HUNTINGTIN INTERACTING PROTEIN 1: INTERACTIONS WITH RECEPTOR TYROSINE KINASES

by

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ABSTRACT

The receptor tyrosine kinase (RTK) signaling pathway is strongly activated in most forms of cancer. As a result, manipulation of the RTK pathway is a common means of promoting oncogenesis. Recently, proteins in the RTK endocytosis pathway have been added to the list of oncoproteins that affect RTK signaling. Among these proteins is Huntingtin Interacting Protein 1 (HIP1), an endocytic adaptor protein that interacts with clathrin, actin, adaptor protein 2 (AP2) and cargo proteins, including RTKs. HIP1 is expressed at high levels in many forms of cancer in a manner that also correlates with RTK overexpression. Additionally, HIP1 overexpression transforms fibroblasts by increasing RTK expression to enable anchorage-independent growth. Though HIP1 has been found to interact with and stabilize multiple RTKs following stimulation, the precise role(s) of HIP1 during the endocytic process remain unclear.

This thesis examines the interaction between HIP1 and RTKs. First, high HIP1 levels were found in a rare, deadly, form of cancer, Merkel cell carcinoma (MCC), which also expresses the RTK c-Kit at high levels. Patients with metastatic MCC also demonstrated high levels of circulating antibody produced against HIP1. Additionally, HIP1 was found to interact with c-Kit and also stabilize this receptor following stem cell factor (SCF) ligand stimulation. These data collectively indicate that targeting HIP1 in MCC may improve patient prognosis. To further investigate a means of targeting HIP1 interaction with RTKs, we found that the EGFR kinase domain was necessary for HIP1 association with this receptor. HIP1 was also found to be tyrosine phosphorylated when

HIP1 and EGFR are co-expressed. This phosphorylation is maintained for 15 to 30 minutes after stimulation, at which time HIP1 and EGFR localize to Rab5 sorting endosomes. Furthermore, a novel form of EGFR was identified that strongly interacts with HIP1 and structurally resembles the oncoprotein EGFRvIII. These findings all provide insight into possible mechanisms of how HIP1-mediates RTK stabilization in cancer.

Chapter 1:
Introduction

Huntingtin interacting protein 1 (HIP1) was identified as a putative oncogene when it was cloned from a leukemogenic translocation discovered in the bone marrow of a patient with chronic myelomonocytic leukemia (CMML) (Ross et al., 1998). This translocation fused the majority of the HIP1 protein to the C-terminal kinase domain of Platelet Derived Growth Factor Receptor β (PDGFRβ), a receptor tyrosine kinase (RTK). Later, it was determined that HIP1 can slow the ligand mediated degradation of these receptors and that HIP1 overexpression can transform NIH-3T3 cells into transplantable tumors that demonstrate anchorage independent growth (Rao et al., 2003). As expected from a protein with these oncogenic properties, HIP1 is upregulated in a number of forms of cancer, including prostate cancer, colon cancer, brain cancer, breast cancer, and lymphomas (Bradley et al., 2007a; Bradley et al., 2007d; Rao et al., 2003; Rao et al., 2002). Additionally, HIP1's expression is associated with poor survival in men with prostate cancer (Rao et al., 2002). HIP1 levels were also found to be especially high in a rare, deadly form of skin cancer called Merkel Cell Carcinoma (MCC) (Rao et al., 2002). Due to our poor understanding of the pathogenesis of MCC, further inquiry into the function of HIP1 in MCC and other cancers could influence assessment and treatment of cancers expressing high levels of HIP1.

While the link of HIP1 to cancer is clear, the mechanism of HIP1-mediated pathogenesis remains a mystery. Since the discovery of HIP1 as an oncogenic protein,

much of the progress regarding the endocytic role of HIP1 has focused on the mechanics of its interactions with clathrin and actin (Gottfried et al., 2010). While it has been shown that HIP1 can interact with and slow the degradation of EGFR and fibroblast growth factor receptor 4 (FGFR4) RTKs (Bradley et al., 2007a; Wang et al., 2008), little is known about how HIP1 is able to slow the degradation of this cargo or if these receptors are able to alter HIP1 function through their signaling. This review will focus on known mechanisms of receptor mediated endocytosis with a focus on RTKs, the role of this process in cancer, and how HIP1 may be able to manipulate endocytosis in a manner that leads to oncogenesis.

1.1 Receptor Mediated Endocytosis

Receptor endocytosis is highly conserved among a number of different receptor types. The most commonly studied receptors in endocytosis, receptor recycling, and trafficking include the low density lipoprotein (LDL) receptor, the transferrin receptor, neurotransmitter-gated ion channels, G-coupled receptors, and RTKs. The majority of proteins in these categories are internalized through the process of clathrin-dependent endocytosis, the most well understood form of receptor endocytosis. During the process of clathrin-dependent endocytosis, clathrin triskelions, composed of a triad of heavy and light chains, are recruited to the cell membrane by adaptor complexes such as the adaptor protein 2 (AP2) complex (Knuehl et al., 2006; Sorkin and von Zastrow, 2009). These triskalions then interlock and form a cage around internally budding membranes. The newly formed clathrin-coated pits are pinched off from the cell membrane by the GTPase dynamin, which allows the newly-formed clathrin-coated vesicles to travel into the cytoplasm (van der Bliek et al., 1993).

