowitz, Waterman et al. 1998; Miyake, Lupher et al. 1998; Lee, Wang et al. 1999). c-Cbl binds the activated RTK and promotes receptor multiple monoubiquitination preventing receptor recycling back to the cell surface by sorting the ubiquitinated receptor for lysosomal degradation. Cbl binds to specific tyrosine residues in CSF-1R, HGFR and EGFR (Klapper, Waterman et al. 2000; Peschard, Fournier et al. 2001; Wilhelmsen, Burkhalter et al. 2002). Cbl can also bind to RTKs indirectly through adaptor proteins, such as APS mediated binding to PDGFR (Yokouchi, Wakioka et al. 1999), and Grb2 and FRS2 mediated binding to FGFR (Wong, Lamothe et al. 2002). In the case of EGFR, Cbl binds activated EGFR at Y1045 and

indirectly via Grb2, a signaling adaptor that is recruited to the receptor upon activation (Waterman, Katz et al. 2002). Cbl monubiquitinates EGFR at multiple lysine sites, a modification that is required for subsequent sorting of internalized EGFR in early endosomes for lysosomal transport. Mutation of the Cbl binding site on the RTK (EGFR-Y1045F and HGFR-Y1003F) prevented RTK degradation and promoted receptor recycling back to cell surface (Jiang and Sorkin 2003; Abella, Peschard et al. 2005).

The role of Cbl in receptor internalization is still controversial. Overexpression of Cbl does not enhance EGF receptor internalization (Levkowitz, Waterman et al. 1998), whereas the internalization of the CSF-1 receptor in cCbl-/- macrophages is delayed, but not blocked (Lee, Wang et al. 1999). By functioning as an adaptor, Cbl recruits the adaptor molecules CIN85 and CD2AP, which triggers the signaling cascades for the initiation of receptor endocytosis (Petrelli, Gilestro et al. 2002; Soubeyran, Kowanetz et al. 2002). CIN85 is associated with endophilins, which can induce negative membrane curvature.

Overexpression of the E3 ligase defective Cbl 70ΔZ delayed EGFR internalization, consistent with a role for the E3 ubiquitin ligase activity of Cbl in EGFR internanlization (Jiang and Sorkin 2003). However, it is still unclear if ubiquitination of EGFR or other accessory proteins by Cbl is important for receptor internalization. The ubiquitination deficient mutants EGFR-Y1045F and HGFR-Y1003F were internalized with the same rate as the wild type receptor countparts, indicating efficient ubiquitination of the receptor itself may not be essential for receptor internalization (Jiang and Sorkin 2003; Abella, Peschard et al. 2005). It was also reported that the E3 ligase activity of Cbl and EGFR ubiquitination were required for receptor recruitment into the clathrin-coated pits by interactions with the UIM of Eps15. In the absence of Cbl E3 ligase activity, EGFR can still be internalized, albeit through a clathrin-dependent pathway not involving Eps15 (de Melker, van der Horst et al. 2004; de Melker, van der Horst et al. 2004). It is not clear if the adaptor function of Cbl plays an important role in receptor internalization when the E3 ligase activity of Cbl is unavailable. Alternatively, multiple and redundant

mechanisms including ubiquitin-dependent and ubiquitin-independent mechanisms could be involved in RTK endocytosis.

Negative regulation of Cbl is associated with cancer

There are at least three mechanisms that negatively regulate Cbl function, thereby leading to malignant growth. First, tyrosine phosphorylation of Cbl by the Src protein tyrosine kinase promotes auto-ubiquitination of Cbl and its degradation via proteasomes. Therefore the oncogenic function of Src may be mediated in part through increased degradation of Cbl (Bao, Gur et al. 2003). Activated cdc42 has also been shown to sequester Cbl from interacting with EGFR and from catalyzing receptor ubiquitination and downregulation (Wu, Tu et al. 2003). Cdc42 is a Rho GTPase, involved in signaling downstream of the Ras, one of the most frequently detected oncogene in cancers. Thus, the negative regulation of Cbl by cdc42 might contribute to cancer. Finally, Cbl can also be inactivated by Spry2, a regulator of branching morphogenesis. Tyrosine phosphorylation of Spry2 upon EGFR activation enables the protein to interact with Cbl, which blocks Cbl binding to the receptor and decreases receptor ubiquitination (Wong, Fong et al. 2002; Fong, Leong et al. 2003; Rubin, Litvak et al. 2003). In human cancers increased expression of RTK is a well-documented occurrence. Therefore, it is becoming increasingly important to investigate the role of Cbl and its regulators in the increased expression of RTKs in human cancers. A detailed understanding of this process could identify novel therapeutic targets for cancer treatment.

THE RECEPTOR TYROSINE KINASE HGFR

In this section I will give an overview of the RTK HGFR, the focus of my dissertation. First, I will discuss differences between the proto-oncogene HGFR and the oncogenic variant Tpr-Met. Second, I will describe two ligands that exist for HGFR, the physiological ligand HGF and the *Listeria* surface protein Internalin B (InlB) (Shen, Naujokas et al. 2000). I will compare these two ligands from distinct aspects, with a particular emphasis on the signal transduction and cell responses from HGFR (HGFR structure and signaling complexes are shown in Figure 2). Finally, I will discuss the

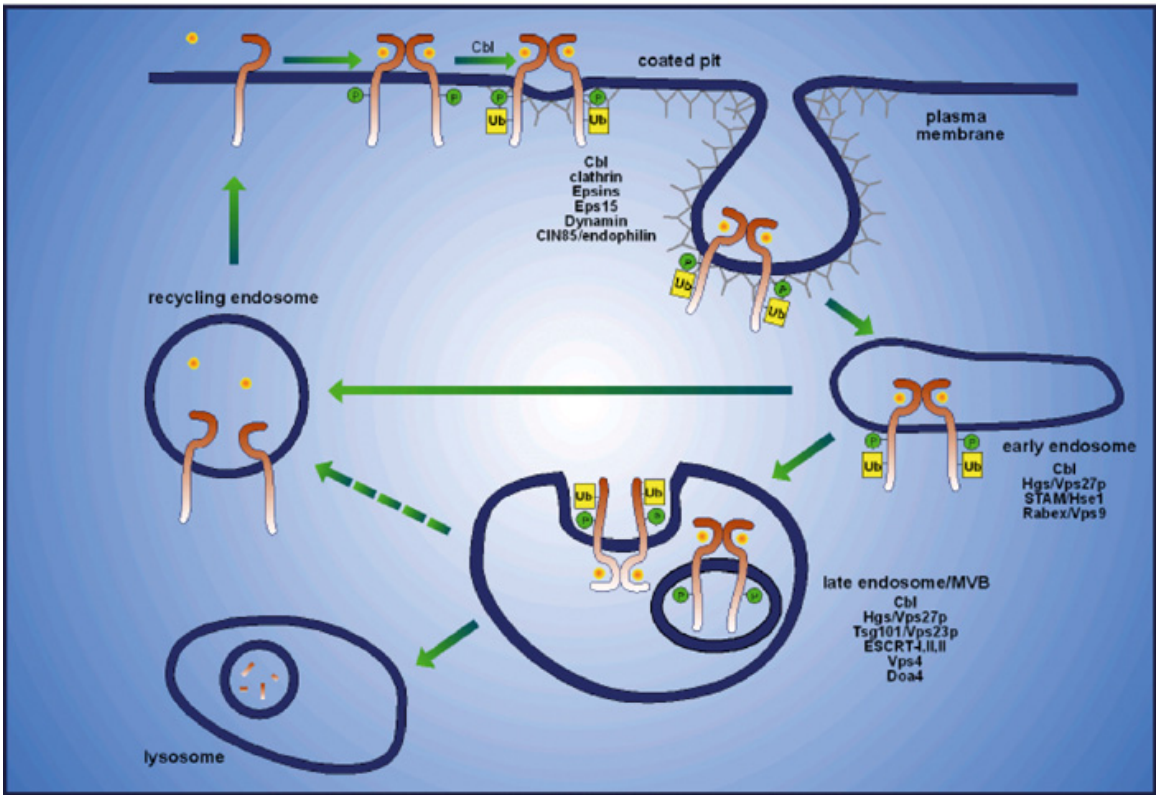

Figure 1. Phosphorylation and ubiquitination are involved in RTK trafficking (EGFR trafficking model). Ligand binding to RTK like EGFR leads to receptor autophosphorylation, recruitment of Cbl and receptor ubiquitination. Ubiquitinated receptor are clustered into clathrin-coated pits by interacting with a multiprotein complex that includes coat adaptor such as Eps15 and Epsin. Besides, Cbl-mediated recruitment of CIN85 and endophilin may promote negative membrane curvature and invagination. EGFR trafficking from early to late endosomes/MVB is dependent on its sustained association with Cbl and ubiquitination. MVB sorting is regulated through recognition of ubiquitinated cargo by Hrs/Hgs/Vps27p, Tsg101/Vps23 and ESCRT-I, II, III complexes. Fusion of the MVB with the lysosome results in degradation of EGFR. Recycling of receptors back to plasma membrane can occur, albeit with decreasing efficiency.

(Marmor MD and Yarden Y, Oncogene. 2004 Mar 15;23(11):2057-70. Review)

possible mechanisms of HGFR induced tumorigenesis and how impaired HGFR downregulation could contribute to cancer.

HGFR was originally identified as an activated oncogene called Tpr-Met. Tpr-Met was the result of a chromosomal translocation that placed the TPR locus (translocated promoter region) encoding a leucine-zipper dimerization motif on chromosome 1 up-stream of the tyrosine kinase domain of HGFR gene found on chromosome 7 (Cooper, Park et al. 1984; Park, Dean et al. 1986) . When expressed in cells, Tpr-Met is constitutively active and is a potent oncogene. Isolation of the Tpr-Met cDNA subsequently led to the identification of full-length HGFR (Park, Gonzatti-Haces et al. 1986).

HGF and InlB

The physiological ligand for HGFR is hepatocyte growth factor/scatter factor (HGF/SF), a disulfide-linked heterodimer produced predominantly by mesenchymal cells and acting primarily on HGFR expressing epithelial cells (Jeffers, Rao et al. 1996). Activation of the HGFR signaling pathway leads to a wide array of cellular responses including cell proliferation, survival, scattering, motility, invasion, angiogenesis, branching morphogenesis, wound healing and tissue regeneration. HGF is a member of the plasminogen-related growth factor family. Like plasminogen, HGF is synthesized as a single-chain precursor and is converted into an active α and β chain heterodimer linked by disulfide bonds like several serine proteinases, including the plasminogen activators uPA (urokinase plasminogen activator) and tPA (tissue-type plasminogen activator). The HGFR is also a disulfide linked heterodimer. The primary HGFR transcript produces a 150-kd polypeptide that is partially glycosylated to produce a 170kd precursor. This 170kd precursor is further glysosylated and then cleaved into a 50kd α chain (entirely extracellular) and 140kd β chain (extracellular domain, transmembrane domain and intracellular domain) (Birchmeier, Birchmeier et al. 2003). The intracellular domain of HGFR β chain contains juxtamembrane domain, kinase domain and carboxyl-terminal tail.

The other ligand for the HGFR is the *Listeria* surface protein InlB. *Listeria monocytogenes* is a food-borne pathogen that causes listeriosis, an infection with a 30% mortality rate in human that is characterized by gastroenteritis, fetoplacental and central nervous system infections (Cossart, Pizarro-Cerda et al. 2003). *Listeria* entry is triggered mainly by two surface internalin proteins: InlA and InlB. InlA and InlB are members of a protein family characterized by leucine-rich repeats (LRRs). InlA specifically interacts with human E-cadherin, while InlB use HGFR as a receptor to trigger bacteria entry. InlB and HGF do not share any similarity in their structure and sequences. InlB is composed of N-terminal Cap, followed by LRR and IR region, which is involved in HGFR binding. At the c-terminal of InlB is the GW domain, which is involved in InlB attachment to either the bacteria surface or proteoglycans (Cossart, Pizarro-Cerda et al. 2003). HGF and InlB seem to bind HGFR at different extracellular sites, because an excess of HGF does not compete with InlB for HGFR binding (Shen, Naujokas et al. 2000). Moreover, it is still unknown if InlB can induce all the cell responses as HGF does, including mitogenesis and morphogenesis.

Signal transduction and cell responses of HGFR

The activation of signal transduction pathways in response to HGF/SF stimulation is mediated by autophosphorylation of specific tyrosine residues in the intracellular region of HGFR. Phosphorylation of two tyrosines (Y1234 and Y1235) located in the activation loop of the HGFR tyrosine kinase domain activates the intrinsic kinase activity of the receptor (Naldini, Vigna et al. 1991; Rodrigues and Park 1994). Subsequent phosphorylation of Y1349 and Y1356 in the c-terminus of HGFR activates the multisubstrate docking site. The multisubstrate docking site is formed by tyrosines 1349 and 1356 and surrounding residues (Y^{1349}VHVNATY^{1356}VNV), resulting in a docking site that binds signal transducers and adaptor proteins containing a Src homology-2 (SH2)

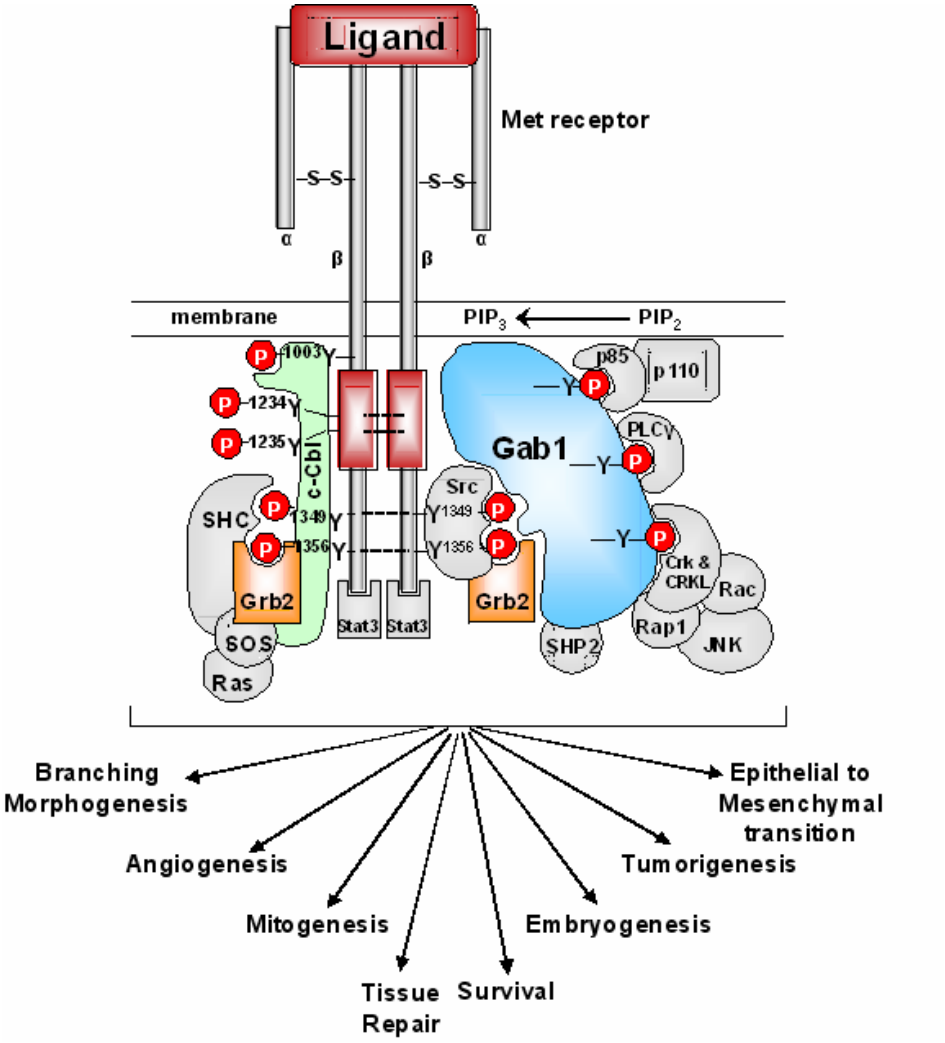

Figure 2. The structure of HGFR and the signaling complex recruited to it on ligand stimulation and receptor dimerization. HGFR is a single-pass disulphide-linked α/β heterodimer formed by proteolytic processing of a precursor. The intracellular domain contains a tyrosine kinase domain (red box) flanked by juxtamembrane domain and C-terminal sequences. A multisubstrate docking site around Y1349 and Y1356 mediates receptor interaction with multiple signal-transducing and adaptor proteins, including Gab1, Grb2, PI3K and PLCγ. Such interactions are important for HGFR induced invasive growth thought to play an essential role in multiple normal physiological processes as well as tumor development and progression.
(Hammond DE et al, Curr Top Microbiol Immunol. 2004;286:21-44. Review)

domain, a phosphotyrosine binding (PTB) domain, or a HGFR binding domain (MBD) (Ponzetto, Bardelli et al. 1994; Pelicci, Giordano et al. 1995; Weidner, Di Cesare et al. 1996). Components of the signaling complex that are recruited to the activated HGFR include the adaptor proteins Grb2, Gab1, SHC and Crk along with other signaling transducers including PI3K, Src, PLC-γ and SHP2.

Several cell responses are evoked in response to HGFR signals. HGFR signals induce cell proliferation and anti-apoptosis in various cell types, and induce cell migration in epithelia. Moreover, when cultured in a collagen matrix MDCK cells form branched tubules in response to HGF (Montesano, Schaller et al. 1991). These cellular responses are essential for embryogenesis since HGF-/- and HGFR-/- transgenic mice are embryonic lethal (Schmidt, Bladt et al. 1995; Uehara, Minowa et al. 1995). Mutation of the multisubstrate docking site (Y1349,1356F) in mouse HGFR resulted in embryonic death, with placental, liver and limb muscle defects, reminiscent of the phenotype of HGFR-/- and HGF-/- mutants (Maina, Casagranda et al. 1996). In adult, HGFR signaling has been shown to be important for organ regeneration. For example, after liver, kidney and heart injury, increased HGF expression has been observed in the damaged organs (Michalopoulos and DeFrances 1997; Nakamura, Mizuno et al. 2000; Matsumoto and Nakamura 2001).

Gab1

Gab1 (Grb2-associated binding protein-1) was first identified as a protein produced from a glial tumor expression cDNA library that binds Grb2 (Holgado-Madruga, Emlet et al. 1996). Gab1 contains an N-terminal pleckstrin homology (PH) domain that binds the membrane lipid phosphoinositol 3, 4, 5-trisphosphate (PIP3), a central region containing multiple tyrosine phosphorylation sites for other signaling molecule binding and a C- terminal proline- rich HGFR Binding Domain (MBD). The MBD domain of Gab1 directly interacts with the multisubstrate docking site of HGFR via pY1349 (Lock, Frigault et al. 2003). Gab1 also binds HGFR indirectly via Grb2. HGFR-Y1356F mutants, in which Grb2 binding is eliminated, show weakened, but not complete loss of Gab1-HGFR interactions (Bardelli, Longati et al. 1997; Maroun, Holgado-

Madruga et al. 1999). In HGF/SF stimulated cells, Gab1 phosphorylation is sustained 3 to 4 times longer than in EGF stimulated cells (Gual, Giordano et al. 2000). Sustained activation of Gab1 may be a major mechanism that mediates HGF/SF induced branching morphogenesis (Rosario and Birchmeier 2003). Gab1 is tyrosine phosphorylated by Src (Chan, Chen et al. 2003), and tyrosine phosphorylation of Gab1 generates multiple docking sites for downstream molecules, including PI3K, Crk, PLC-γ and SHP2 (Gu and Neel 2003).

PI3K plays a significant role in Gab1-mediated signal transduction, since cell treatment with PI3K inhibitors and overexpression of Gab1 mutants unable to bind PI3K prevent Gab1 induced branching morphogenesis (Royal and Park 1995; Niemann, Brinkmann et al. 1998; Maroun, Holgado-Madruga et al. 1999). Expression of a Gab1 mutant with PH domain mutation that reduce the affinity of Gab1 for PIP_3 impaired branching morphogenesis and was not localized to the plasma membrane (Maroun, Holgado-Madruga et al. 1999). PIP_3 is the product of PI3K, therefore recruitment of PI3K via Gab1 sets up a positive feedback loop for Gab1 induced branching morphogenesis (Rodrigues, Falasca et al. 2000). Sustained activation of ERK/MAPK requires Gab1 binding of SHP2, a tyrosine phosphatase. The prolonged activation of ERK contributed by Gab1 is required for cell metastasis and branching morphogenesis. Gab1-/- transgenic mice are embryonic lethal. The phenotypes observed in HGFR-/- mice are also observed in Gab1-/- mice, including reduced liver size, placental defects and impaired migration of myogenic precursor cells, consistent with an essential role for Gab1 in HGFR signaling (Sachs, Brohmann et al. 2000).

Grb2

The adaptor protein Grb2 is composed of an SH2 domain and N- and C-terminal SH3 domain. Grb2 binds activated HGFR via its SH2 domain and recruits other downstream molecules through its N- and C- SH3 domains. In addition to the direct interaction with activated HGFR at Y1356, Grb2 also can be recruited to activated HGFR indirectly via the interaction with tyrosine phosphorylated SHC protein, which binds activated HGFR at Y1349 and Y1356 (Pelicci, Giordano et al. 1995). Several

downstream molecules, including Sos, Gab1 and Cbl, were recruited to HGFR via interactions with Grb2 SH3 domains (Chardin, Camonis et al. 1993; Meisner, Conway et al. 1995; Meisner and Czech 1995; Bardelli, Longati et al. 1997). Grb2 also was found to interact with activated Dynamin, N-WASP through SH3 domain and proline-rich domain interactions (Carlier, Nioche et al. 2000). N-WASP has been shown to be involved in clathrin-coated pit invagination in EGFR endocytosis (Benesch, Polo et al. 2005). Thus, Grb2 is a potential adaptor for receptor endocytosis.

Uncoupling HGFR from Grb2 lowers the HGF-dependent Ras response to about 50% of that evoked by the wild type receptor (Fixman, Fournier et al. 1996). The direct linkage of HGFR with Grb2 is required to promote transformation, but is not essential for triggering cell scatter responses to HGF (Ponzetto, Zhen et al. 1996). Disrupting the consensus binding site for Grb2 binding site (N1358H) in mouse HGFR allowed development to proceed to term, but caused a striking reduction in limb muscle (Maina, Casagranda et al. 1996), suggesting a role for signaling downstream of Grb2 in the development of migratory muscles.

InlB induced signal transduction from HGFR

Like HGF, InlB binds to and activates HGFR, resulting in similar downstream signaling cascades. First, downstream signaling molecules including Gab1, Cbl and Shc are recruited to HGFR and are tyrosine phosphorylated. Second, upon activation by InlB binding PI3K is recruited to HGFR from the cytoplasm to plasma membrane, where it produces $PI(3,4,5)P_3$, a process essential for *Listeria* entry (Ireton, Payrastre et al. 1999). Third, InlB induces membrane ruffles and also cell scattering in certain cell type such as MDCK cells (Shen, Naujokas et al. 2000).

Listeria has been reported to hijack the clathrin-dependent endocytic machinery to invade mammalian cells (Veiga and Cossart 2005). In these studies, the ubiquitin ligase Cbl was shown to be recruited to InlB-activated HGFR, suggesting a role for Cbl mediated-ubiquitination events for bacteria entry. Several major components of the clathrin-dependent endocytic machinery, including clathrin, dynamin, Eps15, CIN85, Cbl, cortactin and Grb2 are important for *Listeria* entry. However, the role of

ubiquitination as a modulator of their endocytic function is unknown. Therefore, through the direct binding of InlB to HGFR, *Listeria* can take advantage of the receptor activated endocytic machinery to invade mammalian cells, an essential key step for pathogen invasion.

HGFR ACTIVATION IN CANCERS

Deregulation of HGF/HGFR signaling has been a crucial feature of many human malignancies, correlating closely with metastatic potential, angiogenesis and poor prognosis. There are a number of mechanisms by which HGFR signaling is activated in human cancers. These include HGFR overexpression by gene amplification, constitutive kinase activation due to gene amplification or HGFR point mutations, paracrine and autocrine activation of HGFR by HGF, and deregulation of HGFR downregualtion. Following is a detailed description of each of these mechanisms that lead to increased HGFR signaling in human cancers.

HGFR overexpression

Elevated HGFR expression has been detected in a number of cancers including lung, renal, pancreatic, colorectal, head and neck, gastric and hepatocellular and a number of sarcomas. Increased HGFR expression can be due to HGFR gene amplification. HGFR expression has also been reported to be upregulated in tumors by epigenetic mechanisms including tumor secreted growth factors, tumor hypoxia and activation of other oncogenes such as Ras (Moghul, Lin et al. 1994; Furge, Kiewlich et al. 2001; Pennacchietti, Michieli et al. 2003).

HGFR structural alterations

Missense mutations in HGFR have been detected in the germ line of hereditary papillary renal cell carcinoma (HPRCC) families (Schmidt, Junker et al. 1998). Kinase domain mutations have been observed in sporadic papillary renal carcinoma, ovarian cancer, and childhood hepatocellular carcinoma (Ma, Maulik et al. 2003).

HGF-dependent autocrine activation

Constitutive HGFR activation can also occur by inappropriate expression of HGF in cells expressing HGFR (or vice versa), forming an autocrine stimulation loop (Rong, Bodescot et al. 1992; Bellusci, Moens et al. 1994; Rong, Segal et al. 1994; Jeffers, Rong et al. 1996).

In addition to these mechanisms, more evidence has shown that impaired endocytosis and downregulation of HGFR can be another novel mechanism for the deregulation of HGFR in cancers.

HGFR endocytosis and degradation

While much is known about HGFR signaling in development, homeostasis and human cancers, little is known about HGFR trafficking. Early studies reported that HGFR internalization requires dynamin, however it was unclear if HGFR internalization is clathrin-dependent or independent (Hammond, Urbe et al. 2001). HGFR internalization was also shown to be sensitive to the proteasome inhibitor lactacystin (Hammond, Urbe et al. 2001). In the presence of lactacystin, HGFR failed to redistribute from the plasma membrane to intracellular compartments (Hammond, Urbe et al. 2001). This data suggested a role for the ubiquitination machinery in HGFR internalization, although the mechanism remained unclear. Cell treatment with proteasome inhibitors has been shown to reduce the size of the free ubiquitin pool in cells, leading to disruption of the ubiquitin machinery and endocytic function in general. It was then found that HGFR internalization requires the E3 ligase Cbl mediated recruitment of CIN85 and endophilin complex (Petrelli, Gilestro et al. 2002). Endophilin has been proposed to promote receptor endocytosis by inducing negative curvature in plasma membranes at the neck of the budding vesicle (Farsad, Ringstad et al. 2001). It is known that HGFR is multiple monoubiquitinated by recruitment of Cbl E3 ligase (Peschard, Fournier et al. 2001), but it is still unknown that the ubiquitination of the receptor or E3 ligase activity of Cbl is involved in the receptor internalization.

Proteasomal activity was shown to be required for HGFR degradation (Hammond, Carter et al. 2003). In this study the proteasome inhibitor lactacystin

promoted the HGFR recycling to the plasma membrane instead of sorting the internalized receptor into late endosomes. In the presence of lactacystin, both HGFR dephosphorylation and tyrosine phosphorylation of Hrs were dramatically reduced (Hammond, Carter et al. 2003). Although the proteasome activity was involved in HGFR degradation, HGFR was shown not to be the target for the proteasomal degradation. A recent report from Dr. Morag Park's group showed that ubiquitination of the HGFR is required for the receptor degradation and also for Hrs tyrosine phosphorylation (Abella, Peschard et al. 2005). They found that the ubiquitination-deficient HGFR-Y1003 is internalized similar to wild type HGFR, but is inefficiently degraded. They also found that the HGFR-Y1003 is tumorigenic *in vivo*. Thus, the endocytic pathway of HGFR may normally function as a tumor suppressor pathway, when inactivated, sustained HGFR signaling could result in cellular transformation.

EXPERIMENTAL RATIONALE AND HYPOTHESIS

The major goal of my thesis is to determine the mechanisms of HGFR endocytosis and degradation. In this introduction, I reviewed our current understanding of HGFR signaling in metastatic cancers and the mechanisms regulating HGFR signaling and trafficking. HGFR is an important RTK, deregulation of which has been linked to tumorigenesis, tumor metastasis and angiogenesis. In addition to several well known mechanisms that amplify HGFR signaling in cancers, impaired inactivation of HGFR by endocytosis and degradation is emerging as a novel mechanism for increased HGFR signaling in cancers. Thus, a detailed study of how endocytic trafficking normally inactivates HGFR signaling should reveal new targets for cancer therapy. Although a lot is known about HGFR signaling, surprisingly little is known about HGFR trafficking and the impact of receptor endocytosis on downstream signaling outputs.

To understand how HGFR trafficking is regulated, I first examined which endocytic route HGFR uses for internalization (chapter 2) (Li, Xiang et al. 2005). I tested if HGFR internalization occurred primarily through a clathrin-dependent pathway using molecular reagents that disrupt this process, including overexpression of dominant

negative Eps15 in cells or using clathrin siRNA to deplete cells of endogenous clathrin. I also demonstrated that soluble InlB mimics HGF induced HGFR internalization through clathrin-coated pits. My studies showed that after internalization, InlB and HGF colocalized with classic calthrin-derived early endosomal markers, including TfnR, EEA1 and Rab5. HGFR internalization was not inhibited by cell treatment with the cholesterol sequestration drug Nystatin, overexpression of Arf6GTPase or caveolin1, confirming that clathrin-independent pathways were not involved in HGFR internalization. These studies were the first to show that HGFR internalization is primarily clathrin-mediated and that InlB mimics HGF mediated HGFR internalization. Next I extended my studies to focus on the molecular mechanisms of how HGFR activation is linked to HGFR internalization (chapter 3). HGFR activation induces the recruitment of multiple signaling molecules and adaptor proteins to the multisubstrate docking site (Y1349 and Y1356). By stably expressing HGFR containing site specific mutations that inhibit HGFR tyrosine kinase activity (K1110A) and downstream signaling molecule recruitment (Y1349,1356F), I demonstrated that the docking site (Y1349 and Y1356) and tyrosine kinase activity of HGFR are both required for the receptor internalization. I extended these findings to examine which adaptor protein (Gab1 or Grb2) is essential for HGFR internalization. Using an SiRNA approach, coupled with dominant negative mutants and a cell line expressing the mutant N1358H-HGFR which is specifically deficient in Grb2 binding I demonstrated that Grb2 played an essential role for HGFR internalization,. Conversely, genetic inactivation or siRNA depletion of Gab1 did not block HGFR internalization. Furthermore, I determined that Grb2 recruited Cbl E3 ligase activity was essential for HGFR internalization. Consistent with this finding, overexpression of ubiquitin mutant unable to bind UIM containing proteins inhibited HGFR internalization. Together, ligand activation of HGFR leads to Grb2 mediated recruitment of the E3 ubiquitin ligase activity to the receptor, which is essential for clathrin-mediated HGFR internalization. Finally, I characterized the mechanism regulating HGFR degradation in lysosomes (chapter 4). Prior to my dissertation studies, HGFR degradation was reported to occur through the proteasomal

pathway involving receptor polyubiquitination (Jeffers, Taylor et al. 1997). My studies showed that Hrs, an essential component for the lysosomal degradation of multiple monoubiquitinated receptors, was essential for HGFR degradation. My data were the first to demonstrate that HGFR was subject to lysosomal degradation rather than involving the proteasome in multiple cell types. Moreover, inhibition of PI3K activity delayed HGFR degradation possibly by interfering the Hrs binding to early endosomes enriched in PI3P, the product of PI3K. Together, HGFR was degraded through lysosomal pathway and PI3K and Hrs are involved in this process.

CHAPTER 2: THE LISTERIA PROTEIN INLB MIMICS HEPATOCYTE GROWTH FACTOR RECEPTOR INTERNALIZATION THROUGH CLATHRIN-COATED PITS

INTRODUCTION

The Hepatocyte Growth Factor Receptor (HGFR or cMet) is a receptor tyrosine kinase that regulates a wide range of cellular responses including cell proliferation and survival, cell motility and invasion, branching morphogenesis and angiogenesis (Furge, Zhang et al. 2000; Birchmeier, Birchmeier et al. 2003). Under physiological conditions HGFR activation and signaling is tightly regulated. However in several human gastrointestinal and hepatic cancers, increased HGFR signaling correlates closely with neoplastic invasion and metastatic potential (Maulik, Shrikhande et al. 2002). A great deal is known about HGFR structure and its signaling properties. For example, human HGFR is a disulphide-linked α/β heterodimer derived by proteolytic cleavage of a 170 KD precursor. The α chain is exclusively extracellular, whereas the 140 KD β chain spans the membrane once and possesses a cytoplasmic tyrosine kinase domain. Ligand binding to the extracellular domain of HGFR activates its tyrosine kinase activity, leading to recruitment and phosphorylation of multiple signaling molecules and adaptor proteins including Grb2, Gab1, Shc and Phosphoinositide 3-Kinase (PI3K) (Furge, Zhang et al. 2000; Birchmeier, Birchmeier et al. 2003). Following activation, HGFR is internalized by endocytosis and eventually inactivated by degradation (Jeffers, Taylor et al. 1997; Hammond, Urbe et al. 2001). As such, endocytosis can be considered an important regulatory mechanism for reducing receptor signaling. This is particularly important as prolonged HGFR activation appears to be a key factor underlying the pathogenesis of HGFR signaling during cellular transformation (Peschard, Fournier et al. 2001) and cell motility (Petrelli, Gilestro et al. 2002).

At least two ligands activate HGFR signaling and endocytosis; the physiological ligand Hepatocyte Growth Factor Receptor (HGF) and the Internalin B (InlB) protein of the bacterium Listeria Monocytogenes (Shen, Naujokas et al. 2000). InlB exists as a form bound to the surface of Listeria as well as a soluble form secreted into culture supernatants (Braun, Dramsi et al. 1997). One major function of InlB is to mediate phagocytic entry of Listeria into host cells by binding directly to HGFR and activating receptor signaling (Shen, Naujokas et al. 2000). Although soluble InlB (herein referred to as InlB) and HGF exhibit similar affinities for HGFR, they share no sequence homology and are structurally unrelated. HGF is a heterodimer formed by □ and □ subunits that are linked by disulphide bonds, whereas InlB is a monomer composed of distinct structural domains (Bierne and Cossart 2002). Like HGF, InlB stimulates the recruitment of Gab1, cSrc, cCbl, Grb2 and PI3K in a HGFR dependent manner (Shen, Naujokas et al. 2000). However, several studies suggest that InlB does not strictly mimic HGF-induced signaling. First, InlB and HGF do not bind to the same site on the extracellular domain of HGFR, since an excess of HGF does not compete with InlB for binding to HGFR (Shen, Naujokas et al. 2000). Second, InlB-induced autophosphorylation of HGFR is more transient than that produced by HGF, which remains consistent after 2 hours (Shen, Naujokas et al. 2000). Third, InlB activates the Ras-MAPK pathway for a considerably longer period than HGF (Copp, Marino et al. 2003). Given the critical role of endocytosis for controlling the signaling intensity and duration of many signaling receptors (Ceresa and Schmid 2000; Di Fiore and De Camilli 2001; Benmerah 2004), the reported differences in InlB- and HGF-induced receptor signaling may reflect differences in the internalization and degradation of HGFR in response to these distinct ligands.

To test this hypothesis, I first examined which endocytic mechanisms InlB and HGF use to regulate the internalization of HGFR. I demonstrate that like HGF, InlB induces rapid internalization of HGFR into clathrin-derived, early endosomes with similar internalization rates. Moreover, over expression of Arf6 mutants, caveolin 1 or treatment with the cholesterol-chelating drug Nystatin to prevent internalization via clathrin-independent mechanisms, did not alter clathrin-mediated HGFR internalization.

Together, my studies indicate that InlB mimics HGF-induced clathrin-mediated HGFR internalization. My findings support the hypothesis that InlB is a bona fide agonist that regulates HGFR endocytosis and hence the growth and motility enhancing properties of HGFR signaling.

MATERIALS AND METHODS

Reagents, Antibodies and Cell lines

All general reagents were obtained from Fisher Scientific or Sigma Aldrich unless indicated otherwise. Recombinant human HGF (PeproTech Inc., NJ); EZ link sulfo-NHS-SS-Biotin, D-Salt Dextran and Spin columns (Pierce Biotechnology Inc., IL); TR-Tfn and Antibody labeling kits (Molecular Probes, OR). The following antibodies were purchased as indicated — anti-human HGF (R&D Systems Inc, MN), EEA1 (BD Biosciences, CA), anti-HA (Roche, NJ), TfnR (Zymed Labs, CA), GFP (Qbiogene Inc., CA), Met C-28 (Santa Cruz Biotechnologies, CA), phospho-Met Y1234, Y1235 (UpState Biotechnology) Dynamin 1/2 (Cell Signaling Technology) JNK, phospho-JNK, p42/p44 MAPK and phospho p42/p44 (Cell Signaling Technology), EGFR (SC-03) and phospho-EGFR (Y1173) (Santa Cruz, CA), Peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Jackson ImmunoResearch Labs Inc, PA), Alexa488 or Alexa594 -labeled goat anti-mouse, anti-rabbit or anti-rat secondary antibodies (Molecular Probes, OR). Human mammary epithelial cells (T47D) stably expressing full length human HGFR (T47D/cMet, M. Park, McGill University) were maintained in DMEM containing 10% FBS, supplemented with 400 μg/ml G418. MDCK and African Green Monkey kidney cells (Vero, ATTC CRL-1587) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. All cell lines were serum starved in DMEM for 6-8 hours before experimentation unless specified otherwise.

Plasmids and Transient Transfections

Eukaryotic expression plasmids for the following proteins were provided as indicated — Human Rab5Q79L in pEGFP-C1 (P. Stahl, Washington University), HA-

tagged Arf6Q67L and Arf6T27N in pXS (J.G. Donaldson, NIH), Eps15-EH29 and Eps15-DIIIΔ2 in pEGFP-C2 (Dr. Alexandre Benmerah, Cochin Institute), HA-tagged wild-type and K44A mutant Dynamin 1 (S. Schmid, Scripps Research Institute), C-terminally GFP-tagged caveolin 1 (R. Anderson, UT Southwestern). Recombinant adenovirus expressing human Dynamin 1 K44A (J. Pessin, Univ. Rochester) or GFP only was prepared, amplified and purified on CsCl gradients. For transfections, T47D/cMet cells were grown on glass poly D-lysine coated cover slips in 24 well plates at least 24 hr prior to transfection and transfected with Fugene 6 reagent (Roche, NJ) as specified by the manufacturer. Confocal microscopy was routinely performed 24-48 hrs following transfection. For studies using recombinant adenoviruses, cells were exposed to virus for 2 hr at an empirically determined MOI to achieve 80% infection before the medium was changed. After 48 hr, the cells were transferred into serum-free media for 6-8 hrs and then incubated with InlB as described in the appropriate figure legends.

Recombinant InlB Purification and Labeling

pKI22 expressing recombinant InlB containing an N-terminal His6-tag was expressed in BL21 DE3 cells and isolated by Ni+ chromatography as previously described (Ireton, Payrastre et al. 1999). A protein fraction enriched in recombinant InlB was desalted (D-Salt Dextran, Pierce, IL) and purified by ion exchange chromatography (HiTrap SP Amersham Pharmacia) eluting with phosphate buffered saline (PBS) containing 0.4-0.5 M NaCl . Pure recombinant InlB was Amicon concentrated to 2-3 mg/ml of protein (BCA Assay, Pierce, IL), exchanged into PBS and stored in aliquots at –80°C. 200-300 μg of purified, recombinant InlB in PBS was routinely labeled with Alexa488 or Alexa594 using a Monoclonal Antibody Labeling Kit (Molecular Probes, Eugene, OR) with the following modifications. 100 μl of InlB (2-3 mg/ml) was mixed with 10 μl of freshly prepared 1.0 M solution of sodium bicarbonate to increase the pH of the reaction mixture for optimal labeling (pH ~ 8.0), and transferred to a single vial containing the reactive Alexa Fluorophore. The reaction was incubated for 1 hr at room temperature in the dark with occasional shaking followed by an overnight incubation at 4°C. The labeling reaction was clarified by centrifugation at 10,000 xg for 10 min and

unreacted fluorophore removed by centrifugation at 1,100 xg for 4 min using P6 micro bio-spin columns pre-equilibrated with PBS (Bio-Rad, Hercules, CA). Labeled InlB was supplemented with 2 mM NaN3, the concentration of incorporated Alexa determined according to the manufacturer's instructions, aliquoted and stored at –80°C.

Biotinylation and HGFR Internalization assay

Cells were surface biotinylated at 4°C for 30 min with EZ-link NHS-SS-biotin. HGFR uptake was induced by incubation in serum-free DMEM containing InlB or HGF at 37°C for the indicated time. Endocytosis was halted by rapid cooling to 4°C and residual cell surface biotin was then stripped by 2-3 washes for 2 hr with washing buffer (150 mM NaCl, 1 mM EDTA, 0.2% BSA, 20 mM Tris, pH 8.6) containing 50 mM MesNa. The cells were rinsed 3-5 times with ice-cold washing buffer, followed by 3-5 washes in PBS containing 1 mM CaCl2 and 1 mM MgCl2 (PBS+CM). Cells were lysed in ice-cold lysis buffer (0.5% Triton X100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris-Cl pH 7.4, 1 mM EDTA, 10 mM NaF, 2 mM Na3VO4, 1 mM PMSF, 1μg/ml of Aprotinin, 1μg/ml of Leupeptin and 1μg/ml of Pepstatin), internalized biotinylated proteins were isolated using streptavidin-agarose beads (Pierce) and identified by Western analysis (Amersham Pharmacia). Resulting digitized blots were quantified in the linear range using AlphaEase v3.1.2 Software (AlphaInnotech) and normalized to the band intensity of the paired biotinylated, but unstripped sample representing total surface receptor at the time of biotinylation. Resulting cell lysates were examined by streptavidin pull downs and Western analyses. Studies were performed in triplicate and all numerical results subjected to a one-way ANOVA with a Neuman-Keuls post-hoc test to determine statistical significance ($P<0.001$) between experimental groups (Prism GraphPad).

Confocal Microscopy and Analysis

For internalization studies using Alexa-InlB, cells were plated on poly lysine D-coated cover slips and incubated in DMEM containing Alexa-InlB at 37°C as indicated in the figure legends. In some cases, cells were cointernalized with TR-Tfn (5 μg/ml). At the appropriate time, cells were washed with 4 °C DMEM, then washed briefly in PBS and fixed for 30 min at room temperature in 4% Para formaldehyde (Ted Pella Inc.) in

PBS, followed by quenching with 50 mM NH_4Cl for 10 min at room temperature. Cells were washed twice with PBS, permeabilized with PBS containing 0.05% saponin and 10% goat serum for 30 min at room temperature. All antibodies were routinely diluted in PBS containing 0.05% saponin and 10% goat serum and incubated on cells for 1 hr at room temperature. Cover slips were mounted on glass slides using FluorSave mounting medium (CalBiochem), and observed by confocal microscopy using a Zeiss LSM 510 confocal microscope with a 63 X oil (1.4 N.A.) immersion objective. Samples were visualized using the 488 nm and 543 nm laser lines and emission filter sets at 505-530 nm for GFP and $Alexa^{488}$ detection or 585-615 nm for Texas Red and $Alexa^{594}$ detection respectively. Figure presentation was accomplished in Adobe Photoshop v6.0. Quantification of colocalization and internal fluorescence intensity were done using Metamorph v5.0 (Universal Imaging Corp., Westchester, PA). All pixel intensity levels were normalized relative to control values, expressed as a percentage ± Standard Error (S.E.) and differences statistically verified by ANOVA using GraphPad Prism Software (GraphPad Prism).

RESULTS

InlB specifically activates HGFR signaling.

Prior to examining the internalization properties of HGFR, I verified the specificity of InlB as a HGFR ligand using T47D/cMet cells (Shen, Naujokas et al. 2000). T47D/cMet cells are a human mammary epithelial cell line that stably expresses human HGFR on their surface that has been used extensively on studies examining HGFR signaling. HGF and InlB induced comparable activation of HGFR, MAPK and JNK (Figure 3). The specificity of InlB was further verified by examining the activation of EGFR. Under these conditions, InlB and HGF did not activate tyrosine phosphorylation of the EGFR in T47D/cMet cells. These studies confirm that InlB is a specific ligand for HGFR and are consistent with previous findings using Vero and other cell lines (Ireton, Payrastre et al. 1999; Shen, Naujokas et al. 2000).

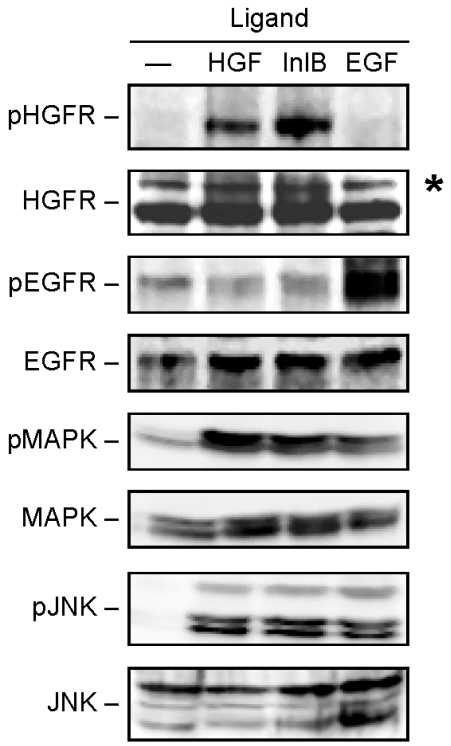

Figure 3. InlB activates HGFR signaling. Serum starved T47D/cMet cells were incubated with HGF (100 ng/ml; 1.5 nM), InlB (150 ng/ml; 2.0 nM), EGF (10 ng/ml; 1.3 nM) or DMEM lacking ligand (-) for 10 min at 37°C. Activation of receptor signaling was examined by Western blot analysis of cell lysates using antibodies for total (HGFR, EGFR, MAPK or JNK) or phosphorylated (pHGFR, pEGFR, pMAPK or pJNK) proteins as indicated. Asterisk (*) denotes the 170 kD HGFR precursor.

InlB triggers rapid internalization of the HGFR

To confirm that InlB induces HGFR internalization, I measured the time course of receptor endocytosis under basal and ligand-stimulated conditions using a surface biotinylation assay. Cell surface proteins were labeled at 4°C with NHS-SS-biotin, that is cleavable by washing with the non-permeable reducing agent MesNa. Control studies performed at 4°C in the absence of InlB verified the efficacy of MesNa washing and the specificity of the streptavidin pull downs. Under these conditions, MesNa washing routinely reduced the ability of streptavidin agarose to isolate biotinylated HGFR by 92-96% (Figure 4A). Constitutive internalization of low levels of HGFR was observed in cells incubated at 37°C; in the absence of ligand, 82.5 ± 1.9% of HGFR remained on the cell surface. Conversely, treatment with InlB at 37°C triggered rapid internalization of 78.0 ± 6.4% of the HGFR. Under these conditions, maximal levels of internalized HGFR

were detected within 15 min of InlB and HGF treatment (Figure 4B). In contrast to the HGFR, 75.5 ± 3.1% of the Transferrin Receptor (TfR) was internalized in the presence or absence of ligand, consistent with the constitutive endocytosis and recycling of this receptor (Figure 4A). To determine if InlB and HGF were comparable at inducing HGFR internalization, I measured the internalization rate of HGFR activated by InlB or HGF binding. Both ligands were directly comparable in their ability to trigger rapid HGFR internalization from the cell surface ($K^e_{InlB} = 0.21 \pm 0.03$ min^{-1}, $K^e_{HGF} = 0.18 \pm 0.02$ min^{-1}) (Figure 4C) implying that InlB and HGF could elicit similar endocytic mechanisms for receptor internalization.

My data indicating that InlB and HGF are equally effective at triggering HGFR internalization suggests that these ligands might colocalize with HGFR following endocytosis. To distinguish internalized HGFR from *de novo* synthesized receptor, I used HGF and InlB as indicators of colocalization with internalized HGFR. InlB was labeled with Alexa^{488} (Alexa-InlB), whereas internalized HGF was detected by costaining with an anti-HGF antibody, and their subcellular localizations were examined by confocal microscopy. Treatment with Alexa-InlB and HGF resulted in colocalization of the ligands in punctate organelles throughout the cytoplasm of the cells, reminiscent of early endosomes (Figure 4D). The ability of Alexa-InlB to activate HGFR and JNK signaling was confirmed by western analysis (data not shown). Taken together, these data indicate that like HGF, InlB remains bound to internalized HGFR.

InlB is internalized into Early Endosomes.

Using Alexa-InlB as a ligand, I examined the fate of internalized HGFR in T47D/cMet cells by confocal microscopy. Alexa-InlB was internalized under steady state conditions at 37°C for 5 min to trigger HGFR internalization. Under these conditions, InlB was detected with cointernalized Texas-Red labeled Transferrin (TR-Tfn) in intracellular punctate structures that costained with antibodies against EEA1 (Figure 5), a marker for clathrin-derived early endosomes . In contrast to the situation with T47D/cMet

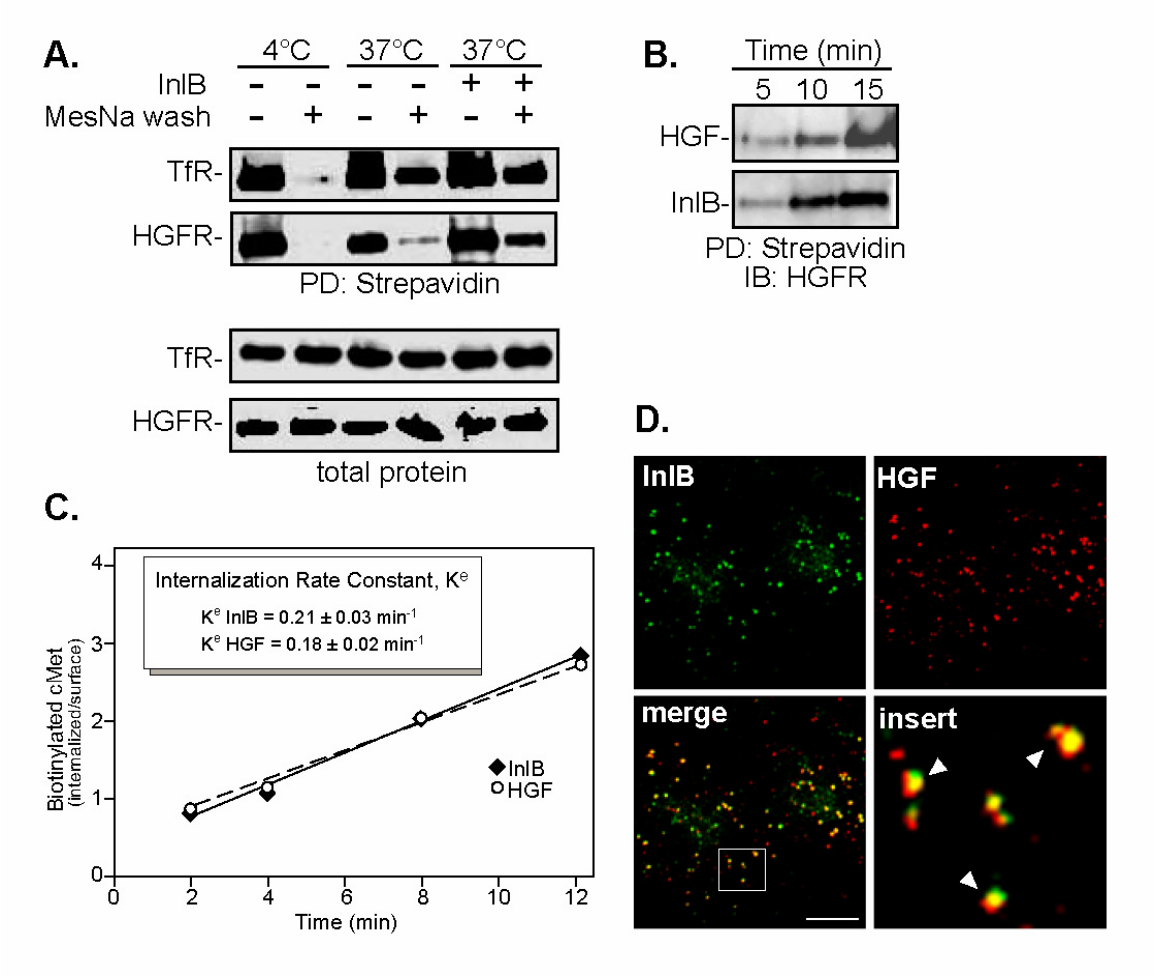

Figure 4. InlB triggers rapid HGFR internalization. A, duplicate plates of surface biotinylated cells T47D/cMet cells were incubated in the absence (-) or presence (+) of 100 ng/ml InlB for 10 min at 4°C or 37°C as indicated. Internalization was stopped by rapid cooling to 4°C. Biotinylated proteins remaining on the cell surface of one set of plates were stripped of biotin by three washes with 50 mM MesNa (+) whereas control plates were washed in saline (-) to measure total biotinylated protein. Biotinylated proteins were isolated by streptavidin pull downs (PD) and analyzed by immunoblot (IB) analysis with antibodies specific for HGFR or the Transferrin Receptor (TfR). A representative example from 3-4 separate studies is shown. B, Surface biotinylated T47D/cMet cells were incubated for 5, 10 or 15 min in media containing 100 ng/ml InlB or HGF (unless stated otherwise) and HGFR endocytosis in MesNa washed cells (+) was examined by streptavidin pull downs (PD) and westerns (IB). C, Surface biotinylated T47D/cMet cells were incubated for 2, 4, 8 or 12 min in media containing 100 ng/ml InlB or HGF, the cells were rapidly cooled to 4°C and residual surface biotin stripped by

MesNa washes. Cells were lysed, proteins isolated by streptavidin pull downs and western analysis as described above. Resulting digitized blots were quantified using AlphaInnotech electrophoresis documentation Software and normalized to the band intensity of a biotinylated, but unstripped sample prepared at 4°C in the absence of HGF or InlB treatment, representing total surface HGFR at the time of biotinylation. The ratio of internalized versus remaining surface HGFR was plotted against time and the specific rate constant for internalization (Ke) calculated as a linear regression coefficient . Data is representative of 3 independent experiments. D, T47D/cMet cells were incubated in DMEM containing Alexa488-labeled InlB (InlB) and HGF at 37°C for 5 min and processed for confocal microscopy using Alexa594-conjugated goat anti mouse secondary antibody to detect internalized HGF. Areas of colocalization in the merged image appear yellow and are shown in the boxed region with arrowheads (lower right panel). Bar 10 μm.

cells, no Alexa-InlB was detected in parental T47D cells in which HGFR is virtually undetectable, confirming that InlB uptake was dependent on the HGFR. To verify that InlB was internalized into early endosomes, I examined the pattern of Alexa-InlB fluorescence in T47D/cMet cells transiently transfected with a GFP-labeled Rab5 mutant. Rab5 functions on early endosomes to regulate early endosome fusion. When overexpressed in cells, the GTPase deficient mutant Rab5Q79L increases the rate of early endosome fusion, resulting in enlarged endosomes . As shown in Figure 3, internalized Alexa-InlB (labeled with $Alexa^{594}$) accumulated in enlarged vesicles coinciding with the localization of GFP-labeled Rab5Q79L. InlB was routinely detected in the lumen surrounded by GFP-Rab5Q79L on the membrane of these vesicles, consistent with internalization into early endosomes. Quantification of the confocal micrographs revealed that 80 ± 5.65% of the ligand was detected in early endosomes within 10 min of internalization. Incubation for longer periods (45-60 min) resulted in colocalization of internalized Alexa-InlB with LAMP 1, a marker for lysosomes (data not shown). However, the overall quantity of Alexa-InlB detected at these later time points was significantly lower than that detected at earlier times, consistent with the dissociation of InlB from HGFR, or lysosomal degradation of InlB and/or HGFR. Together, these data confirm that InlB triggers rapid internalization of HGFR into EEA1- and Rab5 positive early endosomes.

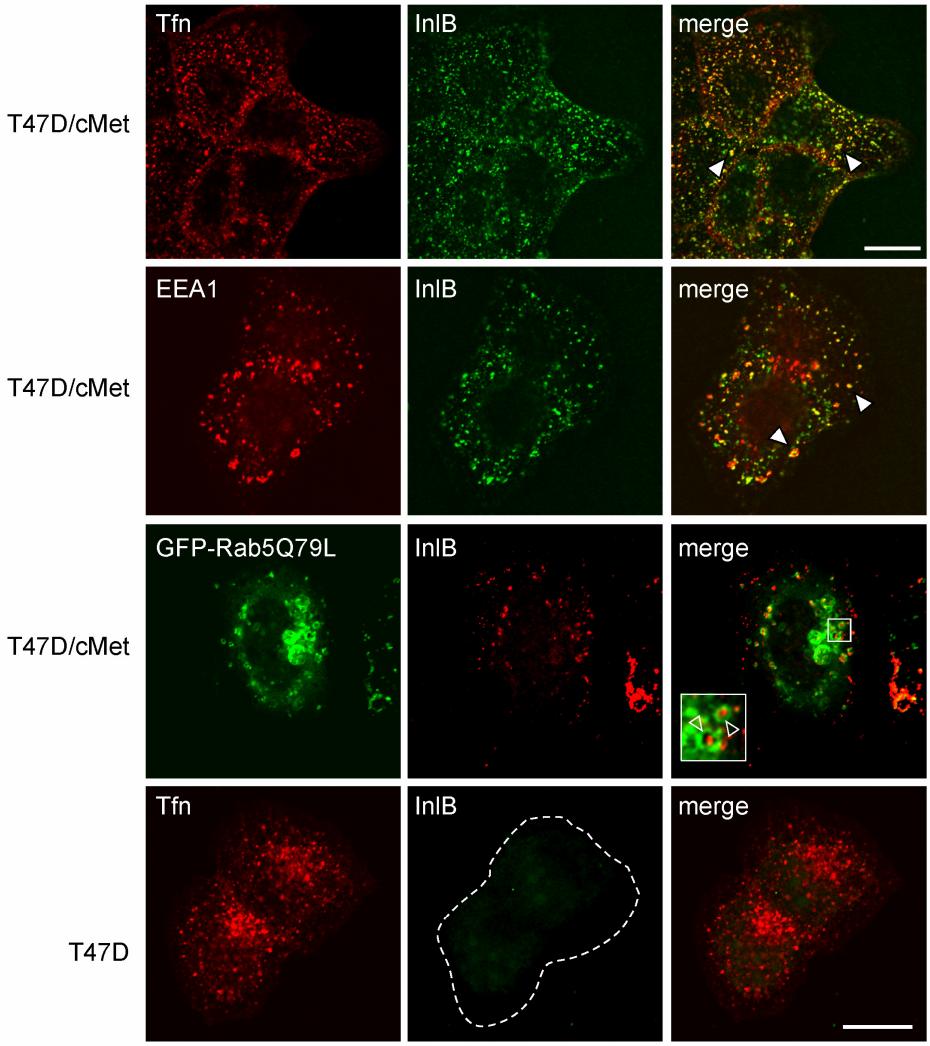

Figure 5. InlB is internalized into early endosomes. T47D cells expressing wild type HGFR (T47D/cMet) or parental T47D cells (T47D) were incubated in media containing TR-Tfn (Tfn) and Alexa^{488}-labeled InlB (InlB) for 5 min at 37°C, and ligand uptake was analyzed by confocal microscopy. T47D/cMet cells internalized with Alexa^{488}-labeled InlB only were stained for endogenous EEA1. Transfected T47D/cMet cells expressing GFP-labeled Rab5Q79L were incubated with Alexa^{594}-labeled InlB (InlB) for 15 min at 37°C. Inset shows InlB detected in the lumen of Rab5-positive vesicles (open arrow). Closed arrows show areas of colocalization (yellow) in the merged images (merge). Bar, 10 μm.

Clathrin-mediated internalization of InlB.

At least four functionally distinct pathways regulate the internalization of cell surface proteins (Conner and Schmid 2003; Nabi and Le 2003; Pelkmans and Helenius 2003). First, clathrin-mediated endocytosis involves the formation of transport vesicles from clathrin-coated pits. Second, caveolae-mediated endocytosis functions independently of clathrin and involves the protein caveolin 1 and lipid rafts enriched in cholesterol. Both clathrin- and caveolae-dependent endocytosis require the activity of the GTPase Dynamin that assembles into a spiral collar around the neck of clathrin- and caveolin-containing pits (Henley, Krueger et al. 1998; Sever, Damke et al. 2000). Third is a lipid raft–mediated pathway involving cholesterol that exists in cells devoid of caveolin 1. Finally, macropinocytosis involves the formation of large vesicles that engulf extracellular fluid via an actin-based mechanism involving PI3K and/or the GTPase Arf6 (Amyere, Payrastre et al. 2000; Donaldson 2003). These pathways appear to intersect downstream of internalization, at the level of clathrin-derived, EEA1-positive early endosomes (Naslavsky, Weigert et al. 2003).

A previous study reported that HGF-induced HGFR internalization required cCbl, CIN85 and endophilin (Petrelli, Gilestro et al. 2002), suggesting that HGFR internalization occurred via clathrin-coated pits. Conversely, a recent study on *Listeria* uptake implicated lipid rafts in InlB-mediated phagocytic entry (Seveau, Bierne et al. 2004). To reconcile these differences, I performed a detailed analysis of the endocytic routes involved in InlB-induced HGFR internalization. To determine if soluble recombinant InlB was internalized via clathrin-dependent or -independent mechanisms, I examined the requirement of internalization on Dynamin activity by confocal microscopy. Expression of the dominant negative mutant Dynamin 1 K44A blocked internalization of Alexa-InlB in 90% of the cells. Under these conditions, faint clusters of InlB were detected on the surface of transfected cells (Figure 6A), akin to non-internalized ligand clustered at internalization sites. To confirm my confocal studies, I used a recombinant adenovirus to overexpress Dynamin 1 K44A, and measured its effect on HGFR internalization using a surface biotinylation assay (Figure 6B). Adenovirus-

mediated expression of Dynamin 1 K44A caused an 82.9% reduction in HGFR internalization following InlB treatment. The inhibitory effect of Dynamin 1 K44A was specific, as InlB triggered internalization of HGFR into T47D/cMet cells infected with a control virus expressing GFP only. Internalization of an InlB-HGFR complex is thus dependent on Dynamin 1 activity (Figure 6B).

Dynamin 1 regulates both clathrin- and caveolae-dependent endocytic pathways. To directly examine the role of clathrin-mediated endocytosis in InlB-induced HGFR internalization, I perturbed the function of Eps15, a key component for assembling functional clathrin-coated pits (Benmerah, Lamaze et al. 1998; Benmerah, Bayrou et al. 1999). Expression of dominant negative Eps15-Δ95/295, but not the inactive variant Eps15-DIIIΔ2 inhibits clathrin-mediated endocytosis of the TfR in many cell types . When transiently expressed in T47D/cMet cells, dominant negative Eps15-Δ95/295 inhibited the internalization of InlB, HGF and Tfn by 79.0%, 82.0% and 90.2% respectively (Figure 6C and D). Conversely, comparable amounts of internalized InlB and Tfn were detected in cells transiently expressing the inactive variant Eps15-DIIIΔ2 and in control untransfected cells (Figure 6D) consistent with the clathrin-dependent internalization of these ligands. A comparable block in InlB internalization was also detected in Vero cells that overexpress dominant negative Eps15-Δ95/295, indicating that clathrin-dependent endocytosis of InlB is not cell type specific. Together, these studies imply that like HGF, InlB induces internalization of the HGFR via clathrin-coated pits.

To confirm that clathrin-independent mechanisms are not involved in InlB-triggered HGFR endocytosis, I examined the role of Arf6, a small GTPase involved in the clathrin-independent endocytosis of many surface proteins, including the Major Histocompatibility Complex class 1 protein (MHC1), the Interleukin 2 receptor subunit (Tac) and E-cadherin (Lamaze, Dujeancourt et al. 2001; Naslavsky, Weigert et al. 2003; Paterson, Parton et al. 2003). In addition, activation of Arf6 by GTP binding occurs in response to HGF, is required for Rac-activated macropinocytosis as well as HGF-induced cell scattering (Donaldson 2003). To examine the requirement for Arf6 in HGFR

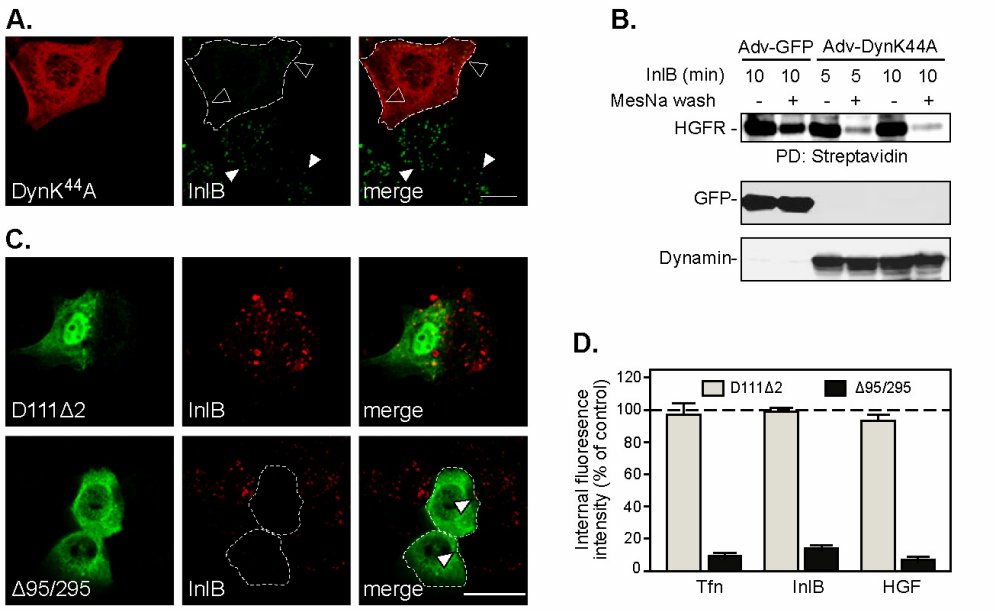

Figure 6. Internalization of HGF and InlB is clathrin-mediated. A, Untransfected T47D/cMet cells or transfected cells expressing HA-tagged Dynamin 1 K44A were allowed to internalize Alexa488-labeled InlB (InlB) for 15 min before processing for confocal microscopy. The presence of InlB on the surface of transfected cells identified by costaining for the HA-epitope is shown in the merged image (open arrowhead). Internalized Alexa-InlB in control cells is shown with a closed arrowhead. Bar, 10 □m. B, T47D/cMet cells were infected with recombinant adenovirus expressing GFP (Adv-GFP) or Dynamin 1 K44A (Adv-DynK44A) for 2 days. Cells were surface biotinylated and incubated in media containing InlB at 37°C for 5 or 10 min. Biotinylated proteins remaining on the cell surface were stripped of biotin with MesNa washes (+) whereas control cells washed in saline (-) were used to measure total biotinylated protein. Cells were lysed and biotinylated HGFR detected by streptavidin pull downs (PD) followed by Western analysis using antibodies specific for HGFR, GFP and Dynamin. Representative blots from 3 independent studies are shown. C, T47D/cMet cells transiently expressing GFP tagged, dominant negative Eps15 (Eps15-Δ95/295) or the inactive variant Eps15-DIIIΔ2 were allowed to internalize TR-Tfn, Alexa594-labeled InlB or HGF for 15 min at 37°C before processing for confocal microscopy as described in the legend to Figure 3. Representative examples of InlB-internalized cells from 3-4 separate experiments are shown. D, The relative amounts of internalized Tfn, InlB and HGF in transfected cells were quantified and expressed as the mean fluorescence intensity of 20 or more randomly selected cells ± S.E. from 3 separate experiments (ANOVA $p < 0.001$).

internalization, T47D/cMet cells were transiently transfected with either Arf6Q67L (a mutant defective in GTPase activity) or the dominant negative mutant Arf6T27N, and Alexa-InlB internalization was quantified by confocal microscopy. Comparable levels of internalized InlB were detected in control, untransfected cells and in cells transiently expressing the Arf6 mutants (Figure 8A and 8B). Consistent with previous reports (Naslavsky, Weigert et al. 2003), Arf6 overexpression did not effect internalization of Tfn, a marker for clathrin-dependent endocytosis (Figure 8B). Control studies confirmed the activity of the Arf6 mutants; expression of constitutively active Arf6Q67L, but not dominant negative Arf6T27N, resulted in the formation of circular, actin-enriched protrusions in transfected cells (see Figure 7), consistent with previous reports (Radhakrishna, Al-Awar et al. 1999). HGF-activated HGFR signaling has been reported to induce coendocytosis of E-cadherin with HGFR (Kamei, Matozaki et al. 1999). Arf6Q67L expression in MCF cells has been shown to restrict endocytosed E-cadherin to large peripheral vesicles devoid of EEA1, by limiting the availability of these vesicles for fusion with classical, clathrin-derived early endosomes. Therefore. I examined if a similar situation occurred with soluble InlB. In T47D/cMet cells overexpressing Arf6Q67L, internalized Alexa-InlB was coincident with endogenous EEA1 (Figure 8C and 8D), in a pattern identical to that observed in untransfected, control cells (refer to Figure 6). These findings confirm that over expression of Arf6 mutants did not alter the internalization of HGFR via clathrin-coated pits.