The routes these different receptors take through the endosomal pathway vary (Figure 1.1). Transferrin receptors, for example, are almost exclusively recycled through a rapid recycling pathway (Jing et al., 1990). RTKs are often recycled but can also be targeted to the late endosome and lysosome through ubiquitination or continue to signal within endosomes (Sorkin and Goh, 2008). Trafficking of the α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic (AMPA) glutamate receptor largely depends on what initiates its endocytosis. Endocytosis caused by binding of the ligand AMPA generally leads to AMPA receptor recycling, while endocytosis of AMPA due to insulin signaling usually targets AMPA to the lysosomal degradation pathway (Lin et al., 2000). All of these mechanisms allow tight control of endocytosis for different receptor types in the optimal manner for cellular survival and normal physiology.

The lysosomal and recycling pathways of clathrin-dependent endocytosis each have canonical forms, but some variations exist. The pathway to the lysosome includes fusion of vesicles into Rab5 positive early endosomes, which is mediated by the GTPase activity of Rab5 (Stenmark et al., 1994). Rab5 activity is also responsible for the shedding of AP2 from the endosome, although clathrin is retained until a later stage (Semerdjieva et al., 2008). Rab5 positive endosomes have early and late components, marked by the APPL1 and EEA1 effector proteins, respectively (Zoncu et al., 2009). During progression to the lysosome, the endosome acidifies, releasing receptor ligand into the lumen of the vesicle. The endosome then fuses with the multivesicular body (MVB) after which endosomes are swallowed into intraluminal vesicles and the receptor ceases contact with the cytoplasm. From there, the receptor progresses to the more acidic and enzyme-filled lysosome, where the receptor is degraded (Sorkin and Goh, 2009).

The recycling pathway is more complicated and less understood, but seems to be composed of two portions: a rapid recycling pathway and a slower recycling route through the endosomal recycling complex (ERC) and Golgi apparatus, marked by the presence of the small GTPase Rab11 (Maxfield and McGraw, 2004). Both of these pathways originate at the sorting endosome, which consists of Rab5 and Rab4 positive early endosomes (Maxfield and McGraw, 2004). These endosomes are deemed sorting endosomes due to the ability of their cargo to either be recycled through fast recycling pathways, through the ERC, or sent to the lysosome for degradation. Prolonged signaling can also occur in these endosomes, which will be discussed later in reference to RTKs.