I also assessed the role of the caveolae/lipid raft pathways on HGFR endocytosis. Using caveolin 1 as a specific marker, I examined the colocalization of Alexa-InlB internalized for 5 min with transiently expressed caveolin 1 (labeled C-terminally with GFP) in T47D/cMet cells. Endogenous caveolin 1 is below the level of detection in T47D/cMet cells by Western analysis or confocal microscopy (data not shown) necessitating the use of an over expression approach. No significant overlap in Alexa-InlB and caveolin 1-GFP was detected under these conditions, indicating that the HGFR was not internalized via a caveolin-mediated pathway (Figure 9A). To confirm

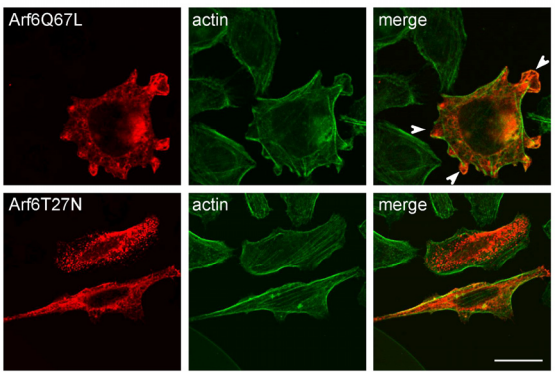

Figure 7. Arf6-GTP expression induces formation of cell protrusions. Hela cells transiently expressing HA-tagged, GDP-bound Arf6T27N or the constitutively active mutant Arf6Q67L were costained with FITC-labeled phalloidin to visualize actin filaments and anti-HA antibodies (Red) before processing for confocal microscopy. Membrane ruffles enriched in actin filaments are indicated (arrows). Bar, 10μm.

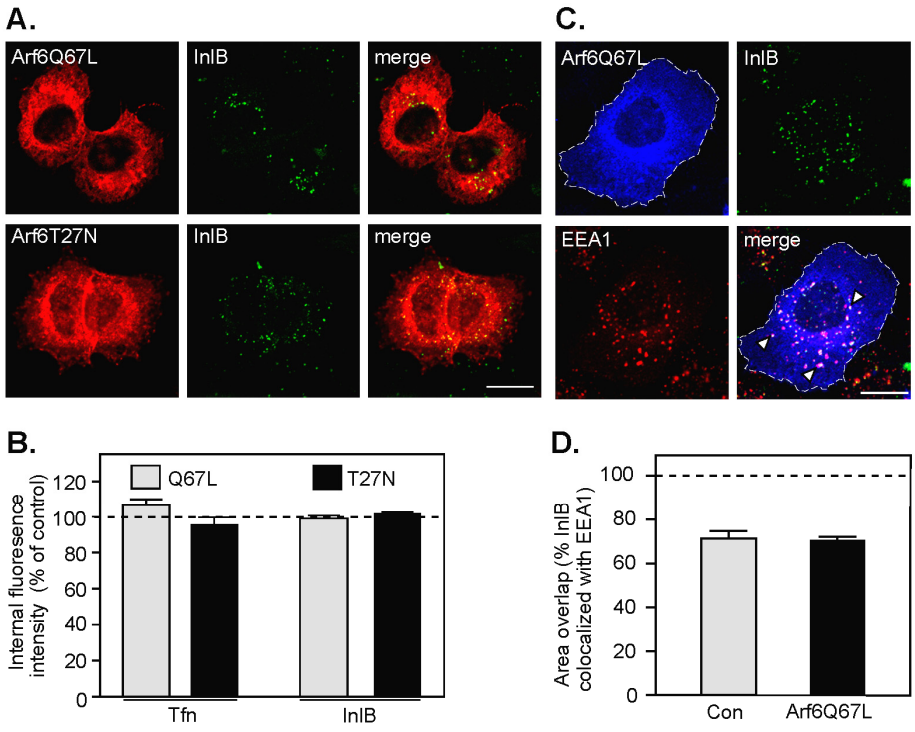

Figure 8. InlB uptake is not regulated by the GTPase Arf6. ***A,*** T47D/cMet cells transiently expressing HA-tagged Arf6T27N or Arf6Q67L were allowed to internalize TR-Tfn (Tfn) or Alexa^{488}-labeled InlB for 15 min before processing for confocal microscopy. Staining with anti-HA antibodies identified cells expressing Arf6 mutants. Representative examples of InlB-internalized cells are shown. Bar, 10 μm. ***B,*** The relative amounts of internalized Tfn and InlB in transfected cells were quantified and expressed as the mean fluorescence intensity of 20 or more randomly selected cells ± S.E.

from 3 separate experiments (ANOVA $p < 0.001$). ***C,*** T47D/cMet cells expressing HA-tagged Arf6Q67L were incubated in DMEM containing Alexa^{488}-labeled InlB (InlB) at 37°C for 15 min and processed for confocal microscopy using the appropriate Alexa-conjugated secondary antibodies to detect endogenous EEA1 (red) and transiently expressed Arf6Q67L (blue). Areas of colocalization between InlB and EEA1 in Arf6Q67L expressing cells appear white in the merged image (arrowheads). ***D,*** The relative percentage (%) of internalized InlB colocalizing with EEA1 was quantified from20 or more randomly selected cells ± S.E. from 3 separate experiments (ANOVA $p < 0.001$).

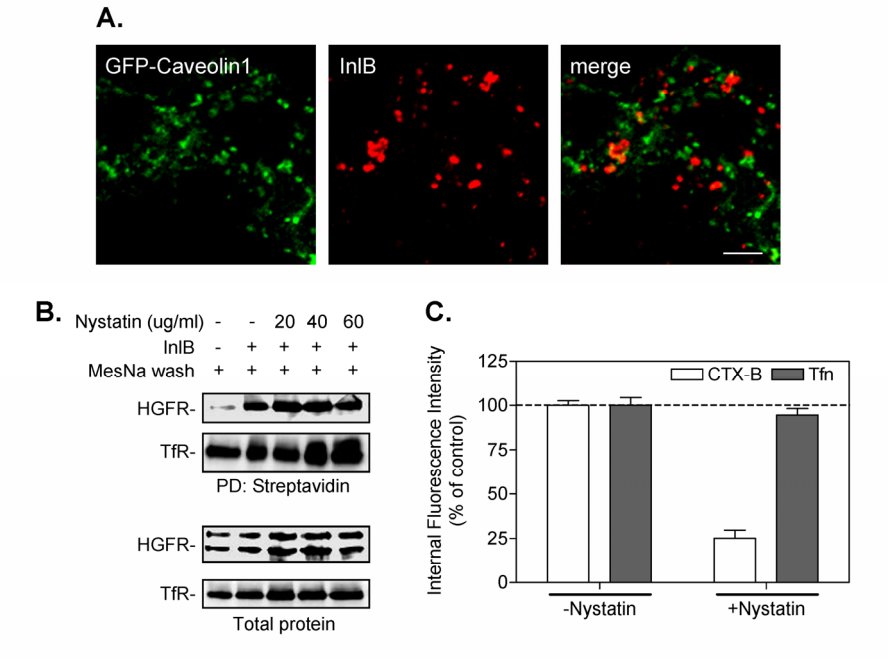

Figure 9. InlB endocytosis does not require the lipid raft/caveolae pathways. A, T47D/cMet cells transiently expressing GFP-labeled caveolin 1 (GFP-caveolin) were incubated with Alexa594-labeled InlB (InlB) at 37°C for 10 min, fixed and processed for confocal microscopy. Representative images from 3 experiments are shown. No colocalization was observed in the merged images (merge). Bar, 10 μm. B, surface biotinylated cells were preincubated in DMEM containing the indicated amount of Nystatin at 37°C for 30 min. Cells were washed and placed in DMEM containing Nystatin in the presence (+) or absence (-) of InlB for 15 min at 37°C. Internalized receptors were detected in lysates prepared from MesNa washed cells by Streptavidin pull downs (PD) and compared to total receptor levels by Western blot analysis using antibodies for HGFR or TfR. C, Control studies in MDCK cells confirmed that 60 μg/ml Nystatin inhibited the internalization of Alexa488-labeled cholera toxin B (CTX-B) via lipid rafts, but not the clathrin-specific marker Tfn (ANOVA, $p<0.001$).

that HGFR internalization does not involve caveolae and lipid rafts, cells were treated with increasing concentrations of Nystatin, a cholesterol-chelating drug that specifically inhibits lipid raft-mediated endocytosis (Ros-Baro, Lopez-Iglesias et al. 2001), but not assembly of clathrin-coated pits when used at low concentrations (20-60 μg/ml) (Rothberg, Heuser et al. 1992; Lisanti, Tang et al. 1993; Subtil, Gaidarov et al. 1999). The cholesterol depleting drug methyl-β-cyclodextrin and the cholesterol sequestering drug filipin (Signoret, Hewlett et al. 2005) have been shown to interfere with the clathrin-mediated uptake of TfR, raising concerns about their specificity for the lipid raft pathway. In my assay system, treatment with 20-60 μg/ml Nystatin did not effect internalization of the TfR (Figure 9B), consistent with previous findings that clathrin-mediated endocytosis is not perturbed under these conditions (Subtil, Gaidarov et al. 1999; Ros-Baro, Lopez-Iglesias et al. 2001). Moreover, internalization of HGFR in T47D/cMet cells was not affected by Nystatin treatment (Figure 9B). In addition, control studies using MDCK cells, which have a prominent lipid raft pathway, confirmed that Nystatin inhibited the internalization of Cholera Toxin B, a specific marker for lipid raft-mediated endocytosis (Harder, Scheiffele et al. 1998). In contrast to other cells that internalize CTX-B through multiple routes, Madin-Darby Canine Kidney (MDCK) cells have a prominent lipid raft pathway (Singh, Puri et al. 2003) and hence were used in this control. As shown in Figure 9C, Nystatin inhibited the lipid raft-mediated internalization of CTX-B and not the clathrin-mediated internalization of Tfn in MDCK cells. Taken together, my findings show that soluble InlB induces HGFR internalization via clathrin-coated pits, and that clathrin-independent routes do not alter this process.

DISCUSSION

I report here that the novel HGFR ligand, InlB, is mechanistically indistinguishable from HGF at inducing clathrin-dependent endocytosis and lysosomal degradation of HGFR. Expression of dominant negative Eps15 prevented InlB-induced HGFR uptake in T47D/cMet cells. The dependence on clathrin for HGFR internalization

was not cell type specific, as InlB internalization in Vero cells was similarly inhibited by dominant negative Eps15. Expression of constitutively active or dominant negative Arf6 mutants that regulate E-cadherin endocytosis and HGF-induced disassembly of Adherens Junctions (AJs) (Palacios, Price et al. 2001; Paterson, Parton et al. 2003) did not alter InlB internalization. Similarly, Nystatin (a compound that disrupts lipid rafts but not clathrin-mediated endocytosis) (Ros-Baro, Lopez-Iglesias et al. 2001) did not prevent the internalization of InlB-HGFR complexes from the cell surface. Thus, my data indicate that the major route for InlB-induced HGFR internalization is clathrin-mediated, a finding consistent with a previous study reporting a role for an endophilin-CIN85-Cbl complex in HGF induced receptor internalization via clathrin-coated pits (Petrelli, Gilestro et al. 2002).

Autophosphorylation of HGFR in response to InlB, has been shown to be more transient than that produced by HGF (Shen, Naujokas et al. 2000; Copp, Marino et al. 2003), raising the possibility that InlB and HGF may exhibit different binding affinities for cell surface associated and/or internalized HGFR, or induce a subtle variation in receptor kinetics. Previous studies reported that InlB- and HGF- induced HGFR phosphorylation occurs at equivalent ligand concentrations, suggesting that the affinity constants of these ligands for HGFR are comparable (Shen, Naujokas et al. 2000). In my studies, both ligands colocalized following their internalization into EEA1- and Rab5-positive early endosomes. At later time points of internalization (i.e. > 60 min) however, Alexa-InlB had largely disappeared and relatively few intracellular vesicles containing InlB colocalized with the late endosomal/lysosomal marker LAMP 1 (data not shown) suggesting that InlB does not dissociate from HGFR until it reaches a lysosomal compartment. Comparable studies on EGFR revealed that EGF also remains bound to its receptor until reaching the lysosome (Burke, Schooler et al. 2001). The transforming Growth Factor α (TGFα) represents a second EGFR agonist. In contrast to EGF, TGFα exhibits a lower affinity than EGF for the receptor following internalization into early endosomes. Whereas EGF-EGFR complexes are efficiently targeted to lysosomes for degradation, dissociation of TGFα from EGFR in early endosomes results in EGFR

dephosphorylation and receptor recycling to the cell surface (Ebner and Derynck 1991; Waterman, Sabanai et al. 1998). Thus my finding that InlB and HGF could remain associated with internalized HGFR, may have important implications for the signaling of this receptor, particularly as HGFR has been shown to undergo minimal recycling back to the cell surface following internalization (Hammond, Carter et al. 2003).

A functional endpoint of HGFR signaling is the activation of the GTPase Arf6 (Palacios, Price et al. 2001) and the internalization of E-cadherin from AJs (Kamei, Matozaki et al. 1999), specialized structures that participate in cell-cell adhesion between neighboring cells. Activation of Arf6 in response to HGFR signaling leads to disassembly of AJs and acquisition of a migratory phenotype (Palacios, Price et al. 2001; Palacios and D'Souza-Schorey 2003). Recently, E-cadherin was shown to be internalized in MCF-7 cells through a clathrin-independent pathway that required Arf6 and Dynamin 1 activity (Paterson, Parton et al. 2003). In MDCK cells, E-cadherin was shown to be co-internalized with HGFR in response to treatment of cells with HGF or the phorbol ester TPA (Kamei, Matozaki et al. 1999). These observations raised the possibility that endocytosis of the HGFR might be regulated by Arf6. However, in my assay system, internalization of HGFR in response to InlB was refractory to regulation by Arf6 and appeared to be solely clathrin-mediated. How can I account for the apparent discrepancies in these findings? One possibility is that E-cadherin internalization occurs via different endocytic routes that are cell type–dependent. Alternatively, the specific pathway used to mediate E-cadherin endocytosis may depend on whether E-cadherin is unbound or engaged in homophilic interactions at AJs. In addition to Arf6-mediated uptake, a distinct clathrin-dependent pathway for E-cadherin internalization has been reported in MDCK cells when cell-cell contact was disrupted by calcium depletion (Le, Yap et al. 1999). Finally, Arf6-positive endocytic transport vesicles have been shown to fuse with clathrin-derived, early endosomes (Naslavsky, Weigert et al. 2003). Thus the fusion of these distinct endocytic organelles may account for the reported colocalization of internalized HGFR and E-cadherin in endocytic vesicles.

In conclusion, my studies demonstrate that soluble, recombinant InlB mimics HGF-induced HGFR internalization via clathrin-coated pits. My data represent the first detailed examination of the internalization properties of HGFR, and reconcile previously reported discrepancies in HGFR signaling in response to InlB and HGF (Shen, Naujokas et al. 2000; Copp, Marino et al. 2003). In this context, it is possible that when anchored to large particles such as opsonized beads or *Listeria*, InlB could divert HGFR from its normal internalization route (i.e clathrin-coated pits) and hence alter downstream signaling events. Consistent with this view, *Listeria* was recently reported to induce the colocalization of host HGFR with the lipid raft marker, ganglioside 1 (Seveau, Bierne et al. 2004), implying a role for lipid rafts in InlB-mediated *Listeria* entry. Similarly, InlB-mediated internalization of *Listeria* does not require host Dynamin 1 (K. Ireton and L. Elferink, unpublished results), confirming that, HGFR internalization in response to soluble and bacterially associated InlB, likely occurs through distinct endocytic mechanisms. Additional studies will be required to determine if this functional dichotomy may be exploited to alter HGFR surface levels, and suppress *Listeria* infections as well as increased HGFR signaling in metastatic cancers.

CHAPTER 3: GRB2-MEDIATED RECRUITMENT OF THE UBIQUITIN LIGASE CBL REGULATES CLATHRIN-DEPENDENT ENDOCYTOSIS OF THE HEPATOCYTE GORWTH FACTOR RECEPTOR

INTRODUCTION

Activation of the receptor tyrosine kinase Hepatocyte Growth Factor Receptor (HGFR) by the binding of its physiological ligand Hepatocyte Growth Factor (HGF) results in increased cell proliferation and cell motility processes critical for embryonic development, tissue regeneration and homoeostasis (Birchmeier, Birchmeier et al. 2003). Persistent HGFR signaling occurs in many human cancers, correlating closely with early stage invasion, dissemination and metastases (Birchmeier, Birchmeier et al. 2003; Gao and Vande Woude 2005). Distinct mechanisms amplify HGFR signaling in human tumors including gene amplification (Ferracini, Longati et al. 1991), point mutations (Olivero, Valente et al. 1999; Park, Dong et al. 1999; Tanyi, Tory et al. 1999) and chromosomal translocations (Dean, Park et al. 1987; Dean, Park et al. 1987; Rodrigues and Park 1993) leading to ligand-independent HGFR activation. Recently, decreased receptor degradation has emerged as a novel mechanism for HGFR induced cell transformation and increased neoplastic growth (Peschard, Fournier et al. 2001; Abella, Peschard et al. 2005). The key pathway for terminating HGFR signaling is ligand-induced endocytosis of HGFR through clathrin-coated pits followed by lysosomal degradation of the receptor (Li, Xiang et al. 2005). Accordingly, receptor endocytosis influences the strength and duration of the signaling outputs. Despite extensive studies on the biological function of HGFR, little is known about its endocytic properties and how receptor endocytosis is regulated.

HGFR is a heterodimer composed of disulphide linked α and β chains. Binding of the ligand to HGFR activates its tyrosine kinase activity leading to autophosphorylation of two key tyrosines in the activation loop (Y1234 and Y1235) and the phosphorylation

of the three additional tyrosine's located at positions 1349 and 1356 in the carboxyl-terminal tail of the β chain and Y1003 in the juxtamembrane region (Furge, Zhang et al. 2000; Birchmeier, Birchmeier et al. 2003). Phosphorylation at Y1003, Y1349 and Y1356 provide docking sites for several adaptor proteins and signaling transducers, including Grb2, Gab1, the ubiquitin ligase Cbl, SHC, Src, PI3K and PLCγ (Furge, Zhang et al. 2000; Birchmeier, Birchmeier et al. 2003). Phosphotyrosines (pYs) 1349 and 1356 comprise a multisubstrate-docking site to which Grb2 and Gab1 bind directly, whereas Cbl binds directly to pY1003. In addition to binding HGFR directly, Gab1 and Cbl associate indirectly with HGFR by binding through Grb2 (Lock, Royal et al. 2000; Peschard, Fournier et al. 2001). Mutations in HGFR that inhibit receptor activation at Y1003, Y1349 and Y1356 prevents the recruitment of these signaling molecules and alters the tumorigenic and motility-promoting properties of HGFR signaling in vitro and in vivo (Bardelli, Longati et al. 1999; Peschard, Fournier et al. 2001).

Once activated by ligand binding, HGFR is internalized via clathrin-coated pits and inactivated by lysosomal degradation (Li, Xiang et al. 2005). However it remains unclear how HGFR activation and endocytosis are mechanistically linked. In the simplest case, signaling molecules bound to HGFR could play a direct role in recruiting receptors into clathrin-coated pits for internalization, or in coupling HGFR directly to the endocytic machinery. Alternatively, increased receptor internalization may be the result of modifications of the endocytic apparatus by the activated receptor or the associated signaling complexes. For example, Grb2 is an adaptor protein important in SOS-Ras-MAPK signaling downstream of activated HGFR (Ponzetto, Bardelli et al. 1994; Fixman, Fournier et al. 1996). Grb2 is composed of a central SH2 domain flanked by amino- and carboxyl-terminal SH3 domains (Lowenstein, Daly et al. 1992). Grb2 binds HGFR directly at pY1356 via its SH2 domain (Ponzetto, Bardelli et al. 1994). Alternatively, Grb2 can bind HGFR indirectly through interactions with tyrosine phosphorylated SHC, which binds HGFR directly at pY1349 and pY1356 (Pelicci, Giordano et al. 1995). In addition to its function as a signaling adaptor, the SH3 domains of Grb2 bind several proteins involved in endocytic trafficking, including the large GTPase Dynamin (Vidal,

Goudreau et al. 1999; Solomaha, Szeto et al. 2005), the actin-polymerizing complex N-WASp-Arp2/3 (Carlier, Nioche et al. 2000; Kempiak, Yamaguchi et al. 2005), and the E3 ubiquitin ligase Cbl (Meisner, Conway et al. 1995; Meisner and Czech 1995). Consistent with these binding properties, a role for Grb2 in the clathrin-mediated endocytosis of the EGF receptor has been established (Yamazaki, Zaal et al. 2002; Jiang, Huang et al. 2003; Stang, Blystad et al. 2004; Huang and Sorkin 2005). However a role for Grb2 in HGFR endocytosis has yet to be established.

The precise role of Cbl ubiquitin activity for HGFR endocytosis remains unresolved. Cbl-mediated ubiquitination of HGFR appears to be critical for HGFR degradation and termination of receptor signaling (Peschard, Fournier et al. 2001; Abella, Peschard et al. 2005). Tpr-Met is an oncogenic form of HGFR generated by chromosomal translocation that deletes the Y1003 binding site for the direct binding of Cbl (Park, Dean et al. 1986; Peschard, Fournier et al. 2001). As Tpr-Met fails to bind Cbl, Tpr-Met is not ubiquitinated or degraded and remains constitutively active (Peschard, Fournier et al. 2001). Cbl has been proposed to regulate HGFR internalization, possibly by linking Cbl associated HGFR to CIN85/Endophilin, a protein complex that drives membrane invagination leading to clathrin-mediated endocytosis (Petrelli, Gilestro et al. 2002). However recent mutagenesis studies on HGFR revealed that although the direct binding of Cbl to pY1003 is important for HGFR degradation, this binding mode was not required for HGFR internalization via clathrin coated pits (Abella, Peschard et al. 2005). Thus the indirect binding of Cbl through Grb2, may account for Cbl function in clathrin-mediated endocytosis of HGFR.

In this study I examined the role of Grb2 and Cbl interactions in clathrin-mediated internalization of HGFR. My data indicates a requirement for the binding of Grb2 to HGFR for receptor internalization. The dependence on Grb2 was specific and does not represent a general impairment in HGFR activation and/or trafficking. Finally, my findings support a key role for Grb2-mediated recruitment of Cbl to HGFR, for clathrin-dependent receptor internalization. These data suggest that attenuation of HGFR

signaling involves distinct Cbl-mediated ubiquitination events that regulate receptor internalization versus receptor degradation.

MATERIALS AND METHODS

Reagents, antibodies and plasmids.

All general reagents were obtained from Fisher Scientific or Sigma-Aldrich unless indicated otherwise. Recombinant human HGF was purchased from PeproTech Inc, Rocky Hill, NJ, USA. EZ-link sulfo-NHS-SS-biotin, D-salt dextran and spin columns were purchased from Pierce Biotechnology Inc (Rockford, IL, USA). Texas Red labeled-Tfn and Antibody labeling kits were obtained from Invitrogen Corp, IL. The following antibodies were purchased as indicated; antihuman HGF (R & D Systems Inc, Minneapolis, MN, USA), TfR (Invitrogen Corp, CA), anti-β-actin and anti-phosphotyrosine clone PY-20 (Sigma-Aldrich, MO), Ub (P4D1), Cbl (c-15), Grb2 (c-23), Met c-12 and Met C-28 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-Gab1 CT, phospho-Met Y1234, Y1235 (UpState Biotechnology, NY), Gab1, phosphor-Gab1, JNK, phospho-JNK, p42/p44 MAPK, phospho p42/p44, MEK and phosphor-MEK (Cell Signaling Technology, MA), the monoclonal GFP antibody (JL-8) (BD Biosciences Clontech, CA), peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA), Alexa488 or Alexa594-labeled goat anti-mouse, anti-rabbit or anti-rat secondary antibodies (Invitrogen Corp, CA). Plasmids encoding wt HGFR and the Y1349.1356F (YF) alleles were previously reported. The HGFR-K1110A allele encodes a receptor lacking kinase activity (Fournier et al., 1996; Zhen et al., 1994). The K1110A mutation was generated through a PCR-based SOEing approach (Horton et al., 1990. Biotechniques 8: 528-535) using wild type HGFR cDNA as a template, Pfu DNA polymerase, and the primers,5’-CAAGCTAGCCACAGCACAGTG-3’,5’CCCATCCTAACTAGTGGGGAC-3’, 5’-CACTGTGCTGTGGCTAGCTTG-3’, and 5’-GCTCTAGAACTAGTGGATCCC-3’. The final ~1.3 kb PCR product was subcloned between the SpeI and XcmI sites of pLXSN-HGFRwt, resulting in a full-

length HGFR gene with the K1110A mutation and verified by DNA sequencing. The various HGFR alleles were subcloned from the plasmid pLXSN into the Murine Stem Cell Virus (MSCV) vector pMSCVpuro (BD Biosciences, CA) for retroviral-mediated transfection in the target cell lines. Grb2/pYFP-N1 and NCSH3mGrb2/pYFP-N1 were kindly provided by Lawrence Samelson (National Institutes of Health) (Yamazaki et al., 2002). Plasmids encoding SH2mGrb2/pYFP, Cbl/SH2 and Cbl/SH2R86A were kindly provided by Alexander Sorkin (University of Colorado Health Sciences Center) (Huang & Sorkin, 2005; Jiang et al., 2003). PcDNA3-Myc-UbR and pcDNA3-Myc-UB8A/I44A were kind gifts from Inger Madshus (Norwegian Radium Hospital, Norway) (Stang et al., 2004). Plasmids encoding HA-tagged wt-Cbl, 70Z-Cbl and Cbl-G306E were kindly provided by Jannie Borst (The Netherlands Cancer Institute, The Netherlands) (de Melker et al., 2004).

Cell lines.

The H10 cell line derived from kidney epithelial cells from embryos of HGFR null transgenic mice (Kjelsberg et al., 1997) was a generous gift of Dr. Lloyd Cantley (Yale University School of Medicine). Mouse embryonic fibroblast (MEF) cell lines derived from Gab1-/- mice and their Gab1+/+ littermates have been previously described (Holgado-Madruga & Wong, 2003). Clonal H10 cell lines stably expressing various HGFR alleles and Gab1+/+ and Gab1-/- MEF cell lines stably expressing wild-type HGFR were generated by retroviral transfection essentially as described (Basar et al., 2004), except that 1-5 micrograms/ml of puromycin was used for selection. Polyclonal H10 cell lines expressing HGFR alleles were made in a similar manner, except that limiting dilution was not performed. Cell lines were analyzed for HGFR expression by Western blotting with the anti-HGFR antibody DL-21 (Upstate Biotechnology, NY), and those cell lines that exhibited similar HGFR levels were chosen for further analysis. The null -/- cell line (cloneH10) was maintained in DMEM/F-12(1:1) with 10 % FBS. H10 polyclonal cell lines stably expressing wild type HGFR, or the mutant HGFR alleles Y1349, 1356F, K1110A or N1358H, were generated via retroviral infection of HGFR null -/- cells and maintained in 5µg/ml puromycin DMEM/F12(1:1) with 10 % FBS.

Mouse embryonic fibroblast cell lines 2BM19 (Gab1-/- with wild type HGFR) and 6BM5 (Gab1+/+ with wild type HGFR) were maintained in DMEM with 10 % FBS containing 5μg/ml puromycin. Human mammary epithelial cells (T47D) stably expressing full-length human HGFR/cMet (T47D/cMet, M. Park, McGill University) were maintained in Dulbecco's modified eagle's medium (DMEM) containing 10 % FBS, supplemented with 400 μg/mL G418.

siRNAs and Cell Transfections.

For small interfering RNAs (siRNA) depletion studies, Cells were grown on coverslips and transfected with empirically determined amounts of Gab1, Grb2 and control siRNAs (Dharmacon, CO) using Lipofectamine 2000 reagent (Invitrogen Corp, CA) according to the manufacturers specifications. For studies using T47D/cMet cells, cells were routinely cultured on coverslips coated with 100 μg/ml poly-lysine prior to experimentation. All experiments were routinely performed 72 hours after siRNA transfection. Cell transfections using plasmids were performed as previously described (Li et al., 2005).

Immunoprecipitation and GST pull down Assays.

For immunoprecipitation (IP) studies, cells were washed twice with ice-cold PBS and lysed on ice in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1 % NP-40, 0.5 % Na-deoxycholate, 0.1 % SDS, 150 mM NaCl, 1 mM EDTA) with protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin) and phosphatase inhibitors (10 mM NaF, 2 mM Na_3VO_4). Cell lysates were cleared by centrifugation at 12,000 xg at 4^oC. 5 μg primary antibody were added to 500-1000 μg cell lysate for IP at 4^o C rotating overnight. Antibody-protein complex were precipitated with 50μl protein A/G agarose solution (Pierce Biotechnology Inc, IL) by rotation at 4^o C for 4 hours. The protein-beads complex were collected by centrifugation at 1000xg for 5min, washed with lysis buffer 3 times and resuspended loading buffer and fractionated by SDS/PAGE. For GST pull downs, the cell lysates were prepared in RIPA buffer as described above. Lysates were incubated with Grb2-GST fusion proteins prebound to glutathione-Sepharose beads

(Sigma) overnight at 4 ^{o}C. Beads were washed three times in RIPA buffer followed by a final wash in 10 mM Tris-Cl pH 7.4, then analyzed by SDS/PAGE.

Cell surface Biotinylation and Western Analysis.

The biotinylation and receptor internalization assay were described previously (Li N et al, 2005). Briefly, cells were surface biotinylated at 4 °C for 30 min with EZ-link NHS-SS-biotin Pierce Biotechnology Inc, IL. Surface expressed proteins were isolated directly using streptavidin-agarose beads as described by the manufacturer (Pierce Biotechnology Inc) and identified by Western analysis. Western analysis was performed using ECL (GE Healthcare Life Sciences, Sweden) and the resulting digitized blots were quantified using AlphaEase v3.1.2 Software (Alpha Innotech Corp, CA).

InlB labeling, Confocal microscopy and Analysis.

The purification and labeling of recombinant His_6-tagged InlB has been described in detail elsewhere (Li et al., 2005). For Confocal microscopy, cells grown on coverslips were incubated in media containing 5.0 μg/ml of Alexa594 labeled Tfn with 100 ng/ml InlB or HGF for 10 min at 37°C, then fixed immediately after each experiment using 4 % paraformaldehyde (Ted Pella Inc, CA) in PBS (137 mM NaCl, 2.7 mM KCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4). Residual paraformaldehyde was quenched using 50 mM NH_4Cl/PBS and cells were then permeabilized with 0.05 % (w/v) saponin (or 0.1 % Triton X-100/PBS for HGF staining) for 20 min and then blocked with 10 % goat serum in PBS. All antibody dilutions were performed in 5 % goat serum/PBS with 0.05 % saponin for 1 hr. In studies staining for HGF, saponin was omitted from the buffers. Coverslips were mounted and observed using a Zeiss LSM 510 confocal microscope with a 63x oil (1.4NA) immersion objective. Samples were visualized with the 488 nm and 543 nm laser lines and emission filter sets at 505-530 nm for YFP and Alexa488 detection or 585615 nm for Texas Red and Alexa594 detection, respectively. Figure presentation was accomplished in Adobe Photoshop V 6.0. Quantification and analysis were done using MetaMorph v5.0 (Universal Imaging Corp, Westchester, PA, USA). Quantification of colocalization and internal fluorescence intensity were done using

Metamorph v5.0 (Molecular Devices, CA). All pixel intensity levels were normalized relative to control values, expressed as a percentage ± Standard Error (S.E.) and differences statistically verified by ANOVA using GraphPad Prism Software (GraphPad Software Inc, CA).

RESULTS

The tyrosine kinase activity and the docking sites Y1349 and Y1356 of HGFR are required for ligand induced HGFR internalization.

Phosphorylation of the docking residues Y1349 and Y1356 in the C-terminal tail of HGFR mediates all of the signaling cascades downstream of activated HGFR (Ponzetto, Bardelli et al. 1994). Mutation of Y1356 (which binds Grb2) decreases HGFR-mediated motility and morphogenesis. The same mutation in oncogenic Tpr-Met impairs its transforming potential (Bardelli, Longati et al. 1997). Conditional knock-in studies in mice using a HGFR mutant (Y1349, 1356F) that is unable to bind Grb2 and Gab1, was embryonic lethal due to placental, liver, limb and muscle defects, reminiscent of HGFR null (-/-) mice (Maina, Casagranda et al. 1996). To determine if HGFR signaling is linked to receptor endocytosis, wild type (wt) or mutant HGFR was expressed in null cells immortalized from the kidneys of HGFR null (-/-) mice. These epithelial HGFR (-/-) cells undergo chemotaxis and branching morphogenesis in response to EGF and TGFβ but not HGF, indicating that HGFR signaling is specifically abrogated in these cells (Kjelsberg, Sakurai et al. 1997). We generated several polyclonal cell lines stably expressing moderate levels of wt or mutant HGFR, including tyrosine kinase deficient (KD-HGFR) K1110A HGFR and the mutant Y1349, 1356F (YF-HGFR), which is deficient in Gab1 and Grb2 binding.

I previously reported that the Internalin B (InlB) protein of *Listeria monocytogenes* mimics HGF-induced HGFR internalization and degradation (Li, Xiang et al. 2005). When anchored to the bacterial surface, InlB induces pathogen entry by triggering the activation and internalization of HGFR (Shen, Naujokas et al. 2000; Veiga and Cossart 2005). HGF and soluble InlB appear indistinguishable in their ability to

activate HGFR signaling and trafficking. Both ligands stimulate the recruitment of Gab1, Cbl, and PI3K (Shen, Naujokas et al. 2000) and induce equivalent endocytic modes for HGFR internalization and degradation (i.e. via clathrin coated pits) (Li, Xiang et al. 2005). Therefore I used InlB and HGF to examine the molecular control of HGFR endocytosis.

Western analysis of cell lysates prepared from the polyclonal cell lines expressing wt or mutant HGFR confirmed that treatment with InlB for 15 min at 37°C caused phosphorylation of wt and the YF-HGFR mutant on residues Y1234 and Y1235 in the activation loop, but not on kinase-dead HGFR (KD-HGFR) (Figure 10A). Ubiquitination of wt-HGFR but not KD-HGFR was detected in response to InlB. A low level of ubiqitinated YF-HGFR was detected under these conditions, consistent with the direct association of Cbl to Y1003 of HGFR , independently of Grb2 binding. Additionally, the reduced level of YF-HGFR ubiquitination suggests that optimal receptor ubiquitination requires Grb2 dependent Cbl recruitment to the receptor. Consistent with this tenet, Cbl phosphorylation was not detected in unstimulated cells or ligand stimulated and KD-HGFR expressing cells, and was impaired in ligand stimulated YF-HGFR expressing cells. Similarly, ligand induced ERK activation was detected in cells expressing wt-HGFR, but was defective in cells expressing the HGFR mutants. Surface biotinylation assays were used to examine the cell surface levels of wt and mutant HGFR in each of the polyclonal cell lines. Cells were surface biotinylated at 4°C for 30 min using NHS-SS-Biotin. Cell lysates were then prepared, biotinylated proteins isolated by Streptavidin pull down and analyzed by Westerns. Comparable levels of wt and mutant HGFR were at the cell surface of the polyclonal cell lines (Figure 10B). No HGFR was detected in the parental null cells, consistent with previous reports (Kjelsberg, Sakurai et al. 1997).

Using a GST pull down assay, I verified that Grb2 bound to wt-HGFR in response to InlB, but not to YF-HGFR or KD-HGFR (Figure 10C). No Grb2 binding was detected in the absence of ligand, confirming that Grb2 binding to wt-HGFR was specific and dependent on HGFR activation. Similarly, Gab1 activation in response to InlB was detected in null cells expressing wt-HGFR, but not in cells expressing the YF or KD

HGFR mutants (Figure 10D) consistent with previous reports that the tyrosine kinase activity of HGFR is critical for efficient Gab1 tyrosine phosphorylation. Together, these data confirm that the recruitment of Gab1 and Grb2 to HGFR is disrupted in the YF and KD-HGFR mutants, but not in cells expressing wt-HGFR.

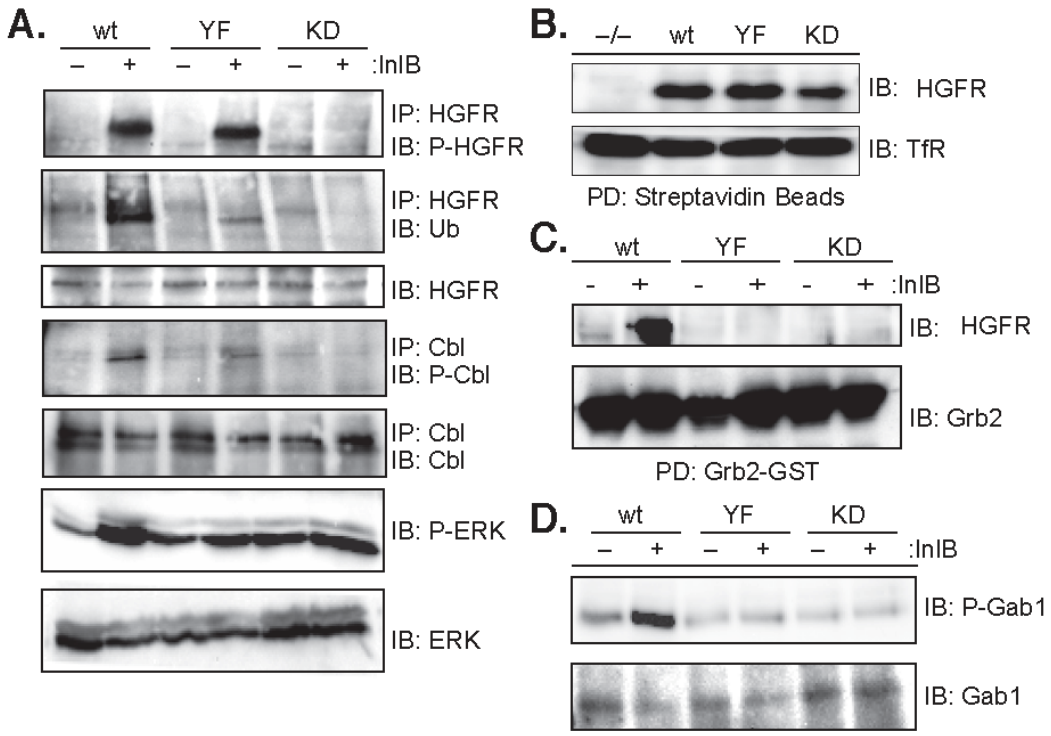

Figure 10. HGFR internalization requires pY1349 and pY1356. A. HGFR signaling in stable HGFR null (-/-) cells expressing wt, kinase dead (KD) or a mutant deficient in Grb2 and Gab1 binding was examined after incubation with (+) or without (-) InlB for 15 min at 37°C by immunoprecipitation (IP) and western analysis (IB) for expression of total protein (HGFR), HGFR and ERK phosphorylation (P-HGFR, P-ERK) and HGFR ubiquitination (Ub). B. Biotinylation assays verified that wt and mutant HGFR were expressed at comparable levels on the cell surface. C. Gbr2 binding was detected in null (/-) cells stably expressing wt but not mutant HGFR in response to treatment with 100 ng/ml InlB. D. Gab1 was phosphorylated in response to treatment with (+) 100 ng/ml InlB in cells expressing wt, but not mutant HGFR.

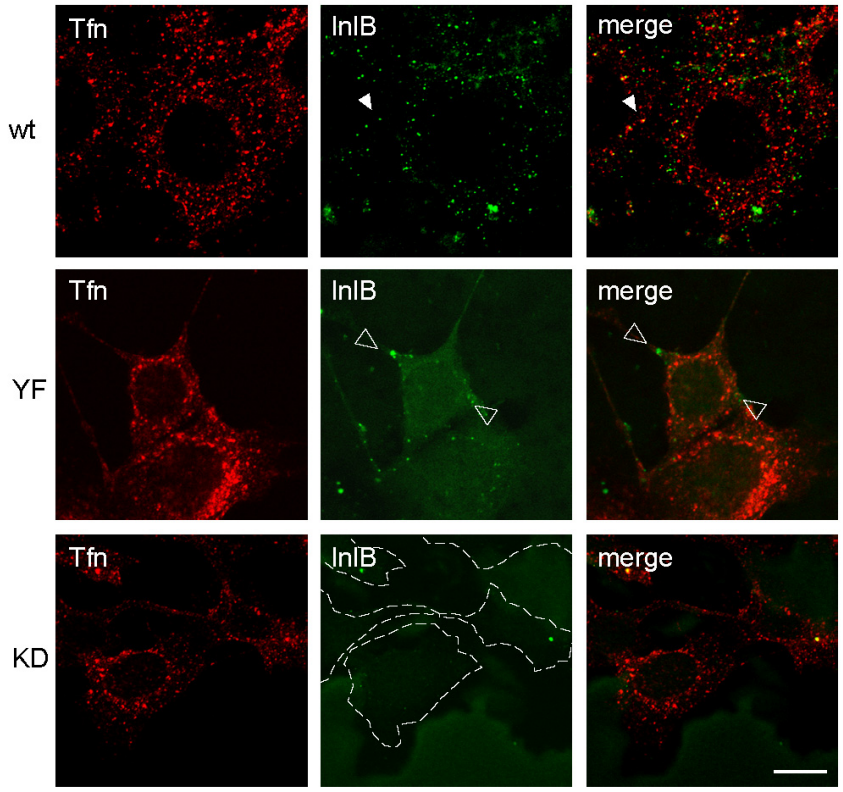

Figure 11. Mutation of the docking site tyrosines pY1349 and pY1356 inhibits HGFR internalization. HGFR null (-/-) cells expressing wt HGFR or the mutants HGFR-K1100A (KD) or HGFR-Y1349, 1356F (YF) were coincubated with Texas Red labeled Transferrin (Tfn) and Alexa488-labeled InlB for 10 min at 37°C and processed for confocal microscopy. Representative examples of internalized InlB (closed arrows) versus surface InlB (open arrows) are shown. Bar 10 μm.

Using Alexa-labeled InlB (Alexa-InlB) as a ligand I examined InlB-induced HGFR internalization of wt and mutant HGFR by confocal microscopy (Figure 11). Alexa-InlB was internalized under steady state conditions at 37°C for 15 min to trigger HGFR internalization. Under these conditions, Alexa-InlB was detected with cointernalized Texas-Red labeled Transferrin (Tfn) in cells expressing wt-HGFR, in intracellular punctate structures reminiscent of early endosomes. SiRNA-mediated depletion of clathrin heavy chain inhibited wt-HGFR internalization confirming that HGFR internalization is clathrin dependent in these cell lines (data not shown) , consistent with my previous studies using T47D and Vero cell lines (Li, Xiang et al. 2005). Conversely, internalized InlB was not detected in cells expressing kinase-dead (KD) HGFR or the HGFR mutant lacking a functional Gab1/Grb2 binding site (YF) (Figure 11). Rather, large spots of InlB fluorescence were detected on the surface of cells expressing YF-HGFR. Clathrin-mediated Tfn uptake was unaffected in null cells expressing YF-HGFR or KD-HGFR, consistent with a specific block in HGFR internalization. The reduced internalization of YF-HGFR and KD-HGFR is not due to saturation of the endocytic machinery, as comparable levels of wt and mutant HGFR were detected at the cell surface (See Figure 10B). Thus the results indicate that one or more of the proteins known to associate with Y1349 and /orY1356 are involved in HGFR endocytosis.

Depletion of Gabl did not inhibit HGFR internalization

Since pY1349 and pY1356 of HGFR provide docking sites for the adaptors Gab1 and Grb2, I examined the requirement for each protein in receptor internalization. Gab1 is the major adaptor protein for HGFR, mediating almost all of the signaling cascades downstream from activated HGFR including PI3K, JNK and ERK signaling (Furge, Zhang et al. 2000). Gab1 is recruited to HGFR by two mechanisms. The primary mechanism involves the indirect recruitment of Gab1 to HGFR via Grb2. The proline rich region of Gab1 binds strongly to the C-terminal SH3 domain of Grb2 (Bardelli, Longati et al. 1997; Lock, Royal et al. 2000). In addition, Gab1 binds directly to HGFR via a novel HGFR Binding Domain (MBD) to pY1349 of HGFR (Lock, Frigault et al. 2003).

To examine the importance of Gab1 in HGFR internalization, I utilized mouse embryonic fibroblasts (MEF) isolated from Gabl null (-/-) mice and a MEF cell line expressing endogenous Gab1 (Gab1 +/+) derived from wt litter mates (Holgado-Madruga and Wong 2003). Since I did not detect endogenous HGFR in these cells, I used a retroviral-transfection approach to stably express low to moderate levels of human wt HGFR in Gab1 null (-/-) and Gab1 (+/+) MEFs (Figure 12A). Western analysis detected comparable levels of Grb2 and HGFR, and HGFR phosphorylation in response to InlB treatment (5 min at 37°C) in Gab1 null (-/-) and Gab1 (+/+) monoclonal cell lines stably expressing wt HGFR. Gab1 expression and phosphorylation were not detected in Gab1 null (-/-) cells expressing wt HGFR in response to InlB, consistent with their Gab1 null phenotype. Conversely, HGFR and Gab1 phosphorylation in response to InlB was readily detected in HGFR expressing Gab1 (+/+) cells. Transient ERK, JNK and MEK phosphorylation in response to InB were comparable between the HGFR expressing Gab1 null (-/-) and Gab1 (+/+) cell lines. In contrast to HGFR-mediated transient ERK activation, the activation of Gab1 downstream of HGFR has been shown to be important for sustained ERK phosphorylation (i.e. > 30 min) (Itoh, Yoshida et al. 2000; Maroun, Naujokas et al. 2000). To determine if the Gab1 null cells were deficient in sustained ERK activation, Gab1 null (-/-) and Gab1 (+/+) cells stably expressing wt HGFR were treated with InlB for 0.5–3.0 hr. Cell lysates were prepared and examined for ERK expression and activation by Western analysis. As shown in Figure 12B, ERK phosphorylation peaked following HGFR activation at 30 min in both cell lines. However, sustained ERK phosphorylation was only detected in cells expressing Gab1, but not in the Gab1 null (-/-) cells. In contrast to the Gab1 (+/+) cell line, ERK phosphorylation returned to basal levels in Gab1 null (-/-) cells following 3 hr treatment with InlB.

I next examined Cbl phosphorylation and HGFR ubiqitination in the Gab1 null (-/-) and Gab1 (+/+) cells stably expressing wt HGFR by western analysis. Cbl is an E3 ubiquitin ligase that binds to HGFR indirectly via Grb2, and directly associates with HGFR at pY1003. Mutation of HGFR at Y1003 results in loss of Cbl-mediated receptor

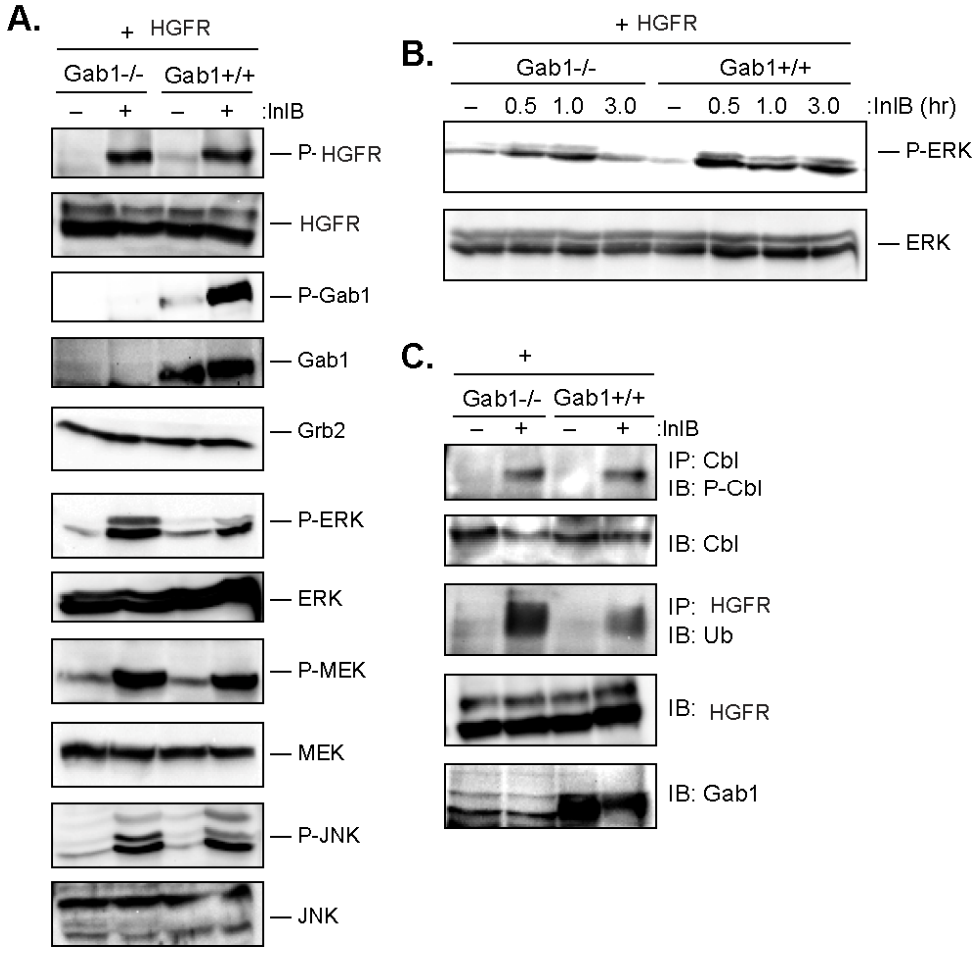

Figure 12. Loss of Gab1 affects sustained ERK activation and HGFR ubiquitination, but not Cbl activation or HGFR phosphorylation. A. Gab1 null (-/-) MEFs and a MEF cell line expressing endogenous Gab1 (+/+) stably expressing wt HGFR (+ HGFR) were treated with (+) or without (-) InlB for 10 min at 37°C. Equal amounts of cell lysates were examined by Western analysis for total protein levels (HGFR, Gab1, ERK, MEK and JNK) and protein phosphorylation (P-HGFR, P-Gab1, P-ERK, P-ERK, P-JNK) as indicated. B. HGFR expressing Gab1 null (-/-) and Gab1 (+/+) MEFs were treated without (-) or with 100 ng/ml InlB for 0.5-30 hr, then examined for total ERK and ERK phosphorylation (PERK) using Western analysis. C. HGFR expressing Gab1 null (-/-) and Gab1 (+/+) MEFs treated with (+) or without (-) InlB were examined by immunoprecipitaion (IP) and Western analysis (IB) for total protein (Cbl, HGFR and Gab1), Cbl phosphorylation (P-Cbl) and HGFR ubiquitination (Ub).

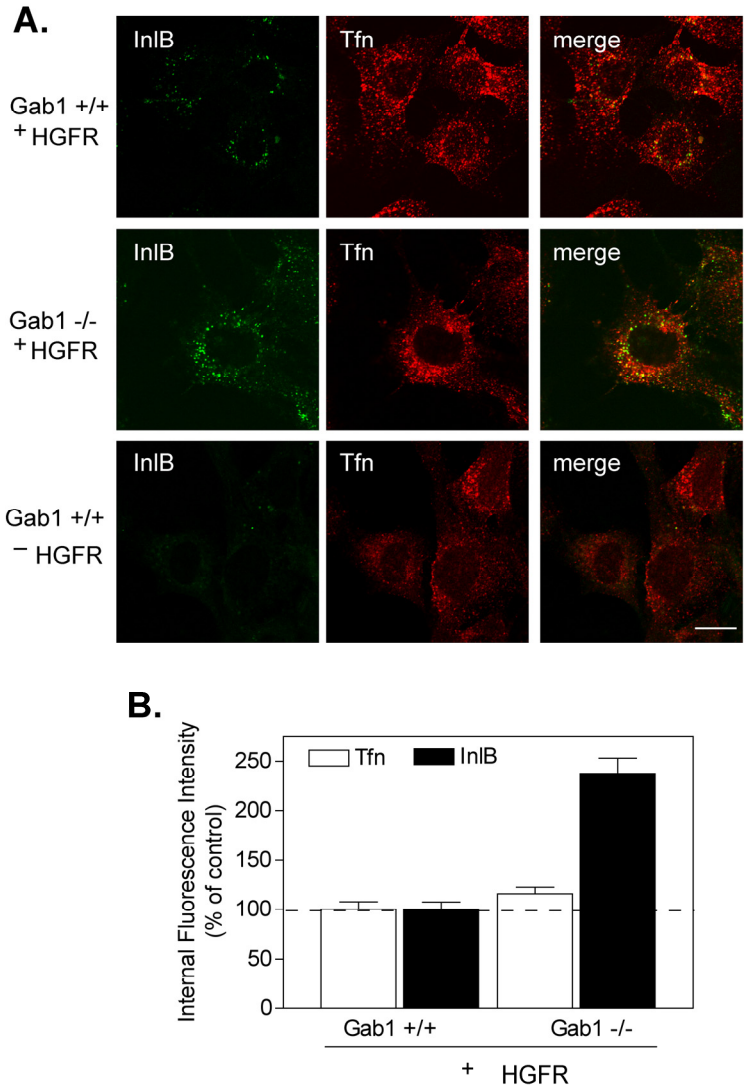

Figure 13. Ligand-induced HGFR internalization proceeds in the absence of Gab1. A. HGFR expressing Gab1 null (-/-) and Gab1 (+/+) MEFs were allowed to cointernalize Texas Red labeled Tfn (Tfn) with Alexa488-labeled InlB (Alexa-InlB) for 10 min at 37°C before processing for confocal microscopy (*see materials and methods*). No internalized Alexa-InlB was detected in parental Gab1 (+/+) cells, in which endogenous HGFR is undetectable. Representative examples of InlB internalized cells from 3-4 separate experiments are shown. Bar 10 um. B. The relative amounts of internalized Tfn and InlB HGFR expressing Gab1 null (-/-) and Gab1 (+/+) MEFs were quantified. Values represent the mean fluorescence intensity ± S.E. from 4 separate experiments and are expressed as a percentage of control values.

ubiquitination (Peschard, Fournier et al. 2001). Although the Gab1 null (-/-) and Gab1 (+/+) cell lines express comparable levels of Cbl, increased HGFR ubiquitination in response to InlB treatment was routinely detected in the Gab1 null (-/-) cells relative to the MEFs expressing endogenous Gab1. The higher ubiqitination of HGFR observed in the Gab1 null (-/-) cells could be the result of a stronger interaction between Cbl and HGFR in these cells.

I next examined the effect of loss of Gab1 on HGFR internalization using confocal microscopy. Gab1 null (-/-) cells and Gab1 (+/+) cells expressing wt HGFR were coincubated in media containing Tfn and Alexa-InlB for 10 min at 37°C to stimulate HGFR activation and endocytosis. The cells were fixed and then processed for confocal microscopy. Internalization of Alexa-InlB was not inhibited in Gab1 null (-/-) cells; rather increased levels of internalized Alexa-InlB were routinely detected in Gab1 null (/-) cells expressing wt HGFR, relative to MEFs expressing endogenous Gab1 (Figure 13A and 13B). No internalized Alexa-InlB was detected in parental Gab1 (+/+) MEF cells in which loss of Gab1. Additional controls using siRNA to knockdown expression of clathrin heavy chain, confirmed that HGFR internalization in the Gab1 null (-/-) and Gab1 (+/+) MEFs expressing wt HGFR is clathrin dependent, consistent with my previous studies using other cell lines.

To confirm that Gab1 is not critical for HGFR internalization, a Gab1 specific siRNA was used to deplete T47D/cMet cells of endogenous Gab1. T47D/cMet cells are a human mammary epithelial cell line that stably expresses human HGFR on their surface that has been used extensively on studies examining HGFR signaling and endocytosis (Shen, Naujokas et al. 2000; Li, Xiang et al. 2005). Western analysis confirmed the specificity of the siRNA-mediated Gab1 knockdown in cells transfected with Gab1 but not a scrambled control (Con) siRNA (Figure 14A). Comparable levels of Cbl, actin, Grb2, transferrin receptor (TfR) and HGFR were detected in Gab1-depleted cells, as well as mock-transfected cells and cells transfected with a control siRNA. Western analysis confirmed that siRNA-depletion of Gab1 did not alter ligand induced HGFR phosphorylation and ubiquitination, or Cbl phosphorylation (Figure 14B). To examine the

effect of Gab1 depletion on HGFR internalization, siRNA-treated cells were coincubated with Texas Red-labeled Transferrin (Tfn) and HGF or InlB (10 min, 37°C) to activate HGFR signaling, and the relative amounts of internalized ligand were measured by confocal microscopy (Figure 14C). Internalized HGF was detected by costaining the cells with an anti-HGF antibody. Comparable levels of cointernalized Tfn were detected in control and Gab1-depleted cells. siRNA-mediated knockdown of Gab1 did not inhibit HGFR internalization in response to InlB or HGF. Rather, Gab1 depletion resulted in

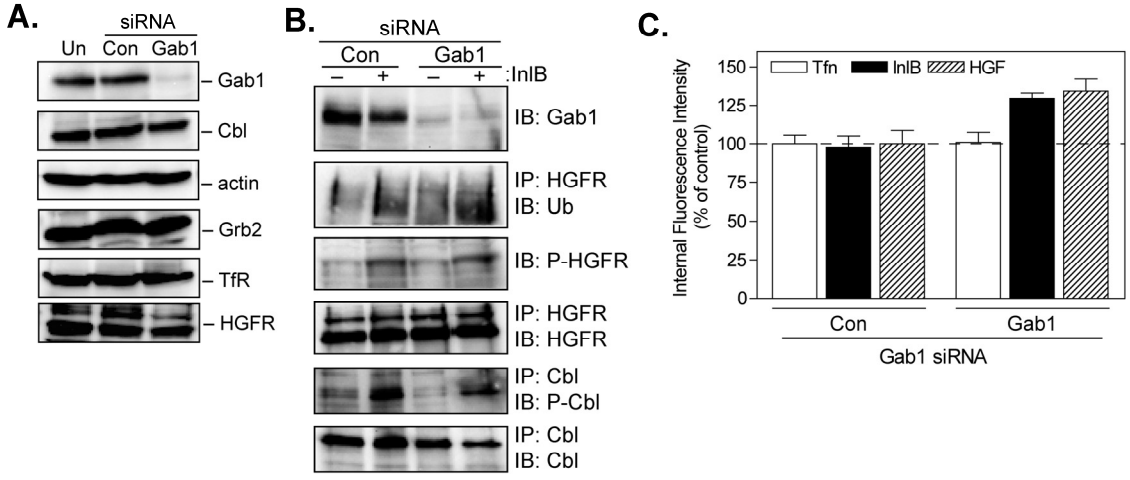

Figure 14. siRNA-mediated Gab1 depletion does not inhibit InlB and HGF induced HGFR internalization. A. Western analysis of lysates from mock-transfected (UN) and T47D/cMet cells transfected with control (Con) or Gab1 siRNAs confirmed specific knock down of Gab1. **B.** T47D/cMet cells were transfected with the indicated siRNAs, following treatment with (+) or without (-) 100 ng/ml InlB for 10 min at 37°C. Equal amounts of lysates were examined by immunoprecipitation (IP) and Western analysis (IB) for total protein (HGFR, Gab1 and Cbl), HGFR and Cbl phosphorylation (P-HGFR, P-Cbl) and HGFR ubiquitination (Ub) as indicated. **C.** The relative amounts of internalized Tfn, InlB and HGF (10 min at 37°C) in cells transfected with control siRNAs (Con) and Gab1-depleted T47D/cMet cells were quantified by confocal microscopy. Internalized HGF was detected by costaining cells with anti-HGF antibodies followed by the appropriate secondary antibody. Values represent the mean fluorescence intensity ± S.E. from 4 separate experiments and are expressed as a percentage of control values.

increased levels of internalized HGFR response to InlB and HGF, consistent with my studies using Gab1 null MEF cells. Thus loss of Gab1 does not lead to a block in clathrin-mediated HGFR internalization.

Grb2 is required for HGFR internalization

My data indicates that the dependence on pY1349 and pY1356 for HGFR internalization does not involve the recruitment of Gab1 leading us to examine the requirement for Grb2 in this process. To test the role of endogenous Grb2, I used siRNAs to deplete T47D/cMet cells of Grb2. Under these conditions, endogenous Grb2 was undetectable by Western analysis (Figure 15A). Conversely, comparable levels of endogenous TfR, actin and HGFR were observed in Grb2-depleted T47D/cMet cells as well as untransfected cells, and T47D/cMet cells transfected with a control siRNA, demonstrating the specificity of the siRNA treatment. siRNA-treated cells were coincubated with Tfn and HGF or Alexa-InlB for 15 min at 37°C and ligand internalization measured by confocal microscopy. HGFR internalization in response to HGF or InlB was reduced 51.3 ± 4.85 % and 74.4 ± 2.87 % respectively in Grb2-depleted cells versus cells transfected with a control siRNA (Figure 15B). Comparable levels of cointernalized Tfn were detected in control and Grb2-depleted cells, indicating that loss of Grb2 led to a specific block in HGFR internalization and not a general defect in clathrin-mediated endocytosis (Figure 15C). siRNA-mediated Grb2 depletion caused a comparable block in HGFR endocytosis in HGFR null (-/-) cells stably expressing wt receptor, indicating that the requirement for endogenous Grb2 for HGFR uptake is not cell type specific (Figure 16). Thus loss of Grb2 led to a specific block in HGF- and InlB-induced HGFR internalization, and not a general inhibition in clathrin-mediated endocytosis.

To confirm a specific role for Grb2 binding to HGFR for receptor internalization, we generated a polyclonal cell line using HGFR-/- null cells expressing moderate levels of the HGFR mutant N1358H (NH-HGFR). N1358H is a well-characterized mutation that specifically interferes with direct Grb2 binding to HGFR. Surface biotinylation

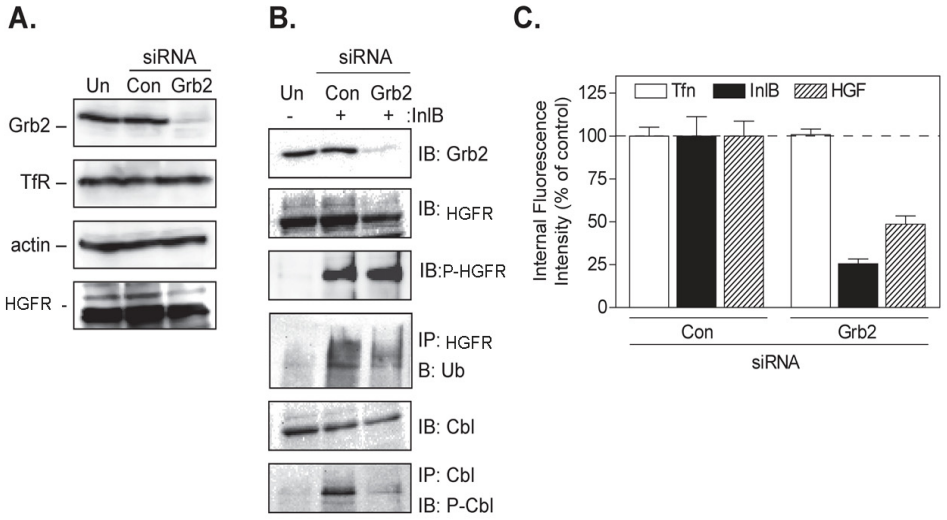

Figure 15. siRNA-mediated Grb2 depletion inhibits InlB and HGF internalization.

A. Lysates from mock transfected (UN) T47D/cMet cells or cells transfected with control(Con) or Grb2 siRNAs were examined by western analysis for Grb2, TfR, actin and HGFR expression. **B**. Mock transfected T47D/cMet cells or cells transfected with the indicated siRNAs were treated for 10 min at 37°C with (+) or without (-) InlB. Cell lysates were examined western analysis (IB) for expression of total protein (HGFR, Grb2, Cbl), phosphorylation of immunoprecipitated (IP) HGFR and Cbl (P-HGFR and P-Cbl) and HGFR ubiquitination (Ub). **C**. The relative amounts of internalized HGF, Alexa-InlB and Tfn (10 min at 37°C) were quantified by confocal microscopy. Values represent the mean fluorescence intensity ± S.E. from 4 separate experiments and are expressed as a percentage of control values.

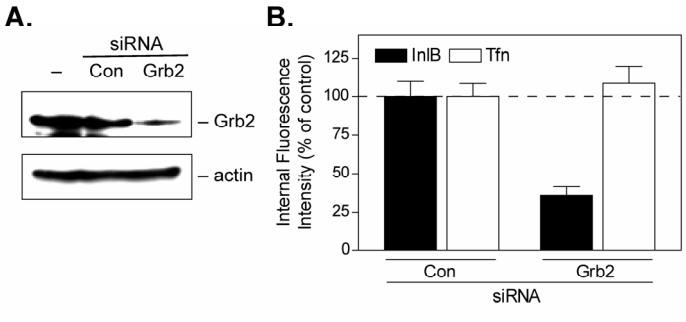

Figure 16. Endogenous Grb2 is required for HGFR internalization in kidney epithelial cells. A. WB confirmed that the Grb2 siRNA was specific for Grb2 and not actin in HGFR null (-/-) cells. B. The internalization of Tfn and Alexa-InlB into HGFR null (-/-) stably expressing wt-HGFR was quantified using confocal microscopy. Values are expressed as the mean fluorescence intensity ± S.E. as a percentage of control values.