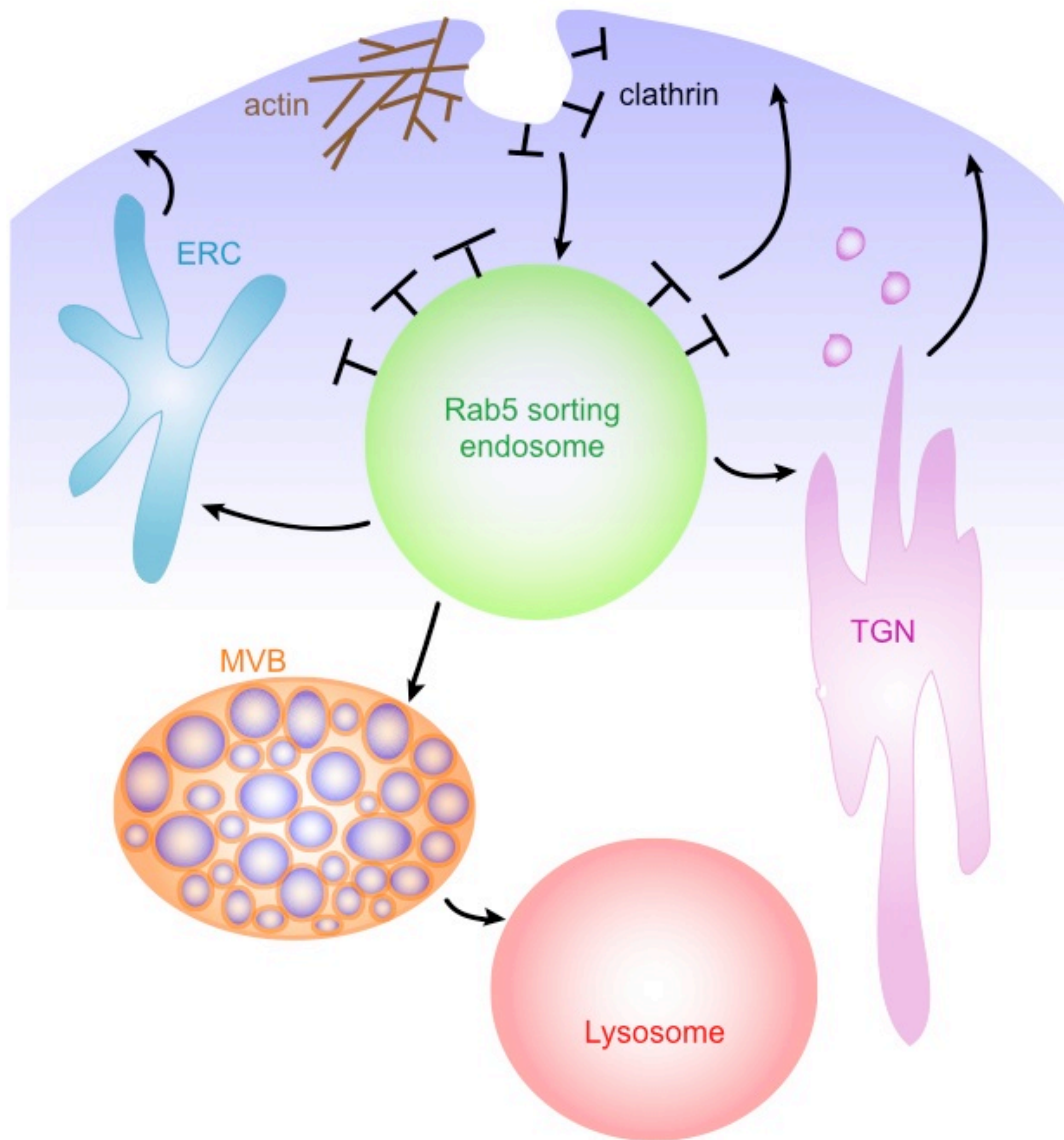


Figure 1.1 Diagram of Normal Endocytosis. In normal receptor-mediated endocytosis, activated or constitutively internalized receptors are endocytosed through clathrin-coated endosomes. These endosomes are then fused into sorting endosomes, from which receptors are either targeted for recycling to the cell surface or degradation in the lysosome. ERC = endosomal recycling complex; TGN = trans golgi network; MVB = multivesicular body.

In most cultured cells, the ERC appears as a series of tubules near the Golgi apparatus (Grant and Donaldson, 2009). The ERC is thought to form as the sorting

endosome matures, dividing itself into both the MVB and the ERC (Maxfield and McGraw, 2004). Though its exact function in receptor recycling is not fully understood, Rab11 seems to act as a driver of ERC recycling activity. Recycling from the ERC back to the plasma membrane is greatly inhibited by dysfunction of Rab11. This is demonstrated by defects in transferrin recycling that occur when the Rab11 dominant negative mutant Rab11S25N is expressed, preventing the delivery of transferrin to the trans-Golgi network for transport back to the plasma membrane (Wilcke et al., 2000). Rab11 dysfunction can also lead to the accumulation of proteins in the ERC that would otherwise be targeted for recycling (Grant and Donaldson, 2009; Maxfield and McGraw, 2004). Like Rab11, the ARF6 GTPase has an important role in promoting recycling from clathrin-independent endosomal pathways, specifically in G-coupled protein receptor trafficking (Grant and Donaldson, 2009).

The fast recycling pathway is not as well understood as the ERC, though this seems to be the default pathway for the majority of receptors that are not targeted for degradation. The small GTPases Rab4 and Rab35 have both been linked to fast endocytic recycling. Rab35 is recruited to clathrin-coated pits through the AP2 binding protein connecdenn (Marat and McPherson, 2010). Knock-down of Rab35 has been found to interfere with recycling of the constitutively recycled transferrin receptor and a *C. elegans* homologue of the LDL receptor (Chua et al., 2010). Rab35 was shown to act in a parallel pathway to Rab11 mediated endocytosis, however it has also been observed localizing to the ERC (Chua et al., 2010). Rab4 also has a confusing role in this process as different forms of inhibition of this protein have different effects. Inhibition with a dominant negative form of Rab4, for example, decreases rapid recycling (McCaffrey et

al., 2001). Knock-down of Rab4 with siRNA, however, was found to increase rapid recycling (Deneka et al., 2003). This could indicate that in the absence of Rab4, alternate recycling pathways are utilized while the presence of a defective Rab4 prevents these other pathways from being used. It also remains unclear whether the fast recycling pathway for constitutively recycled receptors, such as transferrin, is regulated in the same way as other receptors that experience regulated recycling, such as the EGF and AMPA receptors.

Another route for internalized receptors that has recently gained attention is nuclear trafficking. This allows activated transmembrane receptors to directly influence gene transcription. One example of this phenomenon is EGFR signaling in the nucleus. The EGFR family has long been detected localizing to the nucleus (Marti et al., 1991) and EGFR itself has been found to interact with STAT3 as a co-factor in STAT3 mediated transcription (Lo et al., 2005). EGFR family receptors each have a nuclear localization signal on the cytoplasmic region on the protein, adjacent to the plasma membrane (Hsu and Hung, 2007). This corresponds to amino acids 645-667 in EGFR (Hsu and Hung, 2007). Endocytic adaptor proteins have also been observed in nuclear signaling. Nuclear localization signals have been detected in the adaptors HIP1, DAB1 and APPL2 (Honda and Nakajima, 2006; Mills et al., 2005; Nechamen et al., 2007). EPS15, Epsin1, CALM and β-arrestin have also been detected in the nucleus, though no nuclear localization signals have been identified in these proteins (Archangelo et al., 2006; Hyman et al., 2000; Poupon et al., 2002; Wang et al., 2003). Among these nuclear localizing adaptors, APPL protein nuclear function remains one of the most studied. APPL1 and 2 are directed to the nucleus upon EGFR stimulation. Here they associate

with the histone deacetylase NuRD/MeCP1 complex enabling increased DNA synthesis (Miaczynska et al., 2004). The nuclear functions of other endocytic proteins, and whether or not their targeting to the nucleus is linked to their function in the endosome, have yet to be determined.

Adaptor proteins that coat the endosome are thought to be essential to the targeting decisions that are made during the processes of endocytosis and endosomal sorting. Major adaptor proteins involved in endocytosis include clathrin, AP2, and a large number of proteins termed clathrin associated sorting proteins (CLASPs) that bind lipids, cargo, clathrin, and other adaptor proteins, or combinations of two or more of the above (Reider and Wendland, 2011). These CLASPs can be cargo specific, including β-arrestin, which shuttles G-protein coupled receptors and attenuates their signaling, or also the DAB2 protein, which aids in the traffic of LDL receptors (Reider and Wendland, 2011). CLASPs can also be specific to clathrin-mediated traffic or be involved in both clathrin-coated and non-coated endosomes (Reider and Wendland, 2011). Generally, adaptors help shuttle endocytic traffic to specific endpoints through recognition of activation status or post-translational modifications of cargo receptors.