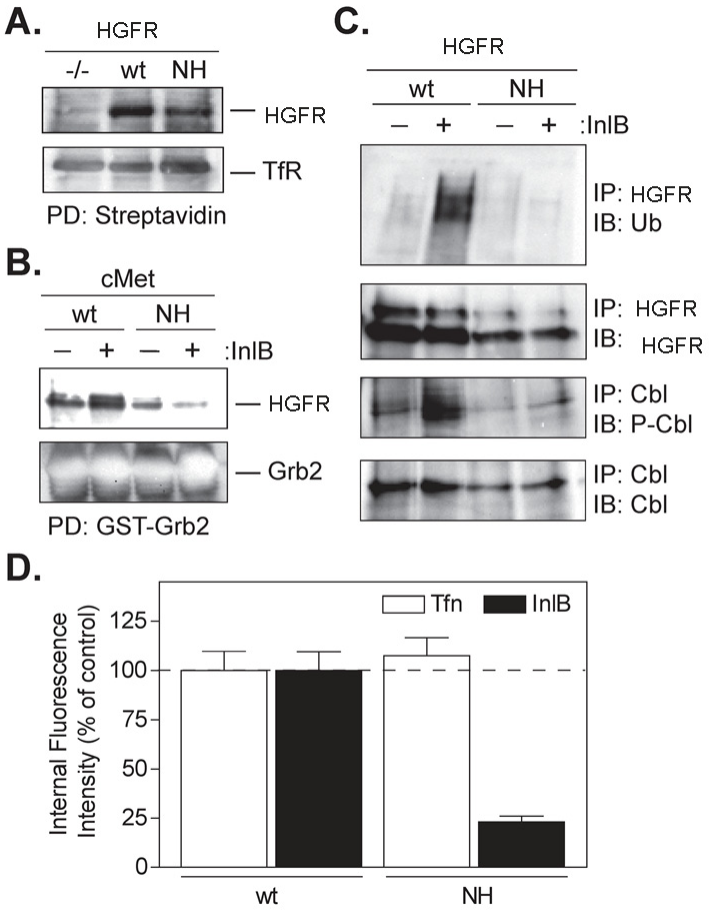

Figure 17. Grb2 is required for HGFR internalization. A. Surface biotinylation assays confirmed that HGFR null (-/-) cells stably express comparable levels of wt HGFR or HGFR-N1358H (NH), a mutant deficient in Grb2 binding, at their surface. B. HGFR null (-/-) cells stably expressing wt or mutant HGFR (NH) were treated with (+) or without (-) 100 ng/ml InlB for 10 min at 37°C. Equal amounts of cell lysates were incubated with recombinant GST-Grb2 and examined by western analysis. Grb2 binding to mutant HGFR-NH was reduced relative to wt HGFR, in response to InlB treatment. C. Total protein (HGFR, Cbl), HGFR ubiquitination (Ub) and Cbl phosphorylation (P-Cbl) in response to InlB (+) were examined in lysates prepared from HGFR null (-/-) cells stably expressing wt or mutant HGFR (NH) by western analysis. D. Confocal microscopy detected reduced levels of internalized Alexa-InlB but not Tfn, in HGFR null (-/-) cells stably expressing mutant HGFR (NH). Values represent the mean fluorescence intensity ± S.E. from 4 separate experiments and are expressed as a percentage of control values.

studies confirmed that NH-HGFR was expressed at the cell surface although at a slightly reduced level relative to a cell line stably expressing wt-HGFR (Figure 17A). GST pull down assays confirmed that recombinant Grb2 bound to wt-HGFR in response to InlB.

Conversely, reduced Grb2 binding to NH-HGFR was detected under these conditions (Figure 17B). HGFR ubiquitination and Cbl phosphorylation in response to HGFR activation was detectable in null cells expressing wt HGFR but reduced in cells expressing the mutant NH-HGFR. These data support the conclusion that the direct binding of Grb2 to docking sites of HGFR is reduced by the mutation N1358H. I next compared the internalization properties of NH-HGFR with the wt receptor. Using Alexa-InlB to stimulate HGFR internalization (10 min, 37°C), I confirmed that HGFR internalization was reduced in null cells expressing NH-HGFR but not in null cells expressing wt-HGFR by confocal microscopy (Figure 17D). Thus my data indicate that the direct binding of Grb2 to activated HGFR is indispensable for receptor internalization through clathrin-coated pits.

Interactions downstream of Grb2 recruitment mediate HGFR internalization.

I used targeted dominant negative Grb2 mutants to verify that the binding of Grb2 to HGFR was important for receptor endocytosis. Grb2 is a cytoplasmic scaffold protein that contains a central SH2 domain flanked by two SH3 domains. The central SH2 domain mediates Grb2 binding to pY1356 in the cytoplasmic region of activated HGFR. The SH3 domains of Grb2 mediate its binding to critical signaling molecules including son-of-sevenless (SOS), Gab1 and cCbl. The mutations P49L and G203R in the NH_2 and C-terminal SH3 domains respectively, block these interactions in a double Grb2 mutant (Grb2-mSH3) that corresponds to loss of function phenotypes in *C. elegans* (Figure 18A). If Grb2 is required for HGFR internalization, I reasoned that wt and mutant Grb2 would bind and colocalize with HGFR when expressed in cells. However, in contrast to wt Grb2, Grb2-SH2 would function as a dominant negative mutant and block the binding of endogenous Grb2 to HGFR, whereas the SH3 double mutant (Grb2-mSH3) would be unable to form productive complexes with downstream effectors. Thus both Grb2 mutants would function to impede HGFR internalization. Control westerns detected

comparable levels of activated HGFR in mock-transfected T47D/Met cells, and in cells expressing wt or mutant Grb2 (Figure 18B) in response to InlB. In contrast to wt Grb2, expression of Grb2mSH3 and to a lesser extent Grb2-SH2, resulted in reduced ERK activation. The 40 % reduction in ERK activation observed in cells expressing Grb2-SH2

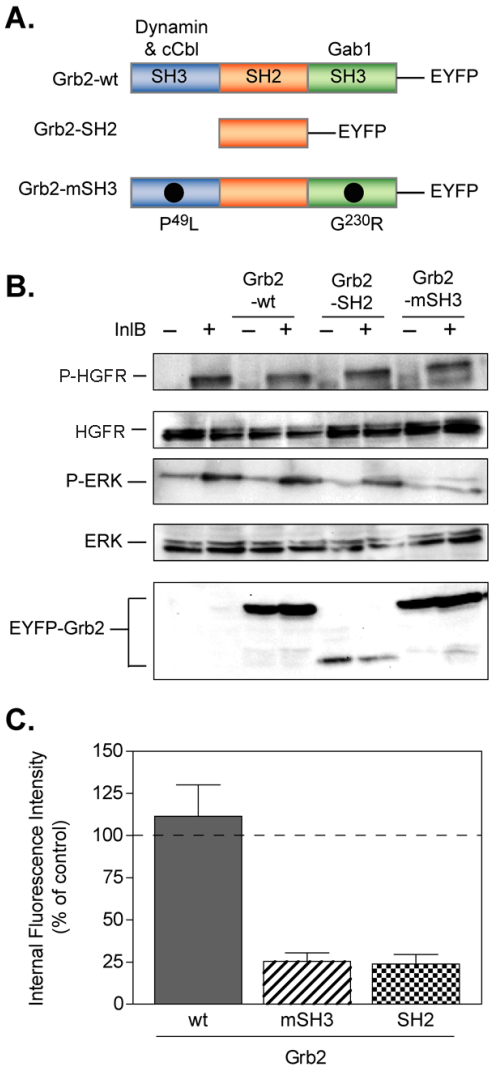

Figure 18. Interactions downstream of Grb2 are involved in HGFR internalization. A. Schematic of EYFP-tagged wt or mutant (mSH2, mSH3) Grb2 and their binding properties. B.T47D/cMet cells expressing EYFP-tagged Grb2 or EYFP only, were treated for 10 min at 37°C with (+) or without (-) InlB. Cell lysates were examined for activation of HGFR and ERK (P-HGFR and P-ERK), for expression of total protein (HGFR and ERK) and for expression of EYFP-tagged Grb2. C. The relative amounts of internalized Tfn and Alexa-InlB (10 min at 37°C) in control T47D/cMet cells (EYFP only) and in cells expressing the indicated EYFP-tagged Grb2 proteins were quantified by confocal microscopy. Values represent the mean fluorescence intensity ± S.E. from 4 separate experiments and are expressed as a percentage of control values.

was consistent with the low transfection rate routinely obtained using this construct.

To test the role of Grb2 in HGFR internalization, T47D/cMet cells were transfected with wt or mutant Grb2 (Grb2-SH2, Grb2-mSH3) labeled at their C-termini with Enhanced Yellow Fluorescent Protein (EYFP) (Figure 18A) to enable detection of the over expressed proteins. The presence of EYFP has been shown not to interfere with the function of wt Grb2 or its mutants (Huang and Sorkin 2005). After treatment with Alexa-InlB for 15 min at 37°C to stimulate HGFR internalization, the effects of wt and mutant Grb2 on Alexa-InlB uptake were quantified by confocal microscopy. Under these conditions, comparable levels of internalized Alexa-InlB were detected in cells expressing wt Grb2 and control cells expressing EYFP only (Figure 18C). Conversely, an 84-90% decrease in the relative amount of internalized Alexa-InlB was detected in cells expressing Grb2-SH2 or Grb2-mSH3, indicating that functional interactions downstream of Grb2 binding are required for HGFR internalization.

Ras activity is not required for HGFR internalization.

Grb2 has been reported to bind several molecules involved in endocytosis including Cbl and the Son-of-Sevenless (Sos), a guanine nucleotide exchange factor for p21 Ras. Cbl has been shown to regulate down regulation of the EGF (EGFR) and Ron receptors (Levkowitz, Waterman et al. 1998; Penengo, Rubin et al. 2003), promotes clustering of endocytic adaptor proteins (Jozic, Cardenes et al. 2005), regulates the internalization of the *Listeria monocytogenes* through clathrin coated pits (Veiga and Cossart 2005) as well was HGFR and EGFR internalization (Petrelli, Gilestro et al. 2002; Soubeyran, Kowanetz et al. 2002). Ras activation has been implicated in the internalization of EGFR and macropinocytosis (Barbieri, Kohn et al. 1998; Tall, Barbieri et al. 2001; Amyere, Mettlen et al. 2002). Consistent with a role in regulating receptor trafficking, the endocytic protein intersectin binds SOS and regulates the Ras/MAP kinase pathway (Tong, Hussain et al. 2000; Tong, Hussain et al. 2000). Therefore, I examined the requirements for Cbl and Ras activity for HGFR internalization. T47D/cMet cells were transfected with HA-tagged, dominant negative Ras (HA-

RasS17N) and then coincubated with Alexa-InB and Tfn for 10 min at 37°C. HA-RasS17N transfected cells were detected by costaining with an anti-HA antibody.

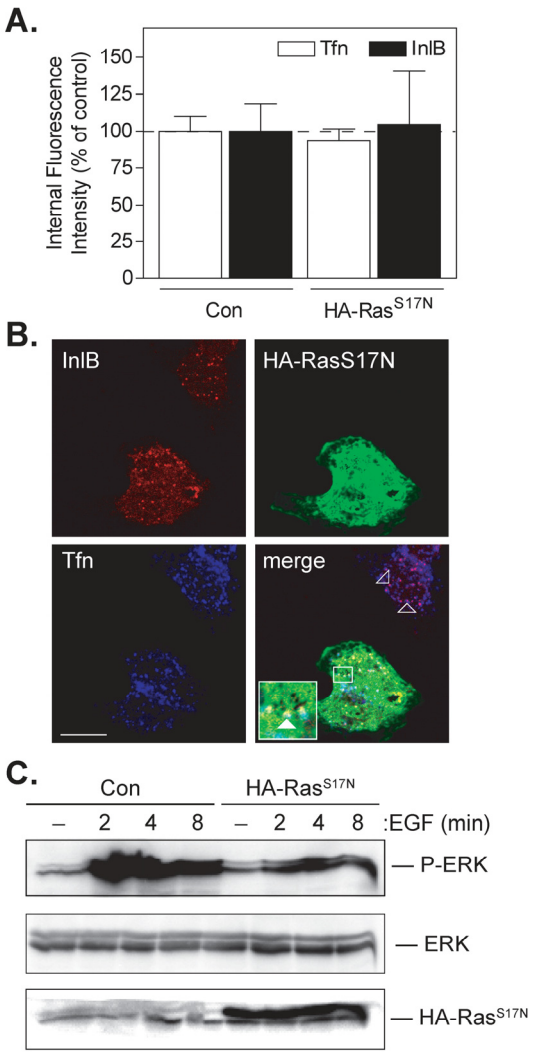

Figure 19. HGFR internalization is not regulated by Ras activity. A. T47D/cMet cells expressing Ha-tagged dominant negative RasS17N and mock-transfected cells (Con) were allowed to cointernalize Tfn and Alexa-InlB for 10 min at 37°C, and processed for confocal microscopy. HA-RasS17N expressing cells were detected by costaining with an antibody for the HA epitope. The relative amounts of internalized ligands are graphed as the mean fluorescence intensity ± S.E. from 4 separate experiments and are expressed as a percentage of control values. B. Representative examples of internalized InlB in mock-transfected cells (open arrows) and cells transiently expressing HA-RasS17N (closed arrows) are shown. Bar, 10 µm. C. HARasS17N expression caused reduced ERK phosphorylation in response to EGF (10 min at 37°C).

Ligand uptake in HA-RasS17N transfected cells and mock transfected cells was quantified by confocal microscopy. Under these conditions, comparable levels of

internalized Alexa-InlB and Tfn were detected in mock transfected cells and cells expressing dominant negative Ras (Figure 19A and 19B). Moreover, internalized Alexa-InlB and Tfn colocalized in intracellular punctate structures reminiscent of clathrin-derived early endosomes (Figure 19B). The activity of HA-RasS17N was confirmed by examining its effect on downstream signaling events. For example, HA-RasS17N expression caused reduced ERK phosphorylation in response to EGF (Figure 19C) in T47D/cMet cells. These data indicate that Grb2-mediated changes in Ras activity are not involved in HGFR internalization.

Cbl ubiquitin ligase activity is required for HGFR internalization.

My earlier data using HGFR null cells expressing targeted HGFR mutants detected reduced levels of Cbl phosphorylation and HGFR ubiquitination in the cell lines expressing YF-HGFR and NH-HGFR (refer to Figure 10A and 17C), HGFR mutants deficient in Grb2 binding. Since the tyrosine kinase activity of the YF and NH mutants is unaffected, these data suggest that the indirect recruitment of Cbl to HGFR via Grb2 in likely disrupted by the Y1349, 1356F and N1358H mutations. Since Grb2 associates constitutively with Cbl (Meisner, Conway et al. 1995; Meisner and Czech 1995), Grb2 may represent the primary mode for Cbl recruitment to HGFR. Therefore I examined if the ubiquitin ligase activity of Cbl was important for HGFR internalization using confocal microscopy. T47D/cMet cells were transfected with wt Cbl or Cbl mutants' defective in either ubiquitin ligase activity (70Z-Cbl), or HGFR pY1003 binding (CblG306E) (Figure 20B). Previous studies have demonstrated that Cbl binds HGFR and induces its monoubiquitination, whereas the ubiquitin ligase deficient mutant Cbl-70Z interacts with HGFR but is unable to induce receptor ubiquitination (Peschard, Fournier et al. 2001; Petrelli, Gilestro et al. 2002; Abella, Peschard et al. 2005). The cells were allowed to cointernalize Tfn with Alexa-InlB or HGF for 10 min at 37°C and the relative amount of internalized ligand examined by confocal microscopy. HGFR internalization was unaffected in mock-transfected control cells and in cells expressing wt Cbl or the receptor binding mutant CblG306E. However, in cells over expressing the ubiquitin ligase deficient mutant 70Z-Cbl, internalized HGF and Alexa-InlB staining was reduced

to 33.5 ± 2.4 % and 50.4 ± 2.6 % of control levels respectively (Figure 20C). Western analysis confirmed that the differences in HGFR internalization were not due to differences in wt and mutant Cbl expression (data not shown). To confirm the requirement of Cbl mediated ubiquitination for HGFR internalization, I took advantage of well-characterized ubiquitin (Ub) mutants that have altered conjugation and binding properties (Figure 20C). Cbl functions to covalently attach monoubiquitin to substrate proteins via C-terminal Glycine residues of Ub. The Ub mutant UbR lacks these glycine residues important for substrate conjugation. However, UbR retains the ability to interact with intracellular proteins harboring Ub-binding domains, including the Ub-interacting motif (UIM). Thus when expressed in cells, UbR functions to block the interaction of ubiquitinated proteins with proteins harboring a UIM. Using confocal microscopy, I examined the effect of transiently expressing UbR on HGFR internalization in T47D/cMet cells. UbR containing the double mutation L8A/I44A (UbRL8A/I44A) that blocks the interaction of UbR with UIMs, was used as a control. As shown in Fig 20D, expression of UbR reduced HGFR internalization 33.7 ± 3.4 % relative to mock transfected control cells or cells expressing the double mutant UbRL8A/I44A. Tfn uptake was unaffected in cells expressing UbR or UbRL8A/I44A. Together, these data indicate that the ubiquitin ligase activity of Cbl is important for HGFR internalization in response to InlB and HGF.

Recruitment of Cbl through the SH2 domain of Grb2 regulates HGFR internalization.

My data showing a requirement for Grb2 and Cbl ubiquitin activity in HGFR internalization is consistent with the hypothesis that Cbl recruitment to HGFR through the SH2 domain of Grb2 is important for HGFR internalization. To mimic Grb2-mediated recruitment of Cbl, I used a chimeric protein in which wt Cbl was tagged with EYFP at its amino terminus and the SH2 domain of Grb2 at its carboxyl-terminus (Cbl-SH2). A second mutant chimeric protein deficient in HGFR binding (Cbl-SH2/R86A) was used as a control (Figure 21A). I examined the ability of these Cbl-Grb2 chimeric proteins to rescue the inhibition of HGFR endocytosis imposed by Grb2 depletion.

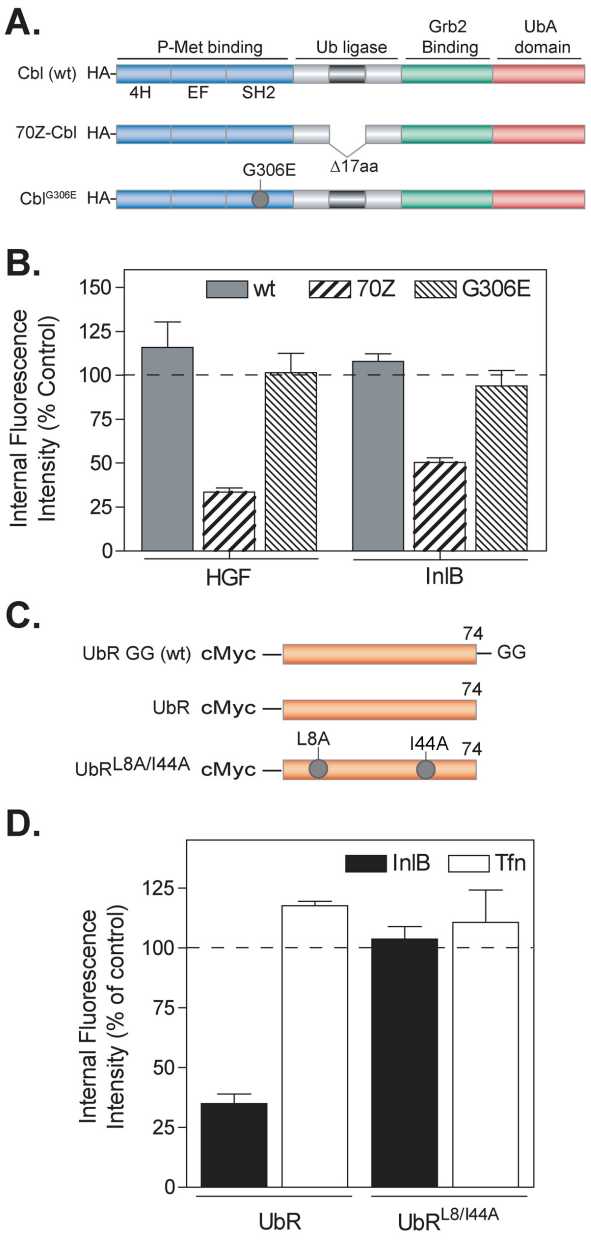

Figure 20. Cbl ubiquitin ligase activity is important for HGFR internalization. A. Schematic of HA-tagged wt or mutant (70Z-Cbl, CblG306E) Cbl and their binding properties. B. Mock transfected T47D/cMet cells and T47D/cMet cells transiently HA-tagged wt or mutant Cbl were allowed to cointernalize InlB and HGF for 10 min at 37°C, and the relative amounts of internalized ligands quantified by confocal microscopy. Values represent the mean fluorescence intensity ± S.E. from 4 separate experiments and are expressed as a percentage of control values. C. Schematic of cMyc-tagged wt Ub, mutant Ub (UbR) and the inactive variant UbRL8A/I44A are shown. D. The relative amounts of internalized Tfn and InlB in mock-transfected control cells and transfected cells expressing the indicated constructs were quantified using confocal microscopy. Values represent the mean fluorescence intensity ± S.E. from 4 separate experiments and are expressed as a percentage of control values.

In these studies, T47D/cMet cells were cotransfected with Grb2-targeted siRNA and with Cbl-SH2, Cbl-SH2/R86A or mock transfected cells as a control. The cells were allowed to cointernalize Alexa-InlB with Tfn for 10 min at 37°C and the relative amounts of internalized ligand examined by confocal microscopy (Figure 21B). Expression of

Cbl-SH2 in Grb-2 depleted cells caused increased uptake of Alexa-InlB in Grb2-depleted cells compared to Grb2depleted cells expressing EGFP only. Conversely, expression of the chimeric protein deficient in HGFR binding (Cbl-SH2/R86A) was not as effective at rescuing HGFR internalization in Grb2-depleted cells. Together, these data indicate that a Cbl/Grb2 chimera that is capable of binding to HGFR rescues HGFR internalization in Grb2-depleted cells.

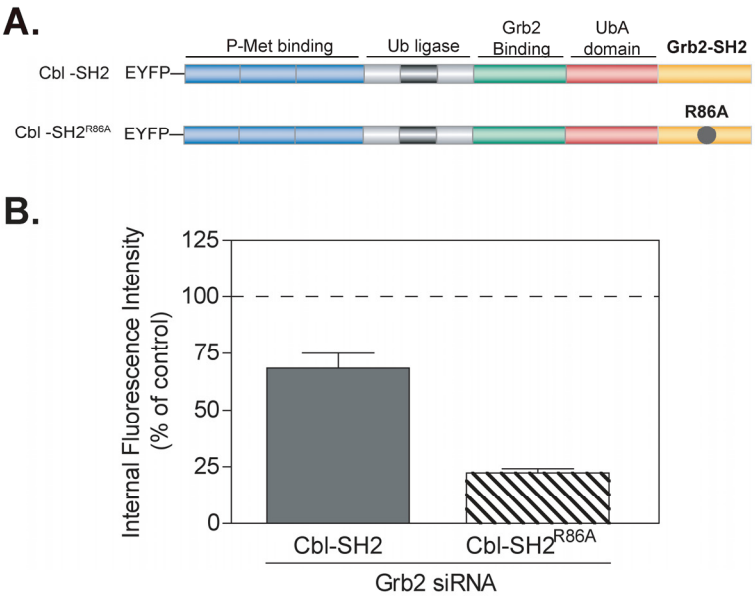

Figure 21. Rescue of HGFR internalization in Grb2-depleted cells using a Cbl-Grb2/SH2 chimeric protein. A. Schematic of EYFP-tagged Cbl fused to the SH2 (HGFR binding) domain of Grb2, or a mutant SH2 domain (SH2R86A) deficient in Grb2 binding. B. T47D/cMet cells were cotransfected with Grb2 specific siRNAs to deplete endogenous Grb2 and EYFP only (control), EYFP-tagged Cbl-SH2 or the mutant deficient in HGFR binding (Cbl-SH2R86A). The relative amounts of internalized Alexa-InlB were quantified by confocal microscopy. Values represent the mean fluorescence intensity ± S.E. from 4 separate experiments and are expressed as a percentage of control values.

DISCUSSION

Endocytic studies on many cell surface receptors confirm that receptor internalization is the key mechanism for attenuating receptor tyrosine kinase signaling by inactivating receptors by degradation. Ligand binding to HGFR normally induces receptor activation, leading to HGFR internalization through clathrin-coated pits and down regulation in lysosomes. Receptor mutations that uncouple activated HGFR from efficient down regulation are tightly associated with human metastatic cancers (Peschard, Fournier et al. 2001; Abella, Peschard et al. 2005). In this study I provide a mechanistic understanding for how HGFR activation is linked to receptor internalization. I demonstrate that clathrin-mediated HGFR internalization is dependent on both the intrinsic kinase activity of HGFR and the phosphorylation of two key tyrosines at positions 1349 and 1356 in the multisubstrate docking site of HGFR. Recruitment of the signaling adaptor Grb2 but not Gab1, to the HGFR multisubstrate-docking site is essential for HGFR internalization. Moreover, I show a requirement for Grb2-mediated recruitment of Cbl ubiquitin ligase activity in the internalization process. Over expression of an Ub mutant that could not be conjugated to substrates yet retained the ability to bind to Ub interacting motifs (UIM), inhibited ligand-induced HGFR internalization. Together, my data support a role for Grb2 as a transducer for Cbl-mediated ubiquitination events in the clathrin-dependent internalization of HGFR.

Efficient RTK internalization requires the formation of large protein complexes within the immediate vicinity of activated receptors. These complexes function to engage activated RTKs with clathrin-coated pit and cytoskeleton components, driving receptor internalization into endocytic vesicles. Not surprisingly, the adaptor protein Grb2 has been shown to interact with several proteins important for clathrin-mediated endocytosis including Cbl (Fukazawa, Miyake et al. 1996), the Ras guanine nucleotide exchange factor SOS, dynamin (Vidal, Montiel et al. 1998; Vidal, Goudreau et al. 1999) and N-WASP (She, Rockow et al. 1997). SOS and Dynamin interact primarily with the N-terminal SH3 domain of Grb2 (Vidal, Montiel et al. 1998), whereas both Grb2 SH3 domains are required for a stable interaction with Cbl (Fukazawa, Miyake et al. 1996). In

my study, the requirement for Grb2 in HGFR internalization was consistent with my earlier report that over expression of dominant negative Dynamin K44A inhibited HGFR internalization (Li, Xiang et al. 2005). Conversely, over expression of dominant negative Ras (S17N) did not interfere with clathrin-mediated HGFR endocytosis, demonstrating that specific Grb2-mediated interactions downstream of activated HGFR regulate this process.

Clathrin-dependent internalization is a multi step process involving clathrin-coated pit formation, plasma membrane invagination, receptor recruitment into clathrin-coated pits, the budding of clathrin-coated vesicle from the plasma membrane and transport to early endosomes. Grb2 has been shown to regulate the internalization of several signaling receptors including the Ron tyrosine kinase (Penengo, Rubin et al. 2003), the FGF receptor (Wong, Lamothe et al. 2002), the CD229 receptor (Martin, Del Valle et al. 2005), MUC1 (Kinlough, Poland et al. 2004) and the EGFR (Wang and Moran 1996; Yamazaki, Zaal et al. 2002; Jiang, Huang et al. 2003). Studies on the EGFR have identified specific requirements for Grb2 in multiple stages of endocytosis including the formation of clathrin-coated pits and the recruitment of EGFR to these sites (Jiang, Huang et al. 2003; Stang, Blystad et al. 2004; Johannessen, Pedersen et al. 2006). Over expression of Grb2-SH3 mutants deficient in binding downstream signaling molecules prevented EGF induced inward translocation of Grb2 with activated EGFR into endocytic structures, presumably endocytic transport vesicles (Yamazaki, Zaal et al. 2002). Consistent with this, the Grb2 interacting protein N-WASP has been shown to regulate EGFR endocytosis at the level of clathrin-coated pit invagination (Benesch, Polo et al. 2005). My data are the first to report a role for Grb2 in HGFR internalization. As an adaptor protein for Cbl, Grb2 could regulate individual steps leading to HGFR internalization, or function to couple distinct stages of the internalization process.

Internalization of a mutant HGFR deficient in Grb2 binding only (N1358H) was partially blocked in response to ligand binding. This contrasts the situation in cells expressing HGFR-YF a mutant defective in both Grb2 and Gab1 binding. Internalized HGF or InlB was not detected in HGFR null cells expressing the Y1349, 1356F mutant.

It is possible that in HGFR null cells expressing the N1358H mutant, a small amount of Grb2 is recruited indirectly to mutant HGFR by association with tyrosine phosphorylated SHC, an adaptor protein that also binds HGFR at pY1349 and pY1356. Indeed, the partial rescue of HGFR internalization in Grb2-depleted cells over expressing a Cbl-Grb2/SH2 chimeric protein is consistent with a role for additional proteins in the internalization process.

Gab1 is an important adaptor molecule for the recruitment and activation of PI3K signaling (Gu and Neel 2003). I previously reported that over expression of a dominant negative mutant for the p85 subunit of PI3K did not inhibit HGFR internalization. Similarly, no differences in the internalization properties of HGFR were observed in T47D/cMet cells treated with the PI3K inhibitors LY294002 or Wortmannin (Li, Xiang et al. 2005). My data showing that the recruitment of the large signaling adaptor Gab1 to HGFR is dispensable for receptor internalization are in agreement with the observation that PI3K signaling is not required for HGFR endocytosis. Interestingly siRNA-mediated depletion and genetic knockout of Gab1 caused increased accumulation of internalized InlB and HGF in T47D/cMet and MEF cells respectively. Gab1 and Cbl interact with the N- and C-terminal SH3 domains of Grb2 through their proline rich domains (Fukazawa, Miyake et al. 1996; Bardelli, Longati et al. 1997). Therefore, it is possible that Grb2/Cbl binding is compromised by the presence of Gab1, perhaps due to steric hindrance. Accordingly, depletion of Gab1 or loss of Gab1 would enable Grb2 to preferentially associate with Cbl, resulting in increased HGFR internalization as observed in my studies.

Alterations that uncouple HGFR from Cbl-mediated ubiquitination are tightly associated with cancer (Peschard, Fournier et al. 2001; Abella, Peschard et al. 2005). In my studies, Grb2 mediated-HGFR internalization was dependent on the ubiquitin ligase activity of Cbl. Reduced HGFR ubiquitination was detected in cells depleted of endogenous Grb2 and in cells stably expressing HGFR-N1358H, a receptor mutant deficient in Grb2 binding. Consistent with my findings, HGFR internalization and HGF-induced signal transduction in HEK293 cells has been shown to depend on the formation

of complex composed of Cbl, the adaptor protein CIN85 and endophilin, an endocytic protein important for plasma membrane invagination and the fusion of clathrin-coated pits (Petrelli, Gilestro et al. 2002). However a recent study reported that a HGFR mutant (Y1003F) defective in Cbl recruitment to HGFR was rapidly internalized in response to HGF with similar kinetics to the wild type receptor (Abella, Peschard et al. 2005). Although the studies using HGFR-Y1003F suggested that Cbl-mediated HGFR ubiquitination was not sufficient for receptor endocytosis, it remains unclear whether the Y1003F mutant is internalized through clathrin-coated pits or via clathrin-independent mechanisms. This suggests that there is no causative relationship between Cbl-mediated HGFR ubiquitination and receptor endocytosis. However, HGFR-Y1003F was weakly ubiquitinated making it difficult to discard a role for HGFR mono-ubiquitination in the internalization process. Taken together, my results and the studies using the Y1003F HGFR mutant are consistent with a scenario in which Grb2-dependent recruitment of Cbl results in HGFR ubiquitination, promoting the association of HGFR with clathrin-coated pit components and subsequent receptor internalization.

Like HGFR, a role for Cbl ubiquitin ligase activity as a regulator of EGFR internalization remains controversial. The E3 ligase activity of Cbl has been shown to regulate EGFR internalization in response to low concentrations of EGF (Jiang and Sorkin 2003; Huang and Sorkin 2005). Low EGF levels (~1-10 ng/ml) promote EGFR internalization via clathrin-coated pits, whereas at high EGF concentrations (>60 ng/ml), clathrin-dependent internalization appears to saturate resulting in additional receptor internalization via caveolae/lipid rafts (Huang and Sorkin 2005; Sigismund, Woelk et al. 2005). A mutant EGFR-Y1045F that cannot efficiently recruit Cbl was internalized at a similar rate to wt EGFR ion response to low levels of EGF (Sigismund, Woelk et al. 2005). The EGFR-Y1045F mutant was shown to be weakly ubiquitinated, especially in cells over expressing Cbl or Grb2 (Waterman, Katz et al. 2002). Moreover EGFR-Y1045F internalization in response to high and low concentrations of EGF was clathrin-mediated, since over expression of a mutant Eps15 which blocks the recruitment of AP2, or an amphiphysin fragment that selectively interferes with clathrin, blocked receptor

internalization. EGFR-Y1045F internalization was insensitive to treatment with filipin, nystatin and genestein, inhibitors that preferentially interfere with non-clathrin internalization (Sigismund, Woelk et al. 2005). More recent studies suggest that high levels of receptor ubiquitination may be an important determinant for EGFR internalization via clathrin-independent mechanisms rather than endocytosis through clathrin-coated pits (Aguilar and Wendland 2005). Consistent with this idea, treatment of cells with high concentrations of EGF (>60 ng/ml) promotes higher levels of receptor ubiquitination and internalization via the caveolin/lipid raft pathway. A chimeric protein composed of mono-Ub fused to a modified form of the Green Fluorescent Protein that is myristylated and palmitylated and hence targeted to the plasma membrane, was internalized via a mechanism that did not require clathrin (Chen and De Camilli 2005). Similarly, a truncated EGFR mutant composed of the extracellular and transmembrane domains is not internalized when expressed in cells and is retained at the cell surface. However, when a cytoplasmically exposed mono-Ub was fused to the truncated EGFR mutant, the chimeric receptor was endocytosed via clathrin-independent mechanisms (Haglund, Sigismund et al. 2003). Thus it remains to be determined whether the requirement for receptor ubiquitination varies between internalization routes, functions in a cell specific context or for different signaling receptors.

My data indicating a role for Grb2-recruited Cbl ubiquitin ligase activity for HGFR internalization are consistent with several models in which a macromolecular complex containing Grb2-Cbl as well as Ub-UIM interactions mediates the clathrin-dependent internalization of activated HGFR (Figure 22). In the first model, Grb2-recruited Cbl would ubiquitinate HGFR at specific cytoplasmic sites, enabling the ubiquitinated receptor to interact with UIM containing endocytic proteins such as Eps15 or CIN85. In support of this model, internalization of the weakly ubiquitinated YF-HGFR mutant deficient in Grb2 binding is inhibited. In the second model, HGFR ubiquitination is not a prerequisite for internalization. Rather Cbl-mediated ubiquitination of other substrates would be required for receptor internalization. In addition to its adaptor function, ubiquitination of other Cbl-mediated ubiquitination of endocytic

machinery components would promote protein-protein interactions required for clustering the receptor to the clathrin-coated pits. One candidate is CIN85, a protein shown to be important for HGFR internalization (Petrelli, Gilestro et al. 2002). Cbl over expression has been shown to promote the clustering of the endocytic adaptor CIN85 in vitro and in vivo, mediating the formation of large macromolecular complexes involved in the regulation of EGFR endocytosis (Jozic, Cardenes et al. 2005). Consistent with this model, over expression of mutant UbR which blocks the binding of UIM containing protein to Ub, impaired HGFR internalization in my studies. The third model is based on a recent study reporting that monoubiquitination of ubiquitin-binding proteins inhibited their capacity to bind to the ubiquitinated targets (Hoeller, Crosetto et al. 2006). In the case of HGFR, a regulatory protein may shield a receptor internalization signal. Grb2 dependent Cbl-mediated ubiquitination of this regulatory protein could promote its dissociation from HGFR, rendering the internalization signal available for inducing clathrin-mediated HGFR endocytosis. Additional experimentation will be required to distinguish between these models.