Clathrin's necessity for receptor internalization or recycling is varied depending on the receptors studied. Chemical cross-linking of clathrin, for example, slows transferrin internalization but does not impact its ability to be recycled. In contrast, clathrin knock-down halves the amount of stimulated EGFR that is recycled to the cell surface (Sigismund et al., 2005). This difference may be due to transferrin's intrinsic targeting to recycling pathways, while EGFR endocytosis involves more complex regulatory controls, such as ubiquitination. Transferrin also does not seem to rely upon

any adaptor proteins to target it for recycling, as deletion of the majority of its cytoplasmic tail has minimal effect on its ability to be recycled (Jing et al., 1990). Without adaptor proteins to shuttle transferrin to the correct location, it is still possible that this targeting could be mediated through lipid interactions within the transferrin transmembrane domain (Mercanti et al., 2010).

Phosphoinositides (PIs) are known to play a major role in endocytic trafficking, as many stages in the endocytic pathway can be identified and regulated through the lipid content of their vesicular bilayer. These lipids compose a small part of the cellular membrane, yet retain profound influence on cellular traffic (Clague et al., 2009). First, the formation of $PI(4,5)P_2$ at the plasma membrane is essential for recruiting AP2 and Dab2 to the plasma membrane to allow endocytosis of their respective cargo (Clague et al., 2009; Jost et al., 1998). Additionally, the presence of PI3P is necessary for the formation of EEA1 positive early endosomes, which contain FYVE domain-containing lipid-binding proteins, such as EEA1 itself, and Hrs, which mediates the transition from the endosome to the MVB (Clague et al., 2009; Katzmann et al., 2003). The presence of these lipids, however, excludes APPL1, an earlier endosomal Rab5 effector that localizes to endosomes with $PI(3,4)P_2$ lipid content (Sorkin and Goh, 2009). Phosphoinositides also participate in endosomal signaling cascades. $PI(3,4,5)P_3$, for example, is an important activator of the Akt pathway by recruiting Akt to the plasma membrane, where it is phosphorylated by the phosphoinositide dependant kinases (PDK1 and PDK2) (Osaki et al., 2004). This cascade also occurs in APPL1 endosomes, which allow specific RTK signaling through GSK3β (Schenck et al., 2008). This regional specificity of

signaling promoted by phosphoinositide lipids is likely a major reason for the endocytosis of cargo that is not initially targeted for degradation.

As demonstrated, endocytosis is a complex and well-controlled process that allows internalized receptors to initiate a large variety of signals in many different parts of the cell. Endocytosis also allows the cell to attenuate signaling through receptors by targeting them for degradation. Lastly, endocytosis allows receptors to be recycled back to the plasma membrane, where they can start the signaling cycle again.

1.2 Receptor Tyrosine Kinase Endocytosis

Receptor tyrosine kinases are unique in the sheer number of downstream signaling pathways they regulate. These receptors, when bound to ligand, dimerize and phosphorylate their own C-terminal tyrosines with their active kinase domain (Baass et al., 1995). Most of these phosphorylated tyrosines recruit and activate downstream signaling proteins that promote cellular proliferation and survival. The two major signaling cascades activated by RTKs are the mitogen activated kinase pathway (MAP kinase) and Akt kinase pathway (Baass et al., 1995). A few of these C-terminal phosphorylated tyrosines have a direct role in recruiting proteins that are important in their internalization and targeting to the lysosomal degradation pathway (Sorkin and Goh, 2009).

EGFR, the most commonly studied RTK in the endocytosis field, begins the process of endocytosis by recruiting the adaptor protein AP2. The μ2 subunit of AP2 binds phosphorylated Y974 on the EGFR C-terminus (Sorkin and Goh, 2009). AP2 is also phosphorylated by EGFR in a manner regulated by the EGFR di-leucine motif LL1010/1011 through an unknown mechanism (Sorkin and Goh, 2009). The growth

factor receptor binding protein 2 (Grb2) is recruited at the phosphorylated EGFR tyrosine Y1068. Grb2 is then involved in the recruitment of the Cbl E3 ubiquitin ligase to EGFR (Sorkin and Goh, 2009). More recently, acetylation of EGFR at lysines K1155, K1158, and K1164 has been implicated in the regulation of endocytosis (Goh et al., 2010). Though removal of these lysines inhibits endocytosis, the mechanism through which EGFR acetylation promotes EGFR endocytosis is unknown. Though all of these EGFR binding domains are important in endocytosis, they initiate a number of redundant mechanisms that allow EGFR to be endocytosed when one pathway is blocked by mutation or inactivation (Goh et al., 2010).

As previously mentioned, ubiquitination is a major regulator of RTK targeting for degradation (Figure 1.2). Ubiquitin is a 76 amino acid protein that can be covalently bonded to lysine residues on receptors that are to be targeted for degradation. Ubiquitination of EGFR, its family members, and several other RTKs is mediated by the Cbl ubiquitin ligase (Acconcia et al., 2009; Schulze et al., 2005). The type and level of ubiquitination that occurs is dependent on the individual receptor (Schulze et al., 2005). ErbB2, for example, is poorly ubiquitinated and is thus usually recycled (Muthuswamy et al., 1999). EGFR, meanwhile, can be monoubiquitinated and also poly-ubiquitinated through Lys63 bound chains by Cbl (Acconcia et al., 2009). Though receptor ubiquitination is not necessary for internalization, it is a necessary cue for RTKs to be shuttled to the lysosome (Huang et al., 2007). Additionally, de-ubiquitinating enzymes (DUBs) can allow a receptor to change course and be recycled instead of degraded (Acconcia et al., 2009).