In summary, my data establish the importance of the Grb2-Cbl complex for HGFR internalization in a variety of cell types. In addition to functioning as a signaling adaptor for Ras activity, Grb2 also initiates HGFR internalization through a process that depends on the recruitment and activation of Cbl. The most likely explanation to reconcile my data with other published findings, is that Grb2-dependent Cbl ubiquitination of receptor associated proteins or clathrin-coated pit components, is required for receptor endocytosis. In this case, Grb2 would function as an adaptor to couple activated HGFR to the endocytic machinery through mechanisms involving Ub-interacting proteins. Thus the identification of the ubiquitinated proteins important for Grb2-mediated HGFR internalization and how their activity is modulated in response to HGFR signaling and Cbl mediated ubiquitination will clearly require additional study.

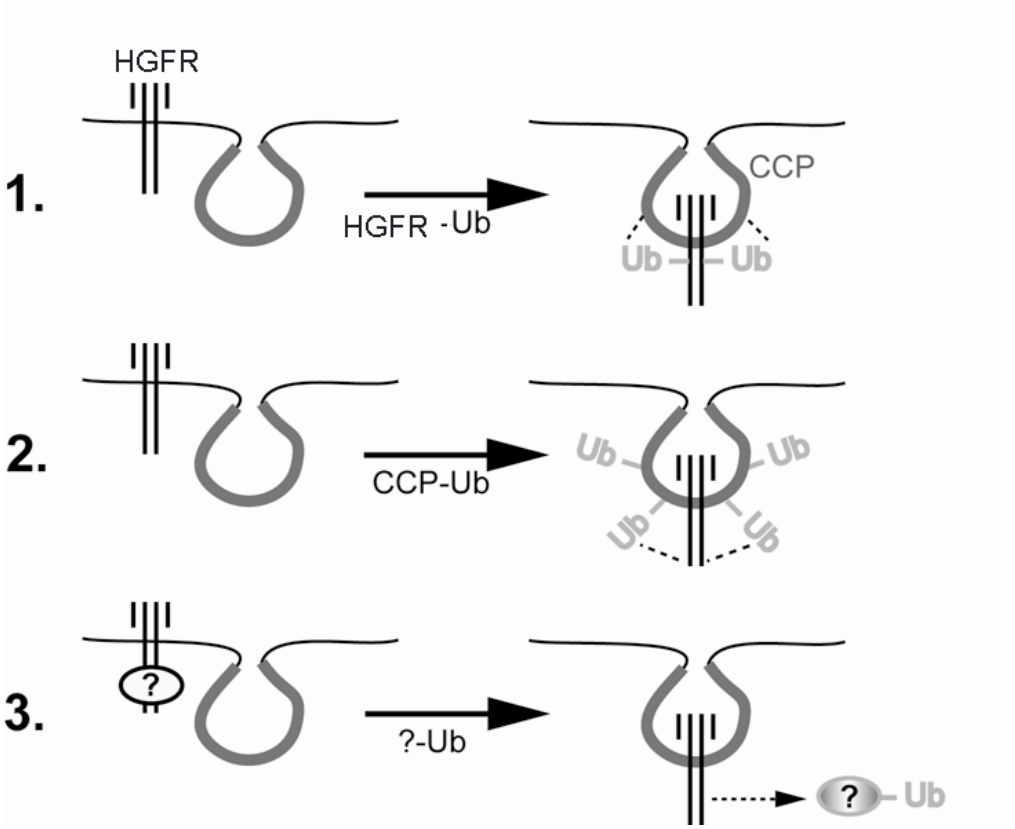

Figure 22. Three models for Grb2-Cbl mediated HGFR internalization. Ubiquitin (Ub), regulatory protein (?), clathrin-coated pits (CCP). See text for details.

CHAPTER 4: PI3K AND HRS REGULATE HGFR DEGRADATION VIA LYSOSOMES

INTRODUCTION

Following ligand stimulation, internalized HGFR can be subjected to two distinct fates: either to recycle back to the plasma membrane or to be degraded via the lysosomal or proteosomal pathway. Degradation of HGFR provides a down-regulation mechanism, that terminates activation of downstream signaling pathways, which if sustained could potentially lead to cellular transformation.

Prior to my study, the mechanism of HGFR degradation was controversial. It was suggested that HGFR was polyubiquitinated and degraded by a ubiquitin-proteasome pathway (Jeffers, Taylor et al. 1997). Consistent with this study, another group also reported that the proteasomal pathway was the major pathway for HGFR degradation (Hammond, Carter et al. 2003; Kermorgant, Zicha et al. 2003). In these studies the proteasome inhibitor, lactacystin, promoted the recycling of internalized HGFR back to the plasma membrane, rather than sorting the receptor to late endosomes. Although proteasomal activity was required for HGFR degradation, HGFR may not be the target for the proteasomal degradation. Multiple monoubiquitination of EGFR has been shown to be important for receptor sorting into the MVB and subsequent degradation in lysosomes (Longva, Blystad et al. 2002). As an alternative explanation, I proposed that HGFR might be degraded via lysosomes involving the ubiquitination machinery. Hrs is an endosomal protein required for the sorting of ubiquitinated receptors targeted for lysosomal degradation(Lu, Hope et al. 2003). Hrs binds to endosomal membranes enriched in PI3P via its FYVE domain as well as a UIM domain that promotes binding to ubiquitinated proteins(Bache, Raiborg et al. 2003). And PI3K is required for the generation of PI3P on the early endosomes (Petiot, Faure et al. 2003). Moreover, Hrs and PI3K are activated downstream of HGFR phosphorylation, suggesting an important role for these proteins in HGFR signaling and hence trafficking.

In my study, I demonstrated that depletion of endogenous Hrs blocked ligand induced HGFR degradation and that the internalized ligand/receptor complex was retained in enlarged endosomes that costained for the early endosomal marker EEA1. Similarly, inhibition of PI3K had no effect on HGFR internalization. Rather inhibition of PI3K activity by expressing a dnPI3K mutant or cell treatment with pharmacological inhibitors specific for PI3K delayed HGFR lysosomal degradation by disrupting Hrs binding to early endosomes.

MATERIALS AND METHODS

Reagents, Antibodies, siRNAs and Cell lines

All general reagents were obtained from Fisher Scientific or Sigma Aldrich unless indicated otherwise. Recombinant human HGF (PeproTech Inc., NJ); EZ link sulfo-NHS-SS-Biotin, D-Salt Dextran and Spin columns (Pierce Biotechnology Inc., IL); TR-Tfn and Antibody labeling kits (Molecular Probes, OR). The following antibodies were purchased as indicated — anti-human HGF (R&D Systems Inc, MN), EEA1 (BD Biosciences, CA), anti-HA (Roche, NJ), TfR (Zymed Labs, CA), Met C-28 (Santa Cruz Biotechnologies, CA), Peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Jackson ImmunoResearch Labs Inc, PA), Alexa^{488} or Alexa^{594} -labeled goat anti-mouse, anti-rabbit or anti-rat secondary antibodies (Molecular Probes, OR). Hrs and control siRNAs were from Dharmacon (Lafayette, CO). Human mammary epithelial cells (T47D) stably expressing full length human HGFR (T47D/cMet, M. Park, McGill University) were maintained in DMEM containing 10% FBS, supplemented with 400 μg/ml G418. MDCK and African Green Monkey kidney cells (Vero, ATTC CRL-1587) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. All cell lines were serum starved in DMEM for 6-8 hours before experimentation unless specified otherwise.

Plasmid, Transient Transfections and siRNA depletion studies.

Eukaryotic expression plasmid provided as indicated —HA-pΔ85 (J. Brugge, Harvard). For transfections, T47D/cMet cells were grown on glass poly D-lysine coated cover slips in 24 well plates at least 24 hr prior to transfection and transfected with Fugene 6 reagent (Roche, NJ) as specified by the manufacturer. Confocal microscopy was routinely performed 24-48 hrs following transfection. siRNA depletion experiments were performed using commercially available control and Hrs siRNAs (Dharmacon, CO) transfected into cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's specifications. The optimal concentration of siRNA required for at least a 95% decrease in mRNA and protein levels was determined empirically, and verified by RT/PCR and western analysis respectively. Confocal analysis on siRNA-depleted cells was routinely performed 48-72 hrs post-transfection.

Biotinylation and HGFR trafficking assays

Cells were surface biotinylated at 4°C for 30 min with EZ-link NHS-SS-biotin. HGFR uptake was induced by incubation in serum-free DMEM containing InlB or HGF at 37°C for the indicated time. Endocytosis was halted by rapid cooling to 4°C and residual cell surface biotin was then stripped by 2-3 washes for 2 hr with washing buffer (150 mM NaCl, 1 mM EDTA, 0.2% BSA, 20 mM Tris, pH 8.6) containing 50 mM MesNa. The cells were rinsed 3-5 times with ice-cold washing buffer, followed by 3-5 washes in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS+CM). Cells were lysed in ice-cold lysis buffer (0.5% Triton X100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris-Cl pH 7.4, 1 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 1μg/ml of Aprotinin, 1μg/ml of Leupeptin and 1μg/ml of Pepstatin), internalized biotinylated proteins were isolated using streptavidin-agarose beads (Pierce) and identified by Western analysis (Amersham Pharmacia). Resulting digitized blots were quantified in the linear range using AlphaEase v3.1.2 Software (AlphaInnotech) and normalized to the band intensity of the paired biotinylated, but unstripped sample representing total surface receptor at the

time of biotinylation. To measure HGFR degradation, duplicate sets of surface biotinylated T47D/cMet cells were incubated in sera free DMEM containing InlB or HGF at 37°C for 15 min as described above. The cells were shifted to ice, washed twice with ice-cold PBS+CM followed by two 40 min MesNa washes to remove residual surface biotin. Then cells were incubated at 37°C for increasing periods of time (1, 2 or 3 hr) to promote receptor degradation in DMEM with or without 100 nM Wortmannin. Resulting cell lysates were examined by streptavidin pull downs and Western analyses. Studies were performed in triplicate and all numerical results subjected to a one-way ANOVA with a Neuman-Keuls post-hoc test to determine statistical significance ($P<0.001$) between experimental groups (Prism GraphPad).

Confocal Microscopy and Analysis

Cells were plated on poly lysine D-coated cover slips and incubated in DMEM containing Alexa-InlB (and at TR-Tfn) 37°C as indicated in the figure legends. At the appropriate time, cells were washed briefly with 4 °C PBS and fixed for 10 min at room temperature in 4% Para formaldehyde (Ted Pella Inc.) in PBS, followed by quenching with 50 mM NH_4Cl for 10 min at room temperature. Cells were washed twice with PBS, permeabilized with PBS containing 0.05% saponin and 10% goat serum for 30 min at room temperature. All antibodies were routinely diluted in PBS containing 0.05% saponin and 10% goat serum and incubated on cells for 1 hr at room temperature. Cover slips were mounted on glass slides using FluorSave mounting medium (CalBiochem), and observed by confocal microscopy using a Zeiss LSM 510 confocal microscope with a 63 X oil (1.4 N.A.) immersion objective. Samples were visualized using the 488 nm and 543 nm laser lines and emission filter sets at 505-530 nm for GFP and $Alexa^{488}$ detection or 585-615 nm for Texas Red and $Alexa^{594}$ detection respectively. Figure presentation was accomplished in Adobe Photoshop v6.0. Quantification of colocalization and internal fluorescence intensity were done using Metamorph v5.0 (Universal Imaging Corp., Westchester, PA). All pixel intensity levels were normalized relative to control values, expressed as a percentage ± Standard Error (S.E.) and differences statistically verified by ANOVA using GraphPad Prism Software (GraphPad Prism).

RESULTS

PI3K signaling is not essential for HGFR internalization.

Activation of PI3K is required for a wide range of growth factor-induced responses including cell proliferation, differentiation and prevention of apoptosis (Wymann, Zvelebil et al. 2003). PI3K signaling regulates internalization of many membrane proteins including the c-Kit receptor (Gommerman, Rottapel et al. 1997), β2-Adrenergic receptor (Naga Prasad, Barak et al. 2001) and Na^+, K^+ ATPase α subunit (Yudowski, Efendiev et al. 2000). In addition, PI3K is needed for several cellular responses downstream of HGFR activation, including HGF-activated membrane ruffling, macropinocytosis, tubulogenesis, epithelial cell scattering and InlB-dependent entry of *Listeria* (Ireton, Payrastre et al. 1996; Khwaja, Lehmann et al. 1998). To determine if PI3K activity is required for ligand-induced HGFR internalization, sera starved T47D/cMet cells were preincubated in media containing the PI3K inhibitor LY294002 (50 μM) for 30 min, followed by treatment with Alexa-InlB for 15 min at 37°C to stimulate HGFR internalization. Under these conditions, InlB accumulated with cointernalized Tfn in enlarged organelles reminiscent of early endosomes (Figure 23A). TfR and InlB internalization was not blocked by LY294002 treatment, indicating that inhibition of PI3K activity did not alter other clathrin-mediated processes. Similarly, internalized Alexa-InlB and Tfn were readily detected in cells treated with 100 nM Wortmannin, another inhibitor of PI3K (Figure 23A). As additional controls, I confirmed the effects of LY29004 and Wortmannin on the subcellular localization of Hrs and EEA1, endocytic proteins that bind to early endosomal PI3P with high affinity via their FYVE domains (Lemmon 2003). As expected, treatment with LY290004 or Wortmannin led to the release of transiently expressed GFP-labeled EEA1 and Hrs from early endosomes (Figure 24). I confirmed that PI3K activity was not essential for HGFR internalization using cell surface biotinylation assays (Figure 23B). Under these conditions, treatment with LY294002 or Wortmannin did not block activation (data not shown) or

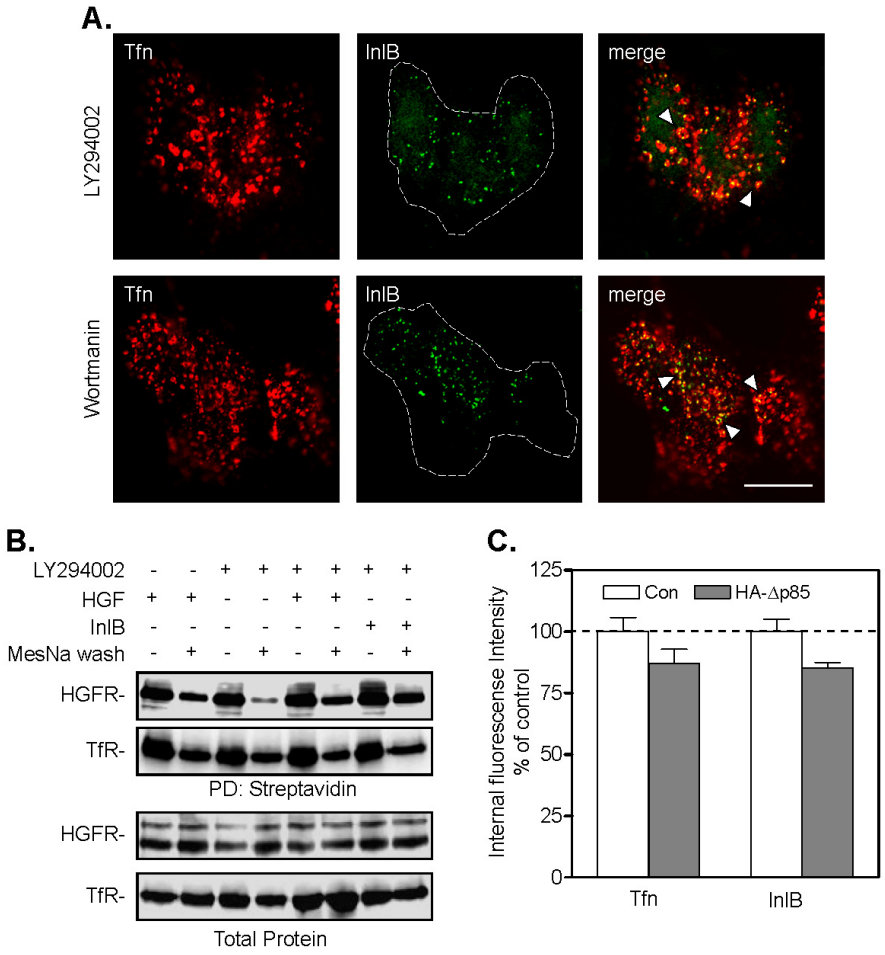

Figure 23. Inhibition of PI3K activity does not prevent HGFR internalization. A, T47D/cMet cells were pretreated with media containing 50μM LY294002 or 100 nM Wortmannin for 30 min at 37°C. Cells were incubated with Alexa488-labeled InlB (InlB) and TR-Tfn (Tfn) in the presence of the inhibitors for a further 15 min at 37°C then analyzed by confocal microscopy. Arrows indicate areas of colocalization (yellow) in the merged images (merge). Bar, 10 μm. B, Surface biotinylated T47D/cMet cells were pretreated with LY294002 (+) or vehicle only (-) for 30 min. InlB or HGF was added as indicated and the cells shifted to 37°C for 15 min to induce HGFR internalization. C, Expression of dominant negative HA-Δp85 does not inhibit internalization of Alexa-InlB or Tfn.

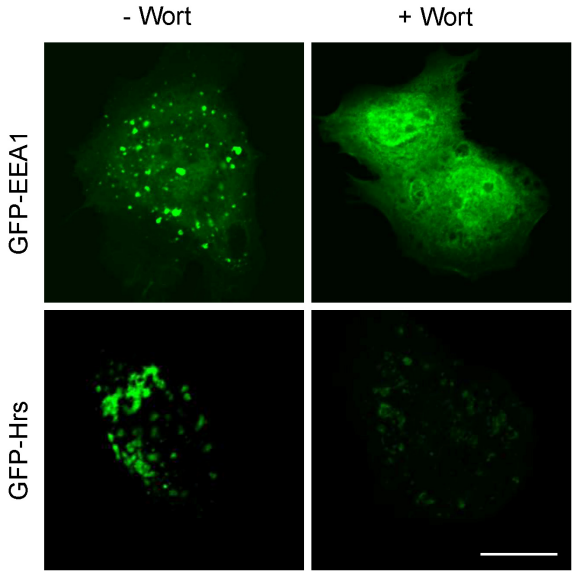

Figure 24. Wortmannin disrupts the binding of EEA1 and Hrs to early endosomal membranes. T47D/cMet cells transiently expressing GFP-EEA1 (A and B) or GFP-Hrs (C and D) were incubated cells in the absence (-) or presence (+) of 100 nM Wortmannin for 1 hr at 37°C. The cells were either fixed directly (GFP-EEA1) or permeablized with 0.05% saponin for 30 min at 37°C to enable removal of soluble protein prior to fixation (GFP-Hrs) and examined using confocal microscopy. Bar, 10 μm.

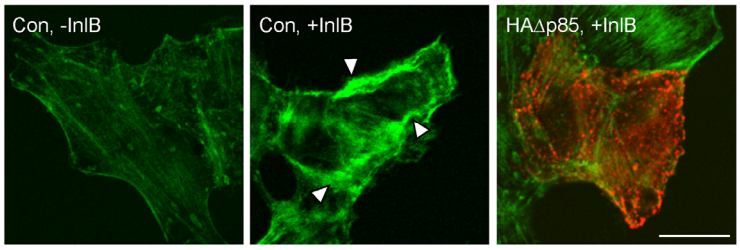

Figure 25. Dominant negative PI3K inhibits InlB-induced membrane ruffling. Control (Con), untransfected T47D/cMet cells and cells transiently expressing dominant negative HA-Δp85 were incubated in the absence (-) or presence (+) of 100 ng/ml InlB for 5 min at 37°C. The cells were fixed and stained with anti-HA antibodies to detect cells expressing HA-Δp85 (Red) and costained with FITC-labeled phalloidin to visualize actin filaments, and examined using confocal microscopy. Membrane ruffles enriched in actin filaments are indicated (arrows). Bar, 10 μm.

internalization of HGFR induced by InlB or HGF. Rather, comparable levels of internalized HGFR were detected in these cells following 15 min incubation at 37°C (Figure 23B). I confirmed my pharmacological studies using a dominant negative mutant of the regulatory p85 subunit (HA-Δp85) of PI3K. HA-Δp85 lacks the SH2 domain critical for the activation of the p110 catalytic subunit of PI3K (King, Mattaliano et al. 1997). Expression of HA-Δp85 did not inhibit internalization of Alexa-InlB or Tfn, consistent with my confocal and biotinylation studies using Wortmannin (Figure 23C). Control studies confirmed that expression of HA-Δp85 inhibited InlB-induced membrane ruffling (Figure 25), consistent with a previous study showing that InlB-induced ruffling was PI3K-dependent(Ireton, Payrastre et al. 1999). Thus, these data indicate that PI3K activity is not required for HGFR internalization.

InlB- and HGF- induced degradation of HGFR requires PI3K activity.

Several lines of evidence implicate PI3K activity in the sorting of internalized receptors for lysosomal degradation at the level of Multivesicular Bodies (MVBs) (Joly, Kazlauskas et al. 1995; Fernandez-Borja, Wubbolts et al. 1999). To examine the requirement of PI3K for HGFR degradation, T47D/cMet cells were treated with Alexa-InlB for 15 min at 37°C to trigger HGFR internalization into early endosomes. The cells were then chased in medium either containing or lacking Wortmannin for increasing time, and internalized Alexa-InlB was then quantified by confocal microscopy (Figure 26A). In the absence of Wortmannin, increasing chase periods resulted in a concomitant decrease in the relative amount of internalized Alexa-InlB, presumably due to ligand dissociation and/or degradation. Conversely, high levels of internalized Alexa-InlB were detected in the presence of Wortmannin, implying that PI3K is required for HGFR degradation (Figure 26B and 26C). To test this directly, cell surface proteins were biotinylated and HGFR was internalized into early endosomes by treatment with InlB or HGF at 37°C for 15 min. The cells were cooled to 4°C to halt endocytosis and washed with MesNa to remove residual surface biotin and ligand. The cells were then shifted to

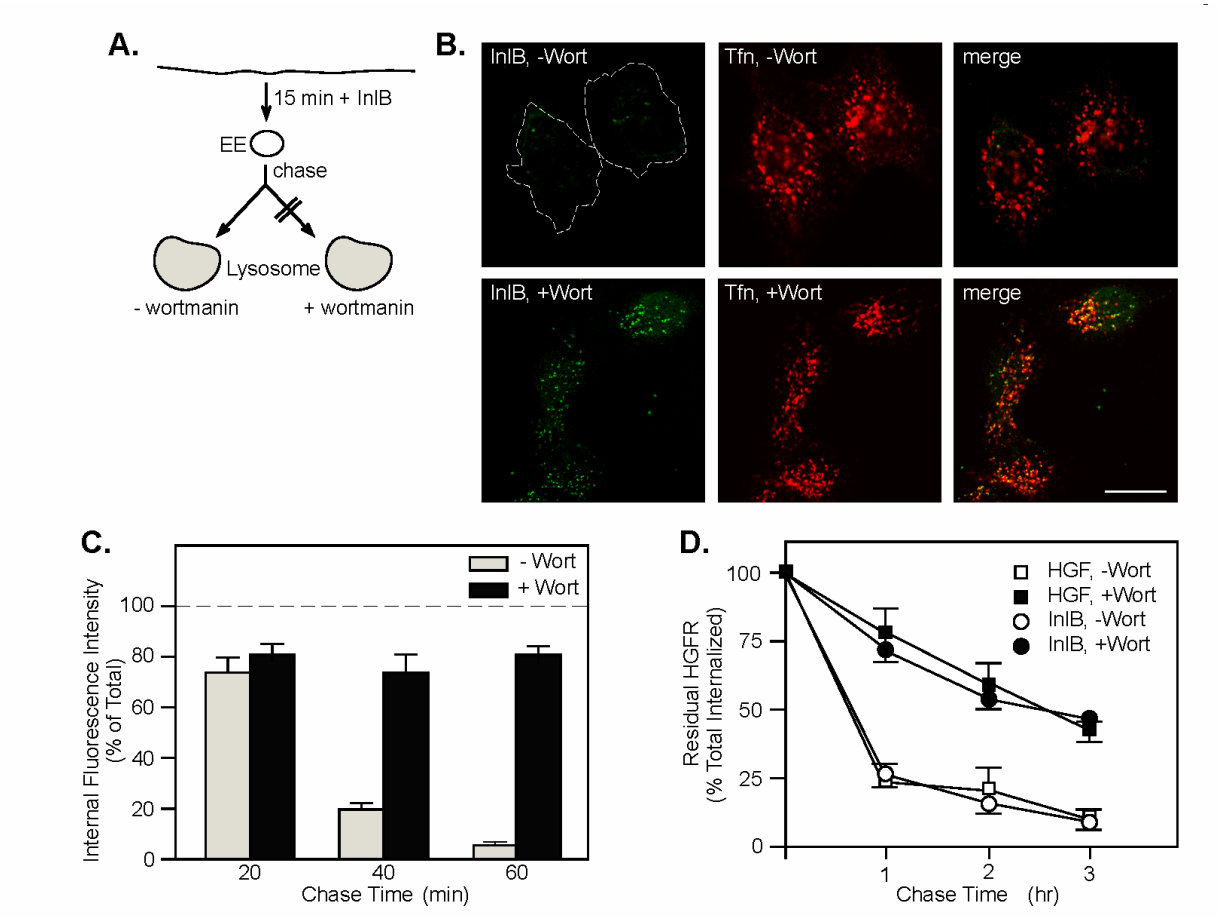

Figure 26. PI3K activity is required for degradation of HGFR. A, Schematic of the assay for examining HGFR degradation. B, Representative examples of T47D/cMet cells containing internalized Alexa488-labeled InlB (InlB) and Alexa594-labeled Tfn (Tfn) followed by a 60 min chase at 37°C in the presence (+) or absence (-) of 100 nM Wortmannin (Wort). Bar, 10 µm. C, The relative amount of internalized Alexa488-labeled InlB was quantified following a 20, 40 or 60 min chase at 37°C as described in the legend to Figure 6C. D, Surface biotinylated T47D/cMet cells were activated with HGF or InlB for 15 min at 37°C, and the relative amount of internalized HGFR in the presence (+) or absence (-) of Wortmannin (Wort) was determined as described in the legend to Figure 4.

37°C for 1, 2 or 3 hr in the presence or absence of Wortmannin, and the relative amount of internalized HGFR remaining after the chase was determined by streptavidin pull downs and Western blotting (Figure 26D). In the absence of Wortmannin, InlB and HGF were comparable in their ability to induce HGFR degradation; 10-12% of internalized HGFR was detected following a 3 hr chase under these conditions. Treatment of cells with Wortmannin resulted in a ~ four-fold increase in the relative amount of internalized HGFR, consistent with an inhibition in HGFR degradation. Taken together, these data indicate an important role for PI3K activity in ligand-induced HGFR degradation.

Hrs is required for InB-induced degradation of HGFR.

My data showing the importance of PI3K activity for HGFR degradation prompted us to examine the role of Hrs on receptor degradation. Hrs is an endosomal protein required for the sorting of ubiquitinated receptors targeted for lysosomal degradation (Lloyd, Atkinson et al. 2002; Raiborg, Bache et al. 2002). Hrs binds to endosomal membranes enriched in PI3P via its FYVE domain as well as a UIM domain that promotes binding to ubiquitinated proteins (Gruenberg and Stenmark 2004). To examine the requirement for Hrs in the lysosomal degradation of HGFR in response to InlB, I used small interfering RNAs (siRNAs) to specifically reduce the endogenous levels of Hrs in T47D/cMet cells. Under these conditions, the endogenous level of Hrs was undetectable by western analysis (Figure 27A). Conversely, comparable levels of EEA1, TfR, HGFR and actin were observed in untransfected control cells as well as T47D/cMet cells transfected with Hrs or control siRNAs, demonstrating the specificity of the siRNA treatment. In addition, I observed no difference in the internalization properties of HGFR or TfR in siRNA treated cells (data not shown).

To examine the requirement of Hrs on InlB-induced HGFR degradation, siRNA-treated T47D/cMet cells were incubated with Alexa-InlB to trigger receptor internalization into early endosomes. Control experiments revealed that InlB colocalized with EEA1 and internalized Tfn in early endosomes in both siRNA-treated and control cells (data not shown), indicating that HGFR internalization from the cell surface is not

altered by the absence of Hrs. Similarly, internalized Alexa-InlB colocalized with transiently expressed, GFP-labeled Hrs on punctate organelles in T47D/cMet cells (Figure 28). To monitor the role of Hrs on InlB-mediated HGFR degradation, the cells were subsequently chased for 60 min in the absence of ligand to initiate lysosomal transport and degradation of internalized InlB-HGFR complexes. In Hrs-depleted cells, the level of internalized Alexa-InlB was not altered during the chase period (Figure 27B). Moreover, internalized InlB remained colocalized with Tfn and EEA1 in enlarged EEA1 positive early endosomes in Hrs-depleted cells (Figure 27C). Conversely, a 90.7 ± 4.0% reduction in the relative amount of internalized InlB was observed in control cells following a 60 min chase. Comparable levels of internalized Tfn were detected in control or Hrs-depleted cells, demonstrating the specificity of the results. These data indicate that InlB-triggered degradation of internalized HGFR is dependent on Hrs.

DISCUSSION

Binding of InlB or HGF to HGFR activates PI3K signaling, a process that is required for membrane ruffling (Ireton, Payrastre et al. 1999), cell scattering (Royal and Park 1995; Shen, Naujokas et al. 2000) as well as InlB-mediated entry of *Listeria* (Ireton, Payrastre et al. 1996). In my studies, PI3K activity was not essential for HGFR internalization by InlB and HGF. Rather, Wortmannin treatment inhibited degradation of activated HGFR, consistent with a requirement for PI3K activity at this post-endocytic step. Under these conditions, the membrane association of Hrs was inhibited and internalized HGFR remained trapped in an endocytic compartment, consistent with early endosomes or MVBs. Similarly, depletion of Hrs inhibited InlB-induced degradation of HGFR. Proteasomal inhibitors were previously reported to block degradation of the HGFR (Jeffers, Taylor et al. 1997; Hammond, Urbe et al. 2001; Kermorgant, Zicha et al. 2003); however a direct role for the proteasome in mediating HGFR degradation has yet

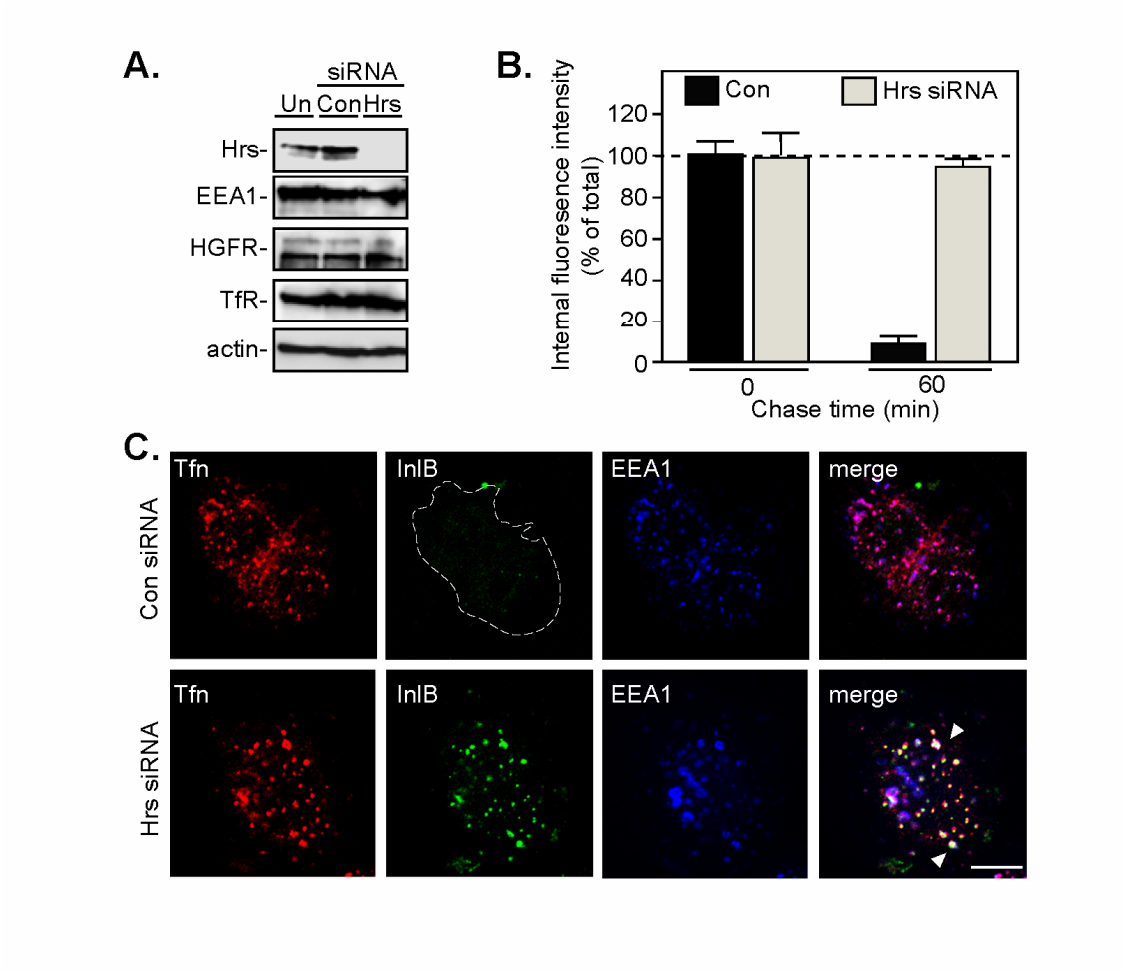

Figure 27. siRNA-mediated depletion of Hrs prevents InlB-induced HGFR degradation. A, Lysates from untransfected (UN), T47D/cMet cells transfected with control (Con) or Hrs siRNAs for 72 hrs were examined by western analysis using antibodies specific for Hrs, EEA1, HGFR, TfR and actin. B, Duplicate sets of control (Con) or Hrs-depleted (Hrs siRNA) T47D/cMet cells containing early endosomal Alexa488-labeled InlB were chased for 0 or 60 min at 37°C and the relative amount of remaining ligand quantified as described in the legend to Figure 9. The data are mean internal fluorescence intensity ± S.E. values from 2-3 experiments. C, Representative examples of Control (Con) or Hrs-depleted (Hrs siRNA) cells containing TR-Tfn (Tfn) and Alexa488-InlB (InlB) internalized into early endosomes, were chased for 60 min at 37°C to promote lysosomal HGFR degradation and then processed for confocal microscopy using antibodies specific for EEA1. Areas of colocalization between EEA1, Tfn and InlB appear white in the merged image (arrows). Bar, 10 μm.