A class of CLASP adaptors, called ubiquitin-binding proteins (UBPs), are necessary for escorting ubiquitinated RTKs to the lysosome. First, UBPs at the cell surface, including Epsin and Eps15, provide cues for RTK internalization (Acconcia et al., 2009). Additionally, tyrosine phosphorylation of Eps15 by the EGFR kinase domain is necessary for some forms of ligand induced endocytosis (Confalonieri et al., 2000). Further down the endocytic pathway, UBPs in the sorting endosome, including Eps15b and Hrs, promote the progression of ubiquitinated RTKs into intraluminar vesicles in the MVB (Acconcia et al., 2009; Katzmann et al., 2003). Together, through their ability to shuttle ubiquitinated receptors to the lysosome as endocytic adaptors, UBPs oppose endocytic recycling.

Signals that promote EGFR and RTK recycling are less understood. Recent work suggests that receptors internalized through clathrin-independent means are usually degraded, while clathrin-coated vesicles are generally recycled. This hypothesis was derived from a study where clathrin knock-down in cells led to decreased signaling (Sigismund et al., 2008; Sigismund et al., 2005). Additionally, clathrin-dependent endocytosis is the primary means of endocytosis in low EGF conditions, when receptors are almost always recycled. Meanwhile, clathrin-independent endocytosis is utilized at higher EGF concentrations, perhaps as a means to attenuate overly strong pro-growth signals (Sigismund et al., 2005). Furthermore, it was found that an engineered protein that fused a ubiquitin amino acid sequence to the cytoplasmic domain of EGFR was primarily internalized through clathrin independent processes (Sigismund et al., 2005). In this model, depletion of the machinery involved in clathrin-mediated endocytosis and

recycling leads to receptor ubiquitination and endocytosis through the clathrin-independent pathway, leading to degradation.

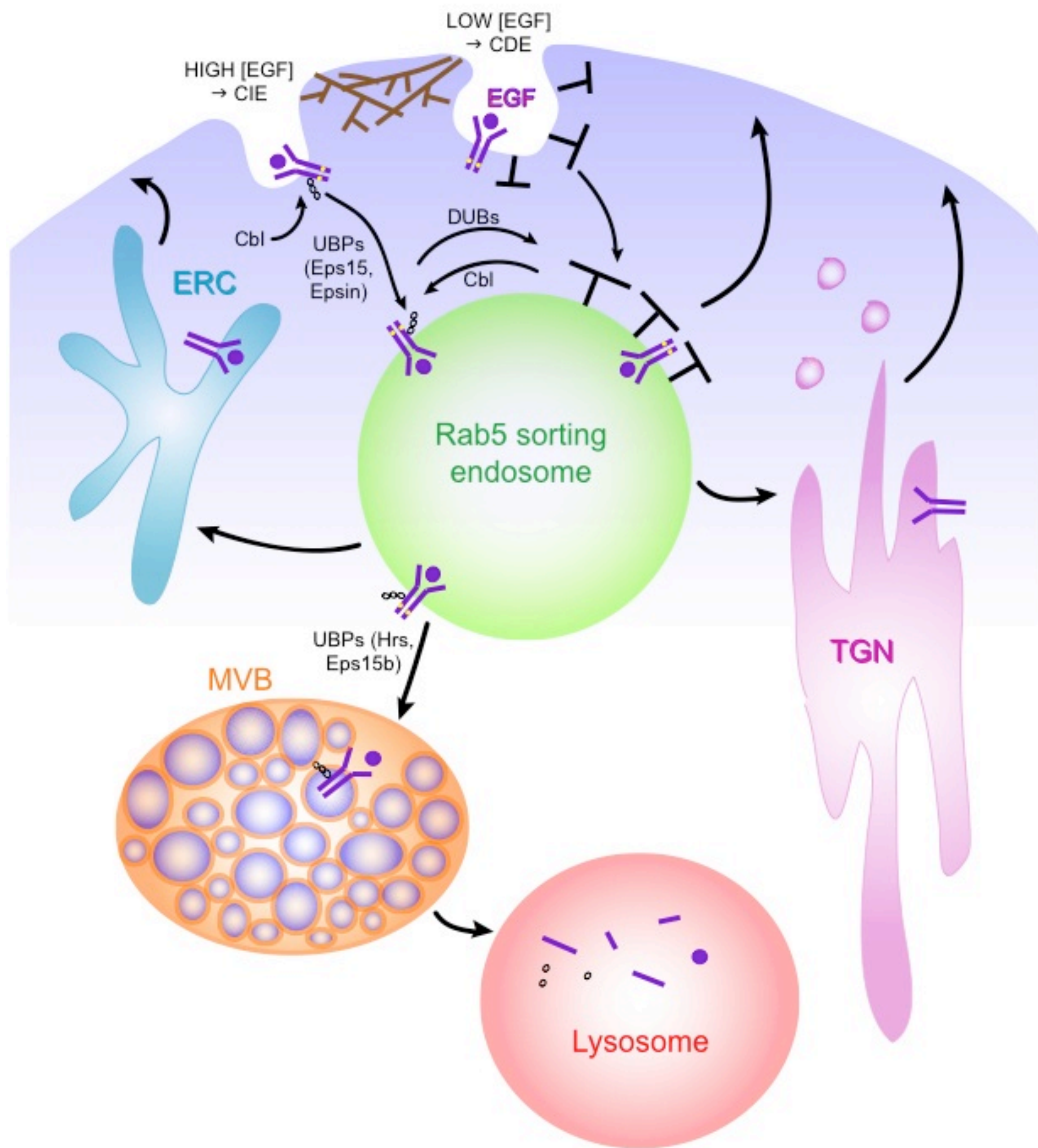


Figure 1.2 Diagram of RTK endocytosis. Activated RTKs can be internalized through either clathrin dependent (CDE) or clathrin independent (CIE) endocytosis. High EGF concentration and EGFR ubiquitination by the Cbl ubiquitin ligase are the major factors influencing the decision for EGFR to be internalized via the CIE pathway and targeted for degradation. Ubiquitinated receptors are shuttled by ubiquiting binding proteins (UBPs) to the lysosome. In contrast, CDE primarily leads to receptor recycling. De-ubiquitinating enzymes (DUBs) allow reversal of RTK degradation before entry into the MVB.

1.3 HIP1's Role in Endocytosis

A substantial amount of insight regarding HIP1's function in endocytosis has been generated through the study of its homologue in yeast, Sla2p (Gottfried et al., 2009). Sla2p was first cloned from a synthetic lethal screen in yeast in 1993 to detect necessary proteins in the absence of the cortical actin binding protein Abp1p (Holtzman et al., 1993). It was subsequently determined that Sla2p has a critical role in actin organization in yeast leading to polarity and budding. Additionally, deletion of the Sla2p light chain binding site slowed the internalization of Sla2p bound patches of membrane (Newpher and Lemmon, 2006).

Sla2p and its mammalian relatives, HIP1 and HIP1r, all have lipid-binding, clathrin light chain-binding, and actin-binding domains. The differences between these homologues seem linked to differential strength in binding to these major binding partners. In mammals, HIP1 and HIP1r mainly differ in that HIP1 displays greater clathrin binding while HIP1r demonstrates greater actin binding and localization to actin-rich membrane ruffles (Legendre-Guillemin et al., 2002). HIP1 may have evolved divergently from Sla2p in its actin-binding and actin organizational capabilities, as no studies have demonstrated a role for HIP1 in actin organization (Gottfried et al., 2010). HIP1 also has a clathrin heavy chain-binding domain and an AP2 binding domain, which HIP1r lacks (Gottfried et al., 2010). Additionally, HIP1r is able to bind cortactin through a proline-rich domain, while HIP1 does not (Le Clainche et al., 2007). It has been hypothesized that through heterodimerization, HIP1 and HIP1r could jointly serve the dual roles of actin organization and clathrin assembly that Sla2p performs. Recent work,

however, demonstrated a preference of homodimerization among these proteins, suggesting that HIP1 and HIP1r function independently (Wilbur et al., 2008).

Due to its direct binding to clathrin, HIP1 is likely primarily to be involved in the process of clathrin-dependent endocytosis, rather than clathrin-independent endocytosis. By definition, HIP1 is a CLASP adaptor protein that can associate with a variety of cargo, but its link to the clathrin-coated vesicle is strong, so it likely is specific for this form of endocytosis, unlike Eps15, which does not bind clathrin (Reider and Wendland, 2011). This indicates that HIP1 is involved in the endocytosis of receptors primarily bound for recycling (Sigismund et al., 2008).

HIP1's role in endocytosis also may vary between receptor types. For example, HIP1 knock-out mice have defects in AMPA receptor internalization but not transferrin receptor internalization (Metzler et al., 2003). HIP1 also interacts with the GluR1 subunit of the AMPA receptor although it is unknown if this interaction is direct or mediated through AP2. Another glutamate receptor, the NMDA receptor, interacts with HIP1 via the NMDAR subunits NR2A and NR2B (Metzler et al., 2007). This interaction was determined to be direct when assessed in a cell-free system where amino acids 219-616 were co-expressed with these NMDA subunits. This region overlaps with the domains mapped for HIP1 interaction with EGFR and FGFR4 (Bradley et al., 2007a; Wang et al., 2008). Together, these data may indicate a consensus binding region for receptor cargo on HIP1 in the vicinity of amino acid 600.

HIP1 is also involved in the targeting of internalized androgen receptor to the nucleus (Mills et al., 2005). As mentioned previously, nuclear trafficking in endocytosis is possibly an important function of adaptor proteins that is only beginning to be

explored. HIP1 interacts with the androgen receptor, which is involved in transcriptional regulation (Mills et al., 2005). Additionally it was found that HIP1 localized to the nucleus, the terminal destination of internalized androgen receptor (Mills et al., 2005). This localization is directed through a nuclear localization signal on HIP1's C-terminus (Mills et al., 2005). Most surprisingly, it was found that HIP1 associated with androgen responsive elements (AREs) through chromatin immunoprecipitation analysis (Mills et al., 2005). This work connects HIP1 with a growing list of endocytic adaptor proteins involved in transcriptional regulation.