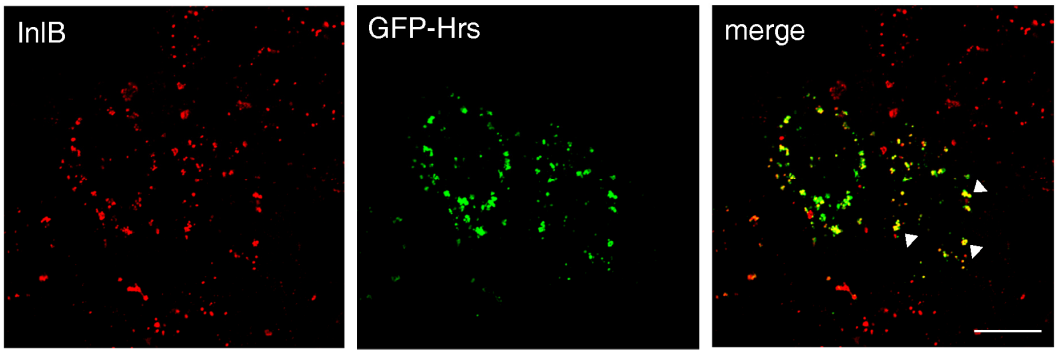

Figure 28. Internalized Alexa-InlB colocalizes with endosomal Hrs. T47D/cMet cells transiently expressing GFP-Hrs (Hrs) were allowed to internalize Alexa594-labeled InlB (InlB) for 15 min before processing for confocal microscopy. Areas of colocalization in the merged image appear yellow (arrowheads). Bar 10 μm.

to be demonstrated. In the case of EGFR, sustained receptor ubiquitination is required for efficient sorting of the EGFR for degradation. Proteasomal inhibitors block the translocation of EGFR from the outer limiting membrane to the inner membrane of MVBs, a sorting step that depends on the sequential recruitment of Hrs and the ESCRT protein complex to early endosomes (Longva, Blystad et al. 2002). Hrs associates with endosomal membranes enriched in PI3P and binds ubiquitinated proteins in early endosomes via a highly conserved Ubiquitin Interacting Motif (Gruenberg and Stenmark 2004). The ubiquitin binding properties of Hrs are essential for the sorting of the EGFR for lysosomal degradation. Since lysosomal degradation of the EGFR depends on proteasomal activity at the level of receptor sorting in MVBs, EGFR itself may not be a target for proteasomal degradation (Lloyd, Atkinson et al. 2002; Longva, Blystad et al. 2002). Thus, I postulate that PI3K likely regulates lysosomal rather than proteasomal degradation of HGFR, through a mechanism involving Hrs-mediated sorting of internalized HGFR in MVBs.

Consistent with my study Dr. Morag Park's group showed that ubiquitination of the HGFR is required for lysosomal degradation of the receptor and also for Hrs tyrosine phosphorylation (Abella, Peschard et al. 2005). In this study, they found that the

ubiquitination-deficient HGFR-Y1003 is internalized as wild type HGFR, but is inefficiently degraded. They also found that the HGFR-Y1003 is tumorigenic *in vivo*. Thus, the endocytic pathway of HGFR may normally function as a tumor suppressor pathway, when inactivated, sustained HGFR signaling could result in cellular transformation.

CHAPTER 5: GENERAL CONCLUSION AND FUTURE DIRECTION

GENERAL CONCLUSION

The RTK HGFR/cMet controls a program of invasive cell growth that combines proliferation with motogenic and morphogenic processes. These cell processes are important for organ development and regeneration, whereas deregulation of HGFR expression and/or downregulation in neoplastic tissues can contribute to cancer progression and metastasis. Ligand activation of HGFR leads to receptor downregulation via endocytosis and lysosomal degradation, the major mechanism for terminating HGFR signaling. Perturbation of HGFR trafficking within the endocytic pathway, either at the level of internalization or during sorting at the early endosome, leads to altered signaling outputs. Therefore, in addition to other well studied mechanisms for increased HGFR signaling in cancers, impaired HGFR trafficking is a novel mechanism for HGFR-induced cancer progression and metastasis. Somatic intronic mutations in HGFR that lead to an alternatively spliced transcript, were recently identified in lung cancer cell lines and patient tumor biopsy samples, that encodes a deletion of the juxtamembrane domain, resulting in the loss of Cbl E3-ligase binding (Kong-Beltran, Seshagiri et al. 2006). The resulting mutant receptor exhibits decreased ubiquitination and delayed down-regulation correlating with elevated HGFR expression in primary lung tumors but not patient matched normal tissue. Thus spontaneous HGFR mutations exist that contribute to cell transformation and cancer progress due to defects in HGFR endocytosis and degradation.

Understanding how HGFR trafficking normally inactivates HGFR signaling can reveal the novel cancer therapeutic targets. Until now, very little was known about HGFR trafficking, compared to the large body of knowledge regarding HGFR signaling. For example, prior to this thesis it was not clear which endocytic route HGFR used for internalization, or how the ligand activation of HGFR promoted the fast internalization of the receptor. It remains unclear how HGFR degradation is regulated and whether

deregulation of this process is a general mechanism for increased HGFR signaling in human cancers. The major goal of my dissertation was to lay the foundation for future studies examining the different mechanism leading to altered HGFR trafficking in human cancers, by determining and characterizing the mechanisms that normally function to regulate HGFR internalization and degradation.

Mutations that disrupt EGFR internalization have been detected in certain cancers (Schmidt, Furnari et al. 2003), indicating the important role of RTK internalization for termination of RTK signaling. In the first part of my dissertation I characterized the HGFR internalization pathway (chapter 2) (Li, Xiang et al. 2005). I found that the two known HGFR agonists InlB and HGF triggered fast internalization of HGFR with similar internalization rates (K_e= 0.2/min). Conversely, the unoccupied HGFR was internalized constitutively at a much slower rate. Multiple lines of evidence showed that ligand induced HGFR internalization was clathrin-mediated, and did not involve caveolae or lipid raft dependent routes. First, ligand activated HGFR was internalized into classic early endosomes after 5 min incubation at 37^{o}C, colocalizing with clathrin-derived early endosomal markers, including TfnR, EEA1, Rab5. Second, macropinocytosis was not involved in HGFR internalization, because dominant negative dynamin K44A blocked HGFR internalization. Third, HGFR internalization was inhibited by overexpression of dominant negative Eps15 or transfection of clathrin siRNA into cells, reagents which specifically disrupt clathrin-mediated endocytosis. Fourth, Arf6 [a GTPase involved in non clathrin-dependent mediated endocytosis (Naslavsky, Weigert et al. 2003)] did not regulate HGFR internalization. Fifth, HGFR internalization was not blocked by cell treatment with Nystatin, a cholesterol sequestration drug, which specifically blocked caveolae or lipid raft mediated endocytosis at a low concentration of 50μg/ml. Furthermore, internalized HGFR did not colocalize with transiently expressed GFP-caveolin 1, an established marker for caveolae and caveosome, indicating caveolae are not involved in HGFR internalization. In summary, my data revealed that soluble InlB and HGF are mechanically equivalent in triggering the fast internalization of HGFR, and that ligand induced HGFR internalization occurs primarily through clathrin-coated pits.

This study represents the first detailed characterization of HGFR internalization and also provides evidence that InlB is an efficient tool for studying HGFR endocytosis.

As a signaling receptor, ligand activation of HGFR leads to fast internalization through clathrin-coated pits followed by inactivation of HGFR via degradation. The second part of my dissertation was to define how ligand activation of HGFR was linked to the receptor internalization (chapter 3). I generated several stable cell lines using HGFR -/- cells (derived from HGFR-/- transgenic mouse kidney epithelial cells), that express wild type or mutant HGFR deficient in the recruitment of signaling adaptors YF-HGFR (Y1349.1356F) and a kinase dead mutant KD-HGFR (K1110A). Ligand induced HGFR internalization was blocked in YF-HGFR and KD-HGFR expressing cells, indicating an essential role of the multisubstrate docking site and tyrosine kinase activity respectively for HGFR internalization. I also demonstrated that HGFR ubiquitination and Cbl phosphorylation was reduced in YF-HGFR expressing cells, suggesting an important role for the receptor docking site in the HGFR and Cbl interaction. The requirement for the two major adaptor proteins Gab1 and Grb2 for HGFR internalization was determined. SiRNA depletion of Grb2 or inhibition of Grb2 recruitment using dominant negative mutants impaired ligand induced HGFR internalization. Conversely, genetic inactivation or siRNA depletion of Gab1 did not block HGFR internalization. Furthermore, HGFR internalization was reduced in N1358H-HGFR expressing cells, a mutation that specifically interfering with Grb2 but not Gab1 binding. This evidence indicated that Grb2 recruitment played an essential role in HGFR internalization. In addition to binding Gab1, Grb2 has been shown to constitutively interact with SOS and Cbl, and that these proteins can be recruited to HGFR indirectly via Grb2 binding (Chardin, Camonis et al. 1993; Meisner, Conway et al. 1995; Meisner and Czech 1995; Bardelli, Longati et al. 1997). Overexpression of dnRas S17N had no effect on HGFR internalization, ruling out a role for SOS pathway in receptor internalization. Cbl, as an adaptor protein, has been shown to be involved in HGFR internalization by the recruitment of the CIN85/Endophilin complex (Petrelli, Gilestro et al. 2002). In my studies, I demonstrated a requirement for the ubiquitination machinery and E3 ligase activity of Cbl in HGFR

internalization. In Grb2 depleted cells both HGFR ubiquitination and Cbl phosphorylation were reduced, indicating that in the absence of Grb2, HGFR and Cbl may not form a stable association. Therefore, I propose that Grb2 functions as a bridge to recruit Cbl E3 ligase activity to the HGFR, to trigger receptor internalization. Taken together, Grb2-mediated recruitment of the E3 ubiquitin ligase Cbl regulates clathrin-dependent endocytosis of the HGFR involving a role for the ubiquitination machinery. Whether ubiquitination of the HGFR or other receptor associated proteins is essential for the receptor internalization will require further studies.

The final part of my dissertation was to characterize the mechanism regarding HGFR degradation (chapter 4) (Li, Xiang et al. 2005). Prior reports showed that HGFR was polyubiquitinated and degraded through the proteasome pathway because proteasome inhibitors were shown to inhibit HGFR degradation (Jeffers, Taylor et al. 1997; Kermorgant, Zicha et al. 2003). Although proteasomal activity is involved in EGFR degradation, EGFR itself was not shown to be the target for proteasomal degradation (Longva, Blystad et al. 2002). Thus, I hypothesized that HGFR was likely degraded via lysosomes and not the proteasome. I tested this hypothesis by examining the role of Hrs and PI3K in HGFR degradation. Hrs is an important molecule in MVB formation and is important for the degradation of multiple monoubiquitinated receptors via the lysosomal pathway. Using an siRNA approach, I found that depletion of endogenous Hrs blocked ligand induced HGFR degradation and that the internalized ligand/receptor complex was retained in the enlarged endosomes. Hrs normally colocalizes on clathrin-derived early endosomes enriched in PI3P via its FYVE domain binding. Because PI3K is required for the generation of PI3P, I proposed that inhibition of PI3K would delay HGFR degradation by disrupting Hrs binding to early endosomes. And as I expected, inhibition of PI3K activity using specific inhibitors delayed ligand induced HGFR degradation. Thus, HGFR degradation occurs primarily through lysosomal degradation in a process dependent on Hrs and PI3K. Consistent with our finding, HGFR was shown to be multiple monoubiquitinated and that HGFR

ubiquitination was required for receptor degradation via lysosome (Abella, Peschard et al. 2005).

In summary, this dissertation provides the first detailed report regarding the mechanisms that regulate HGFR internalization and hence degradation. Clathrin-mediated HGFR internalization is dependent on Grb2-mediated recruitment of the E3 ligase Cbl, which links activated HGFR to the endocytic machinery. In addition to a role as a signaling adaptor important for HGFR signaling, my findings indicate a novel role for Grb2, as a regulator in HGFR endocytosis. Finally, I demonstrated that HGFR degradation occurs via the lysosomal pathway, involving Hrs and PI3K function.

FUTURE DIRECTION

Determine the role of Grb2 in HGFR internalization.

Clathrin-mediated endocytosis is a process that involves multiple steps: newly synthesized clathrin-coated pits, receptor recruitment into clathrin-coated pits, the budding of coated vesicles and receptor transport to early endosomes. Grb2 may function in a specific step leading to HGFR internalization, or couple distinct stages of the internalization process. For example, in addition to binding to Cbl, Grb2 has also been shown to interact with dynamin and N-WASP (Vidal, Goudreau et al. 1999; Carlier, Nioche et al. 2000). Dynamin is a GTPase, that functions to pinch off clathrin-coated pits from the plasma membrane. N-WASP, a molecule involved in actin polymerization, has been shown to be involved in clathrin-coated pit invagination and in EGFR internalization (Benesch, Polo et al. 2005). Cbl E3 ligase activity has been shown to be involved in the recruitment of EGFR to the clathrin-coated pits (Stang, Blystad et al. 2004). In addition, Grb2 was recently also shown to regulate EGF induced clathrin-coated pit formation (Johannessen, Pedersen et al. 2006). Thus, further studies are required to clarify the exact role of Grb2 in HGFR internalization.

How ubiquitination is involved in HGFR internalization?

I envisage three related models that explain the role of Grb2 recruited Cbl Ub ligase activity in HGFR internalization (described in chapter 3). A detailed description of each model for HGFR endocytosis follows.

Is HGFR ubiquitination required for HGFR internalization?

Until now, it remains unclear whether HGFR ubiquitination is required for receptor internalization. Ubiquitination of the Growth-Hormone Receptor and β-adrenergic receptor is not required for receptor internalization, whereas ubiquitination of EGFR is important for Eps15 mediated calthrin-dependent internalization (Govers, ten Broeke et al. 1999; Shenoy, McDonald et al. 2001; de Melker, van der Horst et al. 2004). Conversely, the in frame fusion of a single ubiquitin to the cytoplasmic domain of a truncated EGFR mutant (lacking a cytoplasmic domain) leads to constitutive internalization of the chimeric receptor via lipid rafts (Sigismund, Woelk et al. 2005). Recently, it was found that a mutant HGFR defective in Cbl binding (Y1003F-HGFR) was internalized at a similar rate as the wild type receptor (Abella, Peschard et al. 2005). However, this mutant was weakly ubiquitinated, making it difficult to rule out a role for HGFR ubiquitination for the internalization process. It is possible that HGFR internalization requires monoubiquitination at specific cytoplasmic lysine sites and that different lysine ubiquitination sites may interact with endocytic molecules important for receptor internalization (Eps15) or degradation (Hrs). Further studies will be required to determine the role of HGFR ubiquitination in the receptor endocytosis.

Is the ubiquitination of some receptor associated endocytic molecules required for HGFR internalization?

CIN85 is a substrate of the Cbl E3 ligase. CIN85 monoubiquitination has been shown to play a role in EGFR and HGFR downregulation (Haglund, Shimokawa et al. 2002; Petrelli, Gilestro et al. 2002). Additionally, monoubiquitination of Eps15 plays an important role in EGFR internalization (van Delft, Govers et al. 1997; Polo, Sigismund et al. 2002). Although Eps15 is the substrate of another E3 ligase Nedd4 (Polo, Sigismund et al. 2002), it has been shown that Cbl interacts directly with Nedd4. Cbl may function

in the recruitment of Nedd4 to HGFR. Subsequent ubiquitination of endocytic components associated with HGFR may facilitate the formation of a scaffolding complex, clustering HGFR into clathrin-coated pits for internalization.

Could Cbl regulate HGFR internalization by ubiquitination of a negative regulator which masks the internalization signal motif?

Internalization signal motifs likely exist in the cytoplasmic domain of HGFR, such as an AP-2 binding motif (di-leucine motif or tyrosine based motif) or clathrin binding motif. Further studies should be performed to identify these potential internalization motifs and their role in receptor internalization. I hypothesize that Cbl ubiquitination of an as yet unidentified protein may lead to the exposure of these internalization motifs, which are available to bind AP-2 or/and clathrin, leading to receptor internalization. In summary this study will provide the evidence that HGFR internalization may require multiple mechanisms.

Which molecule recruited to the docking site of HGFR may also be involved in receptor internalization in addition to Grb2?

My studies revealed that the internalization of a HGFR mutant harboring the mutation N1358H is reduced but not blocked. One possibility is that some other protein recruited to the docking site is also involved in HGFR internalization. One promising candidate is Src. Src has been shown to be involved in EGFR internalization by tyrosine phosphorylation of clathrin (Wilde, Beattie et al. 1999). Cbl can be tyrosine phosphorylated by Src, which is essential for Cbl E3 ligase activity and its interaction with CIN85 (Kassenbrock, Hunter et al. 2002; Soubeyran, Kowanetz et al. 2002). Thus, Src may be involved in HGFR internalization by tyrosine phosphorylation of Cbl. On the other hand, Src has also been shown to cause Cbl autoubiquitination and proteasomal degradation (Bao, Gur et al. 2003), which may interfere with receptor endocytosis and degradation, by downregulation of Cbl. Given the fact that Src activity is often increased in cancers, one mechanism for its oncogenic activity may be via interfering with HGFR trafficking through Cbl. Further studies on Src in HGFR trafficking may reveal a novel

mechanism linking the oncogenic activation of Src with the deregulation of HGFR in human cancers.

SUMMARY

Very recently, the somatic intronic mutations of HGFR kinase that lead to an alternatively spliced transcript were identified in a human lung tumor biopsy. The mutation resulted in a deletion of the juxtamembrane domain of HGFR resulting in the loss of Cbl E3-ligase binding (Kong-Beltran, Seshagiri et al. 2006). The mutant receptor exhibited decreased ubiquitination and delayed down-regulation correlating with elevated HGFR expression in primary tumors. I hypothesize that some naturally occurring HGFR mutations implicated may promote or enable cell transformation due to defects in HGFR endocytosis and degradation. The subsequent increase in HGFR signaling would lead to cell transformation, cell metastasis and branching morphogenesis. A detailed study of how impaired endocytosis and/or degradation of HGFR leads to tumorigenesis will contribute to a better understanding of the mechanisms leading to HGFR induced tumorigenesis and tumor metastasis, and reveal novel targets for metastatic cancer therapy.

REFERENCES

Abella, J. V., P. Peschard, et al. (2005). "Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation." Mol Cell Biol **25**(21): 9632-45.

Adams, A., J. M. Thorn, et al. (2000). "Intersectin, an adaptor protein involved in clathrin-mediated endocytosis, activates mitogenic signaling pathways." J Biol Chem **275**(35): 27414-20.

Aguilar, R. C. and B. Wendland (2005). "Endocytosis of membrane receptors: two pathways are better than one." Proc Natl Acad Sci U S A **102**(8): 2679-80.

Amyere, M., M. Mettlen, et al. (2002). "Origin, originality, functions, subversions and molecular signalling of macropinocytosis." Int J Med Microbiol **291**(6-7): 487-94.

Amyere, M., B. Payrastre, et al. (2000). "Constitutive macropinocytosis in oncogene-transformed fibroblasts depends on sequential permanent activation of phosphoinositide 3-kinase and phospholipase C." Mol Biol Cell **11**(10): 3453-67.

Anderson, R. G. (1998). "The caveolae membrane system." Annu Rev Biochem **67**: 199-225.

Babst, M., D. J. Katzmann, et al. (2002). "Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting." Dev Cell **3**(2): 271-82.

Babst, M., D. J. Katzmann, et al. (2002). "Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body." Dev Cell **3**(2): 283-9.

Bache, K. G., C. Raiborg, et al. (2003). "STAM and Hrs are subunits of a multivalent ubiquitin-binding complex on early endosomes." J Biol Chem **278**(14): 12513-21.

Bache, K. G., T. Slagsvold, et al. (2004). "Defective downregulation of receptor tyrosine kinases in cancer." EMBO J **23**(14): 2707-12.

Bao, J., G. Gur, et al. (2003). "Src promotes destruction of c-Cbl: implications for oncogenic synergy between Src and growth factor receptors." Proc Natl Acad Sci U S A **100**(5): 2438-43.

Barbieri, M. A., A. D. Kohn, et al. (1998). "Protein kinase B/akt and rab5 mediate Ras activation of endocytosis." J Biol Chem **273**(31): 19367-70.

Bardelli, A., P. Longati, et al. (1997). "Gab1 coupling to the HGF/Met receptor multifunctional docking site requires binding of Grb2 and correlates with the transforming potential." Oncogene **15**(25): 3103-11.

Bardelli, A., P. Longati, et al. (1999). "A peptide representing the carboxyl-terminal tail of the met receptor inhibits kinase activity and invasive growth." J Biol Chem **274**(41): 29274-81.

Bellusci, S., G. Moens, et al. (1994). "Creation of an hepatocyte growth factor/scatter factor autocrine loop in carcinoma cells induces invasive properties associated with increased tumorigenicity." Oncogene **9**(4): 1091-9.

Benesch, S., S. Polo, et al. (2005). "N-WASP deficiency impairs EGF internalization and actin assembly at clathrin-coated pits." J Cell Sci **118**(Pt 14): 3103-15.

Benmerah, A. (2004). "Endocytosis: signaling from endocytic membranes to the nucleus." Curr Biol **14**(8): R314-6.

Benmerah, A., M. Bayrou, et al. (1999). "Inhibition of clathrin-coated pit assembly by an Eps15 mutant." J Cell Sci **112 (Pt 9)**: 1303-11.

Benmerah, A., C. Lamaze, et al. (1998). "AP-2/Eps15 interaction is required for receptor-mediated endocytosis." J Cell Biol **140**(5): 1055-62.

Bierne, H. and P. Cossart (2002). "InlB, a surface protein of Listeria monocytogenes that behaves as an invasin and a growth factor." J Cell Sci **115**(Pt 17): 3357-67.

Bilodeau, P. S., J. L. Urbanowski, et al. (2002). "The Vps27p Hse1p complex binds ubiquitin and mediates endosomal protein sorting." Nat Cell Biol **4**(7): 534-9.

Birchmeier, C., W. Birchmeier, et al. (2003). "Met, metastasis, motility and more." Nat Rev Mol Cell Biol **4**(12): 915-25.

Blume-Jensen, P. and T. Hunter (2001). "Oncogenic kinase signalling." Nature **411**(6835): 355-65.

Boll, W., I. Rapoport, et al. (2002). "The mu2 subunit of the clathrin adaptor AP-2 binds to FDNPVY and YppO sorting signals at distinct sites." Traffic **3**(8): 590-600.

Braun, L., S. Dramsi, et al. (1997). "InlB: an invasion protein of Listeria monocytogenes with a novel type of surface association." Mol Microbiol **25**(2): 285-94.

Brodsky, F. M., C. Y. Chen, et al. (2001). "Biological basket weaving: formation and function of clathrin-coated vesicles." Annu Rev Cell Dev Biol **17**: 517-68.

Brown, F. D., A. L. Rozelle, et al. (2001). "Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic." J Cell Biol **154**(5): 1007-17.

Burke, P., K. Schooler, et al. (2001). "Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking." Mol Biol Cell **12**(6): 1897-910.

Carlier, M. F., P. Nioche, et al. (2000). "GRB2 links signaling to actin assembly by enhancing interaction of neural Wiskott-Aldrich syndrome protein (N-WASp) with actin-related protein (ARP2/3) complex." J Biol Chem **275**(29): 21946-52.

Ceresa, B. P. and S. L. Schmid (2000). "Regulation of signal transduction by endocytosis." Curr Opin Cell Biol **12**(2): 204-10.

Chan, P. C., Y. L. Chen, et al. (2003). "Src phosphorylates Grb2-associated binder 1 upon hepatocyte growth factor stimulation." J Biol Chem **278**(45): 44075-82.

Chardin, P., J. H. Camonis, et al. (1993). "Human Sos1: a guanine nucleotide exchange factor for Ras that binds to GRB2." Science **260**(5112): 1338-43.

Chen, H. and P. De Camilli (2005). "The association of epsin with ubiquitinated cargo along the endocytic pathway is negatively regulated by its interaction with clathrin." Proc Natl Acad Sci U S A **102**(8): 2766-71.

Chen, W., K. C. Kirkbride, et al. (2003). "Beta-arrestin 2 mediates endocytosis of type III TGF-beta receptor and down-regulation of its signaling." Science **301**(5638): 1394-7.

Claing, A., S. A. Laporte, et al. (2002). "Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins." Prog Neurobiol **66**(2): 61-79.

Conner, S. D. and S. L. Schmid (2003). "Regulated portals of entry into the cell." Nature **422**(6927): 37-44.

Cooper, C. S., M. Park, et al. (1984). "Molecular cloning of a new transforming gene from a chemically transformed human cell line." Nature **311**(5981): 29-33.

Copp, J., M. Marino, et al. (2003). "Multiple regions of internalin B contribute to its ability to turn on the Ras-mitogen-activated protein kinase pathway." J Biol Chem **278**(10): 7783-9.

Cossart, P., J. Pizarro-Cerda, et al. (2003). "Invasion of mammalian cells by Listeria monocytogenes: functional mimicry to subvert cellular functions." Trends Cell Biol **13**(1): 23-31.

de Melker, A. A., G. van der Horst, et al. (2004). "c-Cbl directs EGF receptors into an endocytic pathway that involves the ubiquitin-interacting motif of Eps15." J Cell Sci **117**(Pt 21): 5001-12.

de Melker, A. A., G. van der Horst, et al. (2004). "Ubiquitin ligase activity of c-Cbl guides the epidermal growth factor receptor into clathrin-coated pits by two distinct modes of Eps15 recruitment." J Biol Chem **279**(53): 55465-73.

Dean, M., M. Park, et al. (1987). "Activation of the met proto-oncogene in a human cell line." Haematol Blood Transfus **31**: 464-8.

Dean, M., M. Park, et al. (1987). "Characterization of the rearranged tpr-met oncogene breakpoint." Mol Cell Biol **7**(2): 921-4.

Di Fiore, P. P. and P. De Camilli (2001). "Endocytosis and signaling. an inseparable partnership." Cell **106**(1): 1-4.

Di Guglielmo, G. M., C. Le Roy, et al. (2003). "Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover." Nat Cell Biol **5**(5): 410-21.

Donaldson, J. G. (2003). "Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane." J Biol Chem **278**(43): 41573-6.

Ebner, R. and R. Derynck (1991). "Epidermal growth factor and transforming growth factor-alpha: differential intracellular routing and processing of ligand-receptor complexes." Cell Regul **2**(8): 599-612.

Farsad, K., N. Ringstad, et al. (2001). "Generation of high curvature membranes mediated by direct endophilin bilayer interactions." J Cell Biol **155**(2): 193-200.

Fazioli, F., L. Minichiello, et al. (1993). "eps15, a novel tyrosine kinase substrate, exhibits transforming activity." Mol Cell Biol **13**(9): 5814-28.

Fernandez-Borja, M., R. Wubbolts, et al. (1999). "Multivesicular body morphogenesis requires phosphatidyl-inositol 3-kinase activity." Curr Biol **9**(1): 55-8.

Ferracini, R., P. Longati, et al. (1991). "Identification of the major autophosphorylation site of the Met/hepatocyte growth factor receptor tyrosine kinase." J Biol Chem **266**(29): 19558-64.

Feshchenko, E. A., W. Y. Langdon, et al. (1998). "Fyn, Yes, and Syk phosphorylation sites in c-Cbl map to the same tyrosine residues that become phosphorylated in activated T cells." J Biol Chem **273**(14): 8323-31.

Fixman, E. D., T. M. Fournier, et al. (1996). "Pathways downstream of Shc and Grb2 are required for cell transformation by the tpr-Met oncoprotein." J Biol Chem **271**(22): 13116-22.

Floyd, S. and P. De Camilli (1998). "Endocytosis proteins and cancer: a potential link?" Trends Cell Biol **8**(8): 299-301.

Fong, C. W., H. F. Leong, et al. (2003). "Tyrosine phosphorylation of Sprouty2 enhances its interaction with c-Cbl and is crucial for its function." J Biol Chem **278**(35): 33456-64.

Ford, M. G., B. M. Pearse, et al. (2001). "Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes." Science **291**(5506): 1051-5.

Pelicci, G., S. Giordano, et al. (1995). "The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein." Oncogene **10**(8): 1631-8.

Fukazawa, T., S. Miyake, et al. (1996). "Tyrosine phosphorylation of Cbl upon epidermal growth factor (EGF) stimulation and its association with EGF receptor and downstream signaling proteins." J Biol Chem **271**(24): 14554-9.

Furge, K. A., D. Kiewlich, et al. (2001). "Suppression of Ras-mediated tumorigenicity and metastasis through inhibition of the Met receptor tyrosine kinase." Proc Natl Acad Sci U S A **98**(19): 10722-7.

Furge, K. A., Y. W. Zhang, et al. (2000). "Met receptor tyrosine kinase: enhanced signaling through adapter proteins." Oncogene **19**(49): 5582-9.

Gaidarov, I. and J. H. Keen (1999). "Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits." J Cell Biol **146**(4): 755-64.

Gao, C. F. and G. F. Vande Woude (2005). "HGF/SF-Met signaling in tumor progression." Cell Res **15**(1): 49-51.

Gommerman, J. L., R. Rottapel, et al. (1997). "Phosphatidylinositol 3-kinase and Ca2+ influx dependence for ligand-stimulated internalization of the c-Kit receptor." J Biol Chem **272**(48): 30519-25.

Goodman, O. B., Jr., J. G. Krupnick, et al. (1996). "Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor." Nature **383**(6599): 447-50.

Govers, R., T. ten Broeke, et al. (1999). "Identification of a novel ubiquitin conjugation motif, required for ligand-induced internalization of the growth hormone receptor." EMBO J **18**(1): 28-36.

Gruenberg, J. and H. Stenmark (2004). "The biogenesis of multivesicular endosomes." Nat Rev Mol Cell Biol **5**(4): 317-23.

Gu, H. and B. G. Neel (2003). "The "Gab" in signal transduction." Trends Cell Biol **13**(3): 122-30.

Gual, P., S. Giordano, et al. (2000). "Sustained recruitment of phospholipase C-gamma to Gab1 is required for HGF-induced branching tubulogenesis." Oncogene **19**(12): 1509-18.

Haglund, K., P. P. Di Fiore, et al. (2003). "Distinct monoubiquitin signals in receptor endocytosis." Trends Biochem Sci **28**(11): 598-603.

Haglund, K., N. Shimokawa, et al. (2002). "Cbl-directed monoubiquitination of CIN85 is involved in regulation of ligand-induced degradation of EGF receptors." Proc Natl Acad Sci U S A **99**(19): 12191-6.

Haglund, K., S. Sigismund, et al. (2003). "Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation." Nat Cell Biol **5**(5): 461-6.

Hall, A. B., N. Jura, et al. (2003). "hSpry2 is targeted to the ubiquitin-dependent proteasome pathway by c-Cbl." Curr Biol **13**(4): 308-14.

Hammond, D. E., S. Carter, et al. (2003). "Endosomal dynamics of Met determine signaling output." Mol Biol Cell **14**(4): 1346-54.

Hammond, D. E., S. Urbe, et al. (2001). "Down-regulation of MET, the receptor for hepatocyte growth factor." Oncogene **20**(22): 2761-70.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Harder, T., P. Scheiffele, et al. (1998). "Lipid domain structure of the plasma membrane revealed by patching of membrane components." J Cell Biol **141**(4): 929-42.

Hayes, S., A. Chawla, et al. (2002). "TGF beta receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2." J Cell Biol **158**(7): 1239-49.

Henley, J. R., E. W. Krueger, et al. (1998). "Dynamin-mediated internalization of caveolae." J Cell Biol **141**(1): 85-99.

Hime, G. R., M. P. Dhungat, et al. (1997). "D-Cbl, the Drosophila homologue of the c-Cbl proto-oncogene, interacts with the Drosophila EGF receptor in vivo, despite lacking C-terminal adaptor binding sites." Oncogene **14**(22): 2709-19.

Hinrichsen, L., J. Harborth, et al. (2003). "Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells." J Biol Chem **278**(46): 45160-70.

Hoeller, D., N. Crosetto, et al. (2006). "Regulation of ubiquitin-binding proteins by monoubiquitination." Nat Cell Biol **8**(2): 163-9.

Holgado-Madruga, M., D. R. Emlet, et al. (1996). "A Grb2-associated docking protein in EGF- and insulin-receptor signalling." Nature **379**(6565): 560-4.

Holgado-Madruga, M. and A. J. Wong (2003). "Gab1 is an integrator of cell death versus cell survival signals in oxidative stress." Mol Cell Biol **23**(13): 4471-84.

Huang, F. and A. Sorkin (2005). "Growth factor receptor binding protein 2-mediated recruitment of the RING domain of Cbl to the epidermal growth factor receptor is essential and sufficient to support receptor endocytosis." Mol Biol Cell **16**(3): 1268-81.

Hunter, S., E. A. Burton, et al. (1999). "Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase." J Biol Chem **274**(4): 2097-106.