HIP1 also has a conserved, AP180 N-terminal homology (ANTH) phosphoinositol binding domain. The ANTH domain demonstrates similar binding to phosphoinositides as the Eps15 N-terminal homology (ENTH) domain, but, unlike the ENTH domain, it does not induce curvature in the membranes that it binds (Clague et al., 2009). In HIP1, this domain preferably binds $PI(3,4)P_2$ and $PI(3,5)P_2$ while its yeast homologue Sla2p binds mostly to $PI(4,5)P_2$ (Hyun et al., 2004b). Sla2p lipid-binding also is critical for its ability to organize actin at the cell surface, which is heavily populated by $PI(4,5)P_2$ (Sun et al., 2005). The $PI(3,4)P_2$ and $PI(3,5)P_2$ lipids bound by HIP1 are localized primarily to APPL1 positive early endosomes and late endosomes, respectively (Clague et al., 2009; Zoncu et al., 2009). This unique lipid binding profile may correspond with a more internal vesicular role for HIP1 than its yeast homologue.

Some debate exists regarding the extent to which HIP1 associates with the early endosome. While early data in neuronal trafficking localized HIP1 to EEA1 positive early endosomes in the process of retrograde trafficking, other total internal reflection fluorescent microscopy (TIRF) data has suggested a more transient role for HIP1

associated with vesicles, in which both HIP1 and clathrin uncoat from vesicles soon after they are internalized (Gottfried et al., 2009). Distinguishing among these possibilities is important for defining the function of HIP1 during the process of endocytosis.

These collective data suggest an important role for HIP1 in the endocytosis of several types of receptors. HIP1 is not essential for cellular survival, as HIP1 knockout mice survive and reach young adulthood. HIP1, therefore, is not essential for endocytosis in the same way as the AP2 adaptor (Mitsunari et al., 2005). These HIP1 knockout mice eventually exhibit failure to thrive in adulthood and have short life spans (Hyun et al., 2004a). This suggests that HIP1 has a supplementary role in endocytosis that is necessary for sustenance and growth in adulthood, although not necessary for development.

1.4 Dysregulation of Receptor Tyrosine Kinase Endocytosis in Cancer

Recently, dysregulation of receptor endocytosis has emerged as a common mechanism contributing to cancer. This is due to the ability of endocytosis to alter the availability and signaling properties of the receptors that it regulates. Cbl, for example, serves a tumor suppressor function through its ubiquitination of activated EGFR, leading to its targeting to the lysosome and degradation. Processes such as these, when disrupted, can lead to abnormal EGFR signaling, a hallmark of many types of cancer. Though discovery of these tumor suppressors is helpful for understanding the processes that are dysregulated in cancer and diagnosing molecular subtypes of disease, they are inherently difficult to target in cancer therapy. Conversely, targeted therapy of oncogenes, particularly of RTKs, has been highly successful over previous years, providing an effective and less toxic means of cancer treatment (Collins and Workman, 2006).

Endocytic oncogenes, however, may be valuable targets as they can simultaneously upregulate several types of oncogenic receptor tyrosine kinases. A drug that targets oncogenes in the endocytic pathway may also be a powerful alternative therapy for tumors that gain resistance to RTK inhibitors as a result of upregulation of other RTKs.

Proteins that show oncogenic function in endocytosis are often difficult to detect and prove as causative events in tumorigenesis. In these cases, endocytic oncogenes are usually proteins that slow endocytosis, increase recycling, or rearrange the cytoskeleton. In addition to HIP1, Eps15 is able to induce transform NIH-3T3 cells (Fazioli et al., 1993), however the mechanism for how Eps15 transforms cells is less clear because Eps15 knock-down, rather than overexpression, causes receptor stabilization (Parachoniak and Park, 2009). The definition of Eps15 as an oncoprotein is further confused by the fact that Eps15 aids in targeting RTKs for degradation through its binding to ubiquitin linkages (Acconcia et al., 2009; Roxrud et al., 2008). These functions of Eps15 would indicate that Eps15 serves a tumor suppressor function rather than the oncogenic function demonstrated through its transformation of NIH-3T3 cells. Additionally, upregulation of Eps15 in tumor tissue has not been observed as it has been for HIP1, with the possible exception of phyllodes tumors of the breast (Ahn et al., 2003). In light of these findings, definition of endocytic oncogenes requires three major elements. Endocytic oncogenes must transform cells, but they also must stabilize oncogenic receptors, and be upregulated in cancer. Together, these characteristics help define targetable oncogenes that function in the receptor endocytosis pathway.

Cortactin, a binding partner of HIP1r, has a stronger link to tumor initiation. This protein is highly expressed in advanced and invasive cancers, including invasive breast

cancer (Patel et al., 1998). Cortactin is activated through tyrosine phosphorylation and is responsible for creating free actin barbed ends in cellular invasive protrusions termed invadopodia (Oser et al., 2009). This tyrosine phosphorylation can be promoted by EGFR through the intracellular tyrosine kinases Arg and Src (Mader et al., 2011). Like Sla2p and HIP1r, cortactin has been associated with the organization of actin prior to endocytosis (Kaksonen et al., 2003). It is possible that, in addition to its oncogenic role in cellular invasion, cortactin inhibits EGFR degradation. Some recent work has identified cortactin as an EGFR stabilizing protein (Timpson et al., 2005). Cortactin also interacts with Fgd1, which activates Cdc42, a protein that sequesters and inhibits the Cbl ubiquitin ligase (Hou et al., 2003). These findings indicate that cortactin may have dual role in promoting oncogenesis, by promoting invasion through the formation of invadopodia and through stabilization of EGFR.