Hyun, T. S. and T. S. Ross (2004). "HIP1: trafficking roles and regulation of tumorigenesis." Trends Mol Med **10**(4): 194-9.

Ireton, K., B. Payrastre, et al. (1996). "A role for phosphoinositide 3-kinase in bacterial invasion." Science **274**(5288): 780-2.

Ireton, K., B. Payrastre, et al. (1999). "The Listeria monocytogenes protein InlB is an agonist of mammalian phosphoinositide 3-kinase." J Biol Chem **274**(24): 17025-32.

Itoh, F., N. Divecha, et al. (2002). "The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-beta/Smad signalling." Genes Cells **7**(3): 321-31.

Itoh, M., Y. Yoshida, et al. (2000). "Role of Gab1 in heart, placenta, and skin development and growth factor- and cytokine-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation." Mol Cell Biol **20**(10): 3695-704.

Jeffers, M., M. S. Rao, et al. (1996). "Hepatocyte growth factor/scatter factor-Met signaling induces proliferation, migration, and morphogenesis of pancreatic oval cells." Cell Growth Differ **7**(12): 1805-13.

Jeffers, M., S. Rong, et al. (1996). "Autocrine hepatocyte growth factor/scatter factor-Met signaling induces transformation and the invasive/metastastic phenotype in C127 cells." Oncogene **13**(4): 853-6.

Jeffers, M., G. A. Taylor, et al. (1997). "Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway." Mol Cell Biol **17**(2): 799-808.

Jiang, X., F. Huang, et al. (2003). "Grb2 regulates internalization of EGF receptors through clathrin-coated pits." Mol Biol Cell **14**(3): 858-70.

Jiang, X. and A. Sorkin (2003). "Epidermal growth factor receptor internalization through clathrin-coated pits requires Cbl RING finger and proline-rich domains but not receptor polyubiquitylation." Traffic **4**(8): 529-43.

Johannessen, L. E., N. M. Pedersen, et al. (2006). "Activation of the epidermal growth factor (EGF) receptor induces formation of EGF receptor- and Grb2-containing clathrin-coated pits." Mol Cell Biol **26**(2): 389-401.

Joly, M., A. Kazlauskas, et al. (1995). "Phosphatidylinositol 3-kinase activity is required at a postendocytic step in platelet-derived growth factor receptor trafficking." J Biol Chem **270**(22): 13225-30.

Jozic, D., N. Cardenes, et al. (2005). "Cbl promotes clustering of endocytic adaptor proteins." Nat Struct Mol Biol **12**(11): 972-9.

Kalchman, M. A., H. B. Koide, et al. (1997). "HIP1, a human homologue of S. cerevisiae Sla2p, interacts with membrane-associated huntingtin in the brain." Nat Genet **16**(1): 44-53.

Kamei, T., T. Matozaki, et al. (1999). "Coendocytosis of cadherin and c-Met coupled to disruption of cell-cell adhesion in MDCK cells--regulation by Rho, Rac and Rab small G proteins." Oncogene **18**(48): 6776-84.

Kassenbrock, C. K., S. Hunter, et al. (2002). "Inhibition of Src family kinases blocks epidermal growth factor (EGF)-induced activation of Akt, phosphorylation of c-Cbl, and ubiquitination of the EGF receptor." J Biol Chem **277**(28): 24967-75.

Katz, M., K. Shtiegman, et al. (2002). "Ligand-independent degradation of epidermal growth factor receptor involves receptor ubiquitylation and Hgs, an adaptor whose ubiquitin-interacting motif targets ubiquitylation by Nedd4." Traffic **3**(10): 740-51.

Katzmann, D. J., M. Babst, et al. (2001). "Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I." Cell **106**(2): 145-55.

Keane, M. M., S. A. Ettenberg, et al. (1999). "cbl-3: a new mammalian cbl family protein." Oncogene **18**(22): 3365-75.

Keane, M. M., O. M. Rivero-Lezcano, et al. (1995). "Cloning and characterization of cbl-b: a SH3 binding protein with homology to the c-cbl proto-oncogene." Oncogene **10**(12): 2367-77.

Kempiak, S. J., H. Yamaguchi, et al. (2005). "A neural Wiskott-Aldrich Syndrome protein-mediated pathway for localized activation of actin polymerization that is regulated by cortactin." J Biol Chem **280**(7): 5836-42.

Kermorgant, S., D. Zicha, et al. (2003). "Protein kinase C controls microtubule-based traffic but not proteasomal degradation of c-Met." J Biol Chem **278**(31): 28921-9.

Khwaja, A., K. Lehmann, et al. (1998). "Phosphoinositide 3-kinase induces scattering and tubulogenesis in epithelial cells through a novel pathway." J Biol Chem **273**(30): 18793-801.

Kim, M., T. Tezuka, et al. (2004). "Cbl-c suppresses v-Src-induced transformation through ubiquitin-dependent protein degradation." Oncogene **23**(9): 1645-55.

King, W. G., M. D. Mattaliano, et al. (1997). "Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation." Mol Cell Biol **17**(8): 4406-18.

Kinlough, C. L., P. A. Poland, et al. (2004). "MUC1 membrane trafficking is modulated by multiple interactions." J Biol Chem **279**(51): 53071-7.

Kirkham, M., A. Fujita, et al. (2005). "Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles." J Cell Biol **168**(3): 465-76.

Kjelsberg, C., H. Sakurai, et al. (1997). "Met -/- kidneys express epithelial cells that chemotax and form tubules in response to EGF receptor ligands." Am J Physiol **272**(2 Pt 2): F222-8.

Klapper, L. N., H. Waterman, et al. (2000). "Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2." Cancer Res **60**(13): 3384-8.

Komada, M., R. Masaki, et al. (1997). "Hrs, a tyrosine kinase substrate with a conserved double zinc finger domain, is localized to the cytoplasmic surface of early endosomes." J Biol Chem **272**(33): 20538-44.

Kong-Beltran, M., S. Seshagiri, et al. (2006). "Somatic mutations lead to an oncogenic deletion of met in lung cancer." Cancer Res **66**(1): 283-9.

Korolchuk, V. I. and G. Banting (2002). "CK2 and GAK/auxilin2 are major protein kinases in clathrin-coated vesicles." Traffic **3**(6): 428-39.

Kurzchalia, T. V. and R. G. Parton (1999). "Membrane microdomains and caveolae." Curr Opin Cell Biol **11**(4): 424-31.

Lamaze, C., A. Dujeancourt, et al. (2001). "Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway." Mol Cell **7**(3): 661-71.

Langdon, W. Y., J. W. Hartley, et al. (1989). "v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas." Proc Natl Acad Sci U S A **86**(4): 1168-72.

Le, T. L., A. S. Yap, et al. (1999). "Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics." J Cell Biol **146**(1): 219-32.

Lee, J. H., S. U. Han, et al. (2000). "A novel germ line juxtamembrane Met mutation in human gastric cancer." Oncogene **19**(43): 4947-53.

Lee, P. S., Y. Wang, et al. (1999). "The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation." EMBO J **18**(13): 3616-28.

Lemmon, M. A. (2003). "Phosphoinositide recognition domains." Traffic **4**(4): 201-13.

Levkowitz, G., H. Waterman, et al. (1998). "c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor." Genes Dev **12**(23): 3663-74.

Li, N., G. S. Xiang, et al. (2005). "The Listeria protein internalin B mimics hepatocyte growth factor-induced receptor trafficking." Traffic **6**(6): 459-73.

Lisanti, M. P., Z. L. Tang, et al. (1993). "Caveolin forms a hetero-oligomeric protein complex that interacts with an apical GPI-linked protein: implications for the biogenesis of caveolae." J Cell Biol **123**(3): 595-604.

Lloyd, T. E., R. Atkinson, et al. (2002). "Hrs regulates endosome membrane invagination and tyrosine kinase receptor signaling in Drosophila." Cell **108**(2): 261-9.

Lock, L. S., M. M. Frigault, et al. (2003). "Grb2-independent recruitment of Gab1 requires the C-terminal lobe and structural integrity of the Met receptor kinase domain." J Biol Chem **278**(32): 30083-90.

Lock, L. S., I. Royal, et al. (2000). "Identification of an atypical Grb2 carboxyl-terminal SH3 domain binding site in Gab docking proteins reveals Grb2-dependent and -independent recruitment of Gab1 to receptor tyrosine kinases." J Biol Chem **275**(40): 31536-45.

Longva, K. E., F. D. Blystad, et al. (2002). "Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies." J Cell Biol **156**(5): 843-54.

Lowenstein, E. J., R. J. Daly, et al. (1992). "The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling." Cell **70**(3): 431-42.

Lu, Q., L. W. Hope, et al. (2003). "TSG101 interaction with HRS mediates endosomal trafficking and receptor down-regulation." Proc Natl Acad Sci U S A **100**(13): 7626-31.

Ma, P. C., G. Maulik, et al. (2003). "c-Met: structure, functions and potential for therapeutic inhibition." Cancer Metastasis Rev **22**(4): 309-25.

Magnusson, M. K., K. E. Meade, et al. (2001). "Rabaptin-5 is a novel fusion partner to platelet-derived growth factor beta receptor in chronic myelomonocytic leukemia." Blood **98**(8): 2518-25.

Maina, F., F. Casagranda, et al. (1996). "Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development." Cell **87**(3): 531-42.

Marechal, V., M. C. Prevost, et al. (2001). "Human immunodeficiency virus type 1 entry into macrophages mediated by macropinocytosis." J Virol **75**(22): 11166-77.

Marengere, L. E., C. Mirtsos, et al. (1997). "Proto-oncoprotein Vav interacts with c-Cbl in activated thymocytes and peripheral T cells." J Immunol **159**(1): 70-6.

Marmor, M. D. and Y. Yarden (2004). "Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases." Oncogene **23**(11): 2057-70.

Maroun, C. R., M. Holgado-Madruga, et al. (1999). "The Gab1 PH domain is required for localization of Gab1 at sites of cell-cell contact and epithelial morphogenesis downstream from the met receptor tyrosine kinase." Mol Cell Biol **19**(3): 1784-99.

Maroun, C. R., M. A. Naujokas, et al. (2000). "The tyrosine phosphatase SHP-2 is required for sustained activation of extracellular signal-regulated kinase and epithelial morphogenesis downstream from the met receptor tyrosine kinase." Mol Cell Biol **20**(22): 8513-25.

Martin, M., J. M. Del Valle, et al. (2005). "Identification of Grb2 as a novel binding partner of the signaling lymphocytic activation molecule-associated protein binding receptor CD229." J Immunol **174**(10): 5977-86.

Matsumoto, K. and T. Nakamura (2001). "Hepatocyte growth factor: renotropic role and potential therapeutics for renal diseases." Kidney Int **59**(6): 2023-38.

Maulik, G., A. Shrikhande, et al. (2002). "Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition." Cytokine Growth Factor Rev **13**(1): 41-59.

Meisner, H., B. R. Conway, et al. (1995). "Interactions of Cbl with Grb2 and phosphatidylinositol 3'-kinase in activated Jurkat cells." Mol Cell Biol **15**(7): 3571-8.

Meisner, H. and M. P. Czech (1995). "Coupling of the proto-oncogene product c-Cbl to the epidermal growth factor receptor." J Biol Chem **270**(43): 25332-5.

Michalopoulos, G. K. and M. C. DeFrances (1997). "Liver regeneration." Science **276**(5309): 60-6.

Mineo, C., G. N. Gill, et al. (1999). "Regulated migration of epidermal growth factor receptor from caveolae." J Biol Chem **274**(43): 30636-43.

Mishra, S. K., N. R. Agostinelli, et al. (2001). "Clathrin- and AP-2-binding sites in HIP1 uncover a general assembly role for endocytic accessory proteins." J Biol Chem **276**(49): 46230-6.

Mishra, S. K., P. A. Keyel, et al. (2002). "Disabled-2 exhibits the properties of a cargo-selective endocytic clathrin adaptor." EMBO J **21**(18): 4915-26.

Miyake, S., M. L. Lupher, Jr., et al. (1998). "The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor alpha." Proc Natl Acad Sci U S A **95**(14): 7927-32.

Moghul, A., L. Lin, et al. (1994). "Modulation of c-MET proto-oncogene (HGF receptor) mRNA abundance by cytokines and hormones: evidence for rapid decay of the 8 kb c-MET transcript." Oncogene **9**(7): 2045-52.

Montesano, R., G. Schaller, et al. (1991). "Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors." Cell **66**(4): 697-711.

Nabi, I. R. and P. U. Le (2003). "Caveolae/raft-dependent endocytosis." J Cell Biol **161**(4): 673-7.

Naga Prasad, S. V., L. S. Barak, et al. (2001). "Agonist-dependent recruitment of phosphoinositide 3-kinase to the membrane by beta-adrenergic receptor kinase 1. A role in receptor sequestration." J Biol Chem **276**(22): 18953-9.

Nakamura, T., S. Mizuno, et al. (2000). "Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF." J Clin Invest **106**(12): 1511-9.

Naldini, L., E. Vigna, et al. (1991). "Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-MET." Oncogene **6**(4): 501-4.

Naslavsky, N., R. Weigert, et al. (2003). "Convergence of non-clathrin- and clathrin-derived endosomes involves Arf6 inactivation and changes in phosphoinositides." Mol Biol Cell **14**(2): 417-31.

Niemann, C., V. Brinkmann, et al. (1998). "Reconstitution of mammary gland development in vitro: requirement of c-met and c-erbB2 signaling for branching and alveolar morphogenesis." J Cell Biol **143**(2): 533-45.

Olivero, M., G. Valente, et al. (1999). "Novel mutation in the ATP-binding site of the MET oncogene tyrosine kinase in a HPRCC family." Int J Cancer **82**(5): 640-3.

Owen, D. J. and P. R. Evans (1998). "A structural explanation for the recognition of tyrosine-based endocytotic signals." Science **282**(5392): 1327-32.

Owen, D. J., Y. Vallis, et al. (2000). "The structure and function of the beta 2-adaptin appendage domain." EMBO J **19**(16): 4216-27.

Palacios, F. and C. D'Souza-Schorey (2003). "Modulation of Rac1 and ARF6 activation during epithelial cell scattering." J Biol Chem **278**(19): 17395-400.

Palacios, F., L. Price, et al. (2001). "An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration." EMBO J **20**(17): 4973-86.

Park, M., M. Dean, et al. (1986). "Mechanism of met oncogene activation." Cell **45**(6): 895-904.

Park, M., M. Gonzatti-Haces, et al. (1986). "The met oncogene: a new member of the tyrosine kinase family and a marker for cystic fibrosis." Cold Spring Harb Symp Quant Biol **51 Pt 2**: 967-75.

Park, W. S., S. M. Dong, et al. (1999). "Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas." Cancer Res **59**(2): 307-10.

Paterson, A. D., R. G. Parton, et al. (2003). "Characterization of E-cadherin endocytosis in isolated MCF-7 and chinese hamster ovary cells: the initial fate of unbound E-cadherin." J Biol Chem **278**(23): 21050-7.

Pelicci, G., S. Giordano, et al. (1995). "The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein." Oncogene **10**(8): 1631-8.

Pelkmans, L. and A. Helenius (2003). "Insider information: what viruses tell us about endocytosis." Curr Opin Cell Biol **15**(4): 414-22.

Pelkmans, L., J. Kartenbeck, et al. (2001). "Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER." Nat Cell Biol **3**(5): 473-83.

Penengo, L., C. Rubin, et al. (2003). "c-Cbl is a critical modulator of the Ron tyrosine kinase receptor." Oncogene **22**(24): 3669-79.

Pennacchietti, S., P. Michieli, et al. (2003). "Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene." Cancer Cell **3**(4): 347-61.

Peschard, P., T. M. Fournier, et al. (2001). "Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein." Mol Cell **8**(5): 995-1004.

Petiot, A., J. Faure, et al. (2003). "PI3P signaling regulates receptor sorting but not transport in the endosomal pathway." J Cell Biol **162**(6): 971-9.

Petrelli, A., G. F. Gilestro, et al. (2002). "The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met." Nature **416**(6877): 187-90.

Pike, L. J. and L. Casey (1996). "Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains." J Biol Chem **271**(43): 26453-6.

Polo, S., S. Sigismund, et al. (2002). "A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins." Nature **416**(6879): 451-5.

Ponzetto, C., A. Bardelli, et al. (1994). "A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family." Cell **77**(2): 261-71.

Ponzetto, C., Z. Zhen, et al. (1996). "Specific uncoupling of GRB2 from the Met receptor. Differential effects on transformation and motility." J Biol Chem **271**(24): 14119-23.

Radhakrishna, H., O. Al-Awar, et al. (1999). "ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements." J Cell Sci **112 (Pt 6)**: 855-66.

Raiborg, C., K. G. Bache, et al. (2002). "Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes." Nat Cell Biol **4**(5): 394-8.

Raiborg, C., K. G. Bache, et al. (2001). "Hrs recruits clathrin to early endosomes." EMBO J **20**(17): 5008-21.

Raiborg, C., B. Bremnes, et al. (2001). "FYVE and coiled-coil domains determine the specific localisation of Hrs to early endosomes." J Cell Sci **114**(Pt 12): 2255-63.

Rao, D. S., S. V. Bradley, et al. (2003). "Altered receptor trafficking in Huntingtin Interacting Protein 1-transformed cells." Cancer Cell **3**(5): 471-82.

Rao, D. S., T. S. Hyun, et al. (2002). "Huntingtin-interacting protein 1 is overexpressed in prostate and colon cancer and is critical for cellular survival." J Clin Invest **110**(3): 351-60.

Rapoport, I., Y. C. Chen, et al. (1998). "Dileucine-based sorting signals bind to the beta chain of AP-1 at a site distinct and regulated differently from the tyrosine-based motif-binding site." EMBO J **17**(8): 2148-55.

Razani, B. and M. P. Lisanti (2001). "Caveolins and caveolae: molecular and functional relationships." Exp Cell Res **271**(1): 36-44.

Ricotta, D., S. D. Conner, et al. (2002). "Phosphorylation of the AP2 mu subunit by AAK1 mediates high affinity binding to membrane protein sorting signals." J Cell Biol **156**(5): 791-5.

Rodionov, D. G. and O. Bakke (1998). "Medium chains of adaptor complexes AP-1 and AP-2 recognize leucine-based sorting signals from the invariant chain." J Biol Chem **273**(11): 6005-8.

Rodrigues, G. A., M. Falasca, et al. (2000). "A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling." Mol Cell Biol **20**(4): 1448-59.

Rodrigues, G. A. and M. Park (1993). "Dimerization mediated through a leucine zipper activates the oncogenic potential of the met receptor tyrosine kinase." Mol Cell Biol **13**(11): 6711-22.

Rodrigues, G. A. and M. Park (1994). "Autophosphorylation modulates the kinase activity and oncogenic potential of the Met receptor tyrosine kinase." Oncogene **9**(7): 2019-27.

Roepstorff, K., P. Thomsen, et al. (2002). "Sequestration of epidermal growth factor receptors in non-caveolar lipid rafts inhibits ligand binding." J Biol Chem **277**(21): 18954-60.

Rong, S., M. Bodescot, et al. (1992). "Tumorigenicity of the met proto-oncogene and the gene for hepatocyte growth factor." Mol Cell Biol **12**(11): 5152-8.

Rong, S., S. Segal, et al. (1994). "Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation." Proc Natl Acad Sci U S A **91**(11): 4731-5.

Rosario, M. and W. Birchmeier (2003). "How to make tubes: signaling by the Met receptor tyrosine kinase." Trends Cell Biol **13**(6): 328-35.

Ros-Baro, A., C. Lopez-Iglesias, et al. (2001). "Lipid rafts are required for GLUT4 internalization in adipose cells." Proc Natl Acad Sci U S A **98**(21): 12050-5.

Ross, T. S., O. A. Bernard, et al. (1998). "Fusion of Huntingtin interacting protein 1 to platelet-derived growth factor beta receptor (PDGFbetaR) in chronic myelomonocytic leukemia with t(5;7)(q33;q11.2)." Blood **91**(12): 4419-26.

Rothberg, K. G., J. E. Heuser, et al. (1992). "Caveolin, a protein component of caveolae membrane coats." Cell **68**(4): 673-82.

Royal, I. and M. Park (1995). "Hepatocyte growth factor-induced scatter of Madin-Darby canine kidney cells requires phosphatidylinositol 3-kinase." J Biol Chem **270**(46): 27780-7.

Rubin, C., V. Litvak, et al. (2003). "Sprouty fine-tunes EGF signaling through interlinked positive and negative feedback loops." Curr Biol **13**(4): 297-307.

Sachs, M., H. Brohmann, et al. (2000). "Essential role of Gab1 for signaling by the c-Met receptor in vivo." J Cell Biol **150**(6): 1375-84.

Schmidt, C., F. Bladt, et al. (1995). "Scatter factor/hepatocyte growth factor is essential for liver development." Nature **373**(6516): 699-702.

Schmidt, L., K. Junker, et al. (1998). "Two North American families with hereditary papillary renal carcinoma and identical novel mutations in the MET proto-oncogene." Cancer Res **58**(8): 1719-22.

Schmidt, M. H., F. B. Furnari, et al. (2003). "Epidermal growth factor receptor signaling intensity determines intracellular protein interactions, ubiquitination, and internalization." Proc Natl Acad Sci U S A **100**(11): 6505-10.

Seveau, S., H. Bierne, et al. (2004). "Role of lipid rafts in E-cadherin-- and HGF-R/Met--mediated entry of Listeria monocytogenes into host cells." J Cell Biol **166**(5): 743-53.

Sever, S., H. Damke, et al. (2000). "Dynamin:GTP controls the formation of constricted coated pits, the rate limiting step in clathrin-mediated endocytosis." J Cell Biol **150**(5): 1137-48.

She, H. Y., S. Rockow, et al. (1997). "Wiskott-Aldrich syndrome protein is associated with the adapter protein Grb2 and the epidermal growth factor receptor in living cells." Mol Biol Cell **8**(9): 1709-21.

Shen, Y., M. Naujokas, et al. (2000). "InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase." Cell **103**(3): 501-10.

Shenoy, S. K., P. H. McDonald, et al. (2001). "Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin." Science **294**(5545): 1307-13.

Shih, S. C., D. J. Katzmann, et al. (2002). "Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis." Nat Cell Biol **4**(5): 389-93.

Sigismund, S., T. Woelk, et al. (2005). "Clathrin-independent endocytosis of ubiquitinated cargos." Proc Natl Acad Sci U S A **102**(8): 2760-5.

Signoret, N., L. Hewlett, et al. (2005). "Agonist-induced endocytosis of CC chemokine receptor 5 is clathrin dependent." Mol Biol Cell **16**(2): 902-17.

Singh, R. D., V. Puri, et al. (2003). "Selective caveolin-1-dependent endocytosis of glycosphingolipids." Mol Biol Cell **14**(8): 3254-65.

Slepnev, V. I., G. C. Ochoa, et al. (2000). "Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1 and disruption of clathrin coat function by amphiphysin fragments comprising these sites." J Biol Chem **275**(23): 17583-9.

Solomaha, E., F. L. Szeto, et al. (2005). "Kinetics of Src homology 3 domain association with the proline-rich domain of dynamins: specificity, occlusion, and the effects of phosphorylation." J Biol Chem **280**(24): 23147-56.

Sorkin, A., M. Mazzotti, et al. (1996). "Epidermal growth factor receptor interaction with clathrin adaptors is mediated by the Tyr974-containing internalization motif." J Biol Chem **271**(23): 13377-84.

Soubeyran, P., A. Barac, et al. (2003). "Cbl-ArgBP2 complex mediates ubiquitination and degradation of c-Abl." Biochem J **370**(Pt 1): 29-34.

Soubeyran, P., K. Kowanetz, et al. (2002). "Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors." Nature **416**(6877): 183-7.

Stang, E., F. D. Blystad, et al. (2004). "Cbl-dependent ubiquitination is required for progression of EGF receptors into clathrin-coated pits." Mol Biol Cell **15**(8): 3591-604.

Subtil, A., I. Gaidarov, et al. (1999). "Acute cholesterol depletion inhibits clathrin-coated pit budding." Proc Natl Acad Sci U S A **96**(12): 6775-80.

Tall, G. G., M. A. Barbieri, et al. (2001). "Ras-activated endocytosis is mediated by the Rab5 guanine nucleotide exchange activity of RIN1." Dev Cell **1**(1): 73-82.

Tanyi, J., K. Tory, et al. (1999). "Evaluation of the tyrosine kinase domain of the Met proto-oncogene in sporadic ovarian carcinomas*." Pathol Oncol Res **5**(3): 187-91.

Thien, C. B. and W. Y. Langdon (2005). "Negative regulation of PTK signalling by Cbl proteins." Growth Factors **23**(2): 161-7.

Thien, C. B., F. Walker, et al. (2001). "RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation." Mol Cell **7**(2): 355-65.

Tong, X. K., N. K. Hussain, et al. (2000). "Intersectin can regulate the Ras/MAP kinase pathway independent of its role in endocytosis." J Biol Chem **275**(38): 29894-9.

Tong, X. K., N. K. Hussain, et al. (2000). "The endocytic protein intersectin is a major binding partner for the Ras exchange factor mSos1 in rat brain." EMBO J **19**(6): 1263-71.

Uehara, Y., O. Minowa, et al. (1995). "Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor." Nature **373**(6516): 702-5.

van Delft, S., R. Govers, et al. (1997). "Epidermal growth factor induces ubiquitination of Eps15." J Biol Chem **272**(22): 14013-6.

Veiga, E. and P. Cossart (2005). "Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells." Nat Cell Biol **7**(9): 894-900.

Vidal, M., N. Goudreau, et al. (1999). "Molecular and cellular analysis of Grb2 SH3 domain mutants: interaction with Sos and dynamin." J Mol Biol **290**(3): 717-30.

Vidal, M., J. L. Montiel, et al. (1998). "Differential interactions of the growth factor receptor-bound protein 2 N-SH3 domain with son of sevenless and dynamin. Potential role in the Ras-dependent signaling pathway." J Biol Chem **273**(9): 5343-8.

Wang, Z. and M. F. Moran (1996). "Requirement for the adapter protein GRB2 in EGF receptor endocytosis." Science **272**(5270): 1935-9.

Wanker, E. E., C. Rovira, et al. (1997). "HIP-I: a huntingtin interacting protein isolated by the yeast two-hybrid system." Hum Mol Genet **6**(3): 487-95.

Warren, R. A., F. A. Green, et al. (1997). "Saturation of the endocytic pathway for the transferrin receptor does not affect the endocytosis of the epidermal growth factor receptor." J Biol Chem **272**(4): 2116-21.

Warren, R. A., F. A. Green, et al. (1998). "Distinct saturable pathways for the endocytosis of different tyrosine motifs." J Biol Chem **273**(27): 17056-63.

Watarai, M., I. Derre, et al. (2001). "Legionella pneumophila is internalized by a macropinocytotic uptake pathway controlled by the Dot/Icm system and the mouse Lgn1 locus." J Exp Med **194**(8): 1081-96.

Waterman, H., M. Katz, et al. (2002). "A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling." EMBO J **21**(3): 303-13.

Waterman, H., I. Sabanai, et al. (1998). "Alternative intracellular routing of ErbB receptors may determine signaling potency." J Biol Chem **273**(22): 13819-27.

Weidner, K. M., S. Di Cesare, et al. (1996). "Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis." Nature **384**(6605): 173-6.

Weissman, A. M. (2001). "Themes and variations on ubiquitylation." Nat Rev Mol Cell Biol **2**(3): 169-78.

Wells, A., J. B. Welsh, et al. (1990). "Ligand-induced transformation by a noninternalizing epidermal growth factor receptor." Science **247**(4945): 962-4.

Wilde, A., E. C. Beattie, et al. (1999). "EGF receptor signaling stimulates SRC kinase phosphorylation of clathrin, influencing clathrin redistribution and EGF uptake." Cell **96**(5): 677-87.

Wiley, H. S. (1988). "Anomalous binding of epidermal growth factor to A431 cells is due to the effect of high receptor densities and a saturable endocytic system." J Cell Biol **107**(2): 801-10.

Wilhelmsen, K., S. Burkhalter, et al. (2002). "C-Cbl binds the CSF-1 receptor at tyrosine 973, a novel phosphorylation site in the receptor's carboxy-terminus." Oncogene **21**(7): 1079-89.

Wolf, A. A., Y. Fujinaga, et al. (2002). "Uncoupling of the cholera toxin-G(M1) ganglioside receptor complex from endocytosis, retrograde Golgi trafficking, and downstream signal transduction by depletion of membrane cholesterol." J Biol Chem **277**(18): 16249-56.

Wong, A., B. Lamothe, et al. (2002). "FRS2 alpha attenuates FGF receptor signaling by Grb2-mediated recruitment of the ubiquitin ligase Cbl." Proc Natl Acad Sci U S A **99**(10): 6684-9.

Wong, E. S., C. W. Fong, et al. (2002). "Sprouty2 attenuates epidermal growth factor receptor ubiquitylation and endocytosis, and consequently enhances Ras/ERK signalling." EMBO J **21**(18): 4796-808.

Wu, H., D. A. Windmiller, et al. (2003). "YXXM motifs in the PDGF-beta receptor serve dual roles as phosphoinositide 3-kinase binding motifs and tyrosine-based endocytic sorting signals." J Biol Chem **278**(42): 40425-8.

Wu, W. J., S. Tu, et al. (2003). "Activated Cdc42 sequesters c-Cbl and prevents EGF receptor degradation." Cell **114**(6): 715-25.

Wymann, M. P., M. Zvelebil, et al. (2003). "Phosphoinositide 3-kinase signalling--which way to target?" Trends Pharmacol Sci **24**(7): 366-76.

Yamabhai, M. and R. G. Anderson (2002). "Second cysteine-rich region of epidermal growth factor receptor contains targeting information for caveolae/rafts." J Biol Chem **277**(28): 24843-6.

Yamazaki, T., K. Zaal, et al. (2002). "Role of Grb2 in EGF-stimulated EGFR internalization." J Cell Sci **115**(Pt 9): 1791-802.

Yao, D., M. Ehrlich, et al. (2002). "Transforming growth factor-beta receptors interact with AP2 by direct binding to beta2 subunit." Mol Biol Cell **13**(11): 4001-12.

Yokouchi, M., T. Wakioka, et al. (1999). "APS, an adaptor protein containing PH and SH2 domains, is associated with the PDGF receptor and c-Cbl and inhibits PDGF-induced mitogenesis." Oncogene **18**(3): 759-67.

Yoon, C. H., J. Lee, et al. (1995). "Similarity of sli-1, a regulator of vulval development in C. elegans, to the mammalian proto-oncogene c-cbl." Science **269**(5227): 1102-5.

Yudowski, G. A., R. Efendiev, et al. (2000). "Phosphoinositide-3 kinase binds to a proline-rich motif in the Na+, K+-ATPase alpha subunit and regulates its trafficking." Proc Natl Acad Sci U S A **97**(12): 6556-61.

Zhang, J., S. S. Ferguson, et al. (1996). "Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization." J Biol Chem **271**(31): 18302-5.

VITA

Ning Li was born to parents Guimin Wang and Junhan Li in Jining, China on March 16, 1973. She graduated from FuDan University Medical Center (formerly Shanghai Medical University) in 1995, receiving her Bachelor Degree of Medicine. She then attended graduate school and received her Master Degree of Medicine in Pharmacology. She applied her skills at the Chinese Academy of Sciences, Shanghai Institute of Material Medical working as a research scientist. During this time she realized she should go abroad to learn new advanced technologies and sciences. She was accepted in the Ph.D. program in biomedical sciences at University of Texas Medical Branch. She joined Dr. Lisa Elferink's lab to help establish a new project exploring the Hepatocyte Growth Factor Receptor trafficking and signaling mechanisms.

Ning gained significant working experience while working as a small project leader at Shanghai Institute of Material Medical. She also obtained considerable teaching experience while attending FuDan University Medical Center graduate school, teaching medical school students a pharmacology laboratory course. While in Dr. Lisa Elferink's lab, she also helped to mentor several rotation students and summer students and gained valuable experience in manuscript preparation and grant writing.

Education

09/1990-08/1995	FuDan University Medical Center, China
	(Formerly Shanghai Medical University)
	Bachelor Degree of Medicine
9/1995-08/1998	FuDan University Medical Center, China
	(Formerly Shanghai Medical University)
	Master Degree of Medicine in Pharmacology

Publications

1. **Li, N.**, G. S. Xiang, et al. (2005). "The Listeria protein internalin B mimics hepatocyte growth factor-induced receptor trafficking." Traffic **6**(6): 459-73.

2. **Li, N.**, K. Ireton, et al. (2006). "Grb2-mediated recruitment of the ubiquitin ligase Cbl regulates clathrin-dependent endocytosis of the cMet receptor." Oncogene (submitted)

Abstracts

1. **Li N.,** Ireton K., Elferink L. A. "The signaling adaptor Grb2 is required for cMet internalization." The American Association for Cancer Research 97th Annual Meeting, Washington DC, 2006.

2. **Li N.,** Ireton K., Elferink L. A. "The signaling adaptor Grb2 is required for cMet internalization." The American Society for Cell Biology 45th Annual Meeting, San Francisco, CA. 2005.

3. Daniel Gomez, Kristen Hill, **Ning Li,** Lisa Elferink. "The role of Cbl in clathrin-mediated endocytosis of cMet." Annual Biomedical Research Conference for Minority Students, Atlanta, GA, 2005. Also presented in Summer Undergraduate Research Program, Poster Section, UTMB, Galveston, TX, 2005

4. **Li, N.,** Ireton K., Elferink, L. A. "*Listeria* Surface Protein InlB Mimics HGF-triggered Endocytic Trafficking of the Receptor Tyrosine Kinase cMet." Sealy Center of Molecular Biology & Sealy Center of Cancer Cell Biology Annual Science Forum, Galveston, TX, 2005.

5. **Li, N.,** Ireton K., Elferink, L. A. "*Listeria* Surface Protein InlB Mimics HGF-triggered Endocytic Trafficking of the Receptor Tyrosine Kinase cMet." The American Society for Cell Biology 44th Annual Meeting, Washington, DC, 2004.

6. **Li, N.,** Elferink, L. A. "Endosomal EGFR activates ERK and JNK pathway." National Science Research Forum, Galveston, TX, 2003.