Other oncoproteins have also been more extensively associated with inhibition of the Cbl tumor suppressor protein, namely COOL-1 (cloned-out of library 1) and Src. COOL-1 is also involved in the Cdc42-mediated sequestration of Cbl, preventing it from ubiquitinating RTKs for degradation (Feng et al., 2006). Additionally, COOL-1 is an EGFR substrate that, when phosphorylated, forms a complex with both Cbl and Cdc42 (Feng et al., 2006). To prolong Cbl sequestration, Src activity sustains COOL-1 phosphorylation (Feng et al., 2006). COOL-1, also known as betaPIX, has been observed to have strong expression in breast cancer (Ahn et al., 2003). Src, meanwhile, is one of the first oncogenes identified, due to its role as an intracellular tyrosine kinase present in the chicken Rous sarcoma virus (Levinson et al., 1980). It is also overexpressed in lung, breast, colon, pancreatic, and prostate cancers (Kim et al., 2009). These proteins,

together, coordinate to inhibit Cbl-mediated ubiquitination, allowing several types of oncogenic RTKs to be stabilized.

Genomic amplification of endocytic proteins, such as Rab25 and RAB coupling protein (RCP, Rab11FIP1), has also been observed (Agarwal et al., 2009; Zhang et al., 2009). RCP is a positive effector of Rab11 and thus promotes receptor recycling. It was identified in the 8p11–12 chromosomal region that is frequently amplified in breast cancer and is able to increase Erk signaling upon overexpression (Zhang et al., 2009). RCP also interacts with Rab25, which is amplified in colon, breast, and ovarian cancers. Rab25 is located in recycling endosomes and is closely related to Rab11 in both localization and homology (Agarwal et al., 2009). While Rab25 and RCP both seem to increase receptor recycling by enhancing or mimicking Rab11 activity, it is unknown why Rab11 itself has not been identified as an oncogene. Perhaps Rab11 is too important for cellular function to be intrinsically altered in tumorigenesis, or its genetic locus is resistant to amplification.

GOLPH3 is also a novel oncogene that affects the trafficking of receptors. This protein is localized to the Golgi apparatus and cytosol and is genomically upregulated in lung cancer through a 5p13 copy number amplification (Scott et al., 2009). Upregulation of GOLPH3 is associated with mTOR phosphorylation and is thought to regulate this pathway through an unknown mechanism (Scott et al., 2009). GOLPH3 interacts with Vps35, which helps escort proteins from endosomes to the TGN through the retromer complex and also transports acid hydrolase to endosomes to enable ligand dissociation. Knock-down of Vps35 leads to decreased endocytosis, increased signaling and increased surface localization of transmembrane receptors. One currently held hypotheses for the

oncogenic function of GOLPH3 is that it increases receptor recycling through the TGN through its interaction with Vps35 (Scott and Chin, 2010). Additionally, GOLPH3 could inhibit the retromer-mediated transfer of acid hydrolase to the endosome and thus slow endosome acidification, increasing endosomal signaling (Bonifacino and Hurley, 2008).

Mutations in receptor tyrosine kinases that intrinsically slow their degradation are also common in cancer. For example, a large deletion in the N-terminal portion of the EGFR receptor yields an endocytosis defective receptor called EGFRvIII. This mutant is present in glioblastomas, colorectal cancers, and breast cancer (Loew et al., 2009). Although EGFRvIII lacks the ability to bind ligand, it is constitutively active (Zeineldin et al., 2010). The EGFR dimerization arm is also deleted in this mutant, but it still retains the ability to dimerize with wild type EGFR and ErbB2 (Zeineldin et al., 2010). It also retains the ability to bind Cbl, but poor ubiquitination of this receptor is observed, likely due to poor phosphorylation of Y1045 (Grandal et al., 2007). This degradation defect results in increased observed receptor localization at the plasma membrane (Zeineldin et al., 2010). Additionally, other ErbB family members, including the HER2/neu oncogene, have decreased endocytosis as compared to EGFR (Baulida et al., 1996).

One point of confusion for these poorly-endocytosed oncogenic receptors is that their signaling levels are often similar to or lower than wild type EGFR. This is particularly true for the EGFRvIII oncogene (Huang et al., 1997). This raises the question of whether oncogenesis though the process of impaired receptor degradation is potent because it *increases* signaling strength or because it *stabilizes* pro-growth signaling. This differentiation would require a systems analysis of whether low but stable RTK signals have the same or greater impact on cell growth over time versus strong

signals. Moderate but stabile signals, for example, may initiate fewer negative feedback events than strong signals.

In summary, oncogenes and tumor suppressors can derail endocytosis at multiple levels (Figure 1.3). First, as in the case of cortactin, proteins involved in the process of cytoskeleton organization for endocytosis can also increase cellular process formation and invasion. Secondly, oncoproteins such as COOL-1 and Src can suppress signals, such as Cbl-mediated ubiquitination, that direct receptors for degradation. Thirdly, the process of receptor recycling can be promoted by oncoproteins such as Rab25, RCB and possibly GOLPH3. Lastly, and most importantly, receptors themselves can be mutated in a way that allows them to evade degradation in the lysosome. Interestingly, no oncogene or tumor suppressor has been identified that completely prevents the initiation of endocytosis. This is likely due to the fact that a number of signaling processes depend on the endocytosis of activated receptors to the cytoplasm. This is most elegantly demonstrated through the expression of the dominant negative dynamin mutant K44A, that prevents the dissociation of newly formed endosomes from the plasma membrane and reduces EGFR signaling through both MAP kinase and PI3P kinase pathways (Vieira et al., 1996). Still, targeting endocytic oncogenes in each of these endocytic pathways could have beneficial and possibly synergistic effects in cancer treatment when used with RTK inhibitors.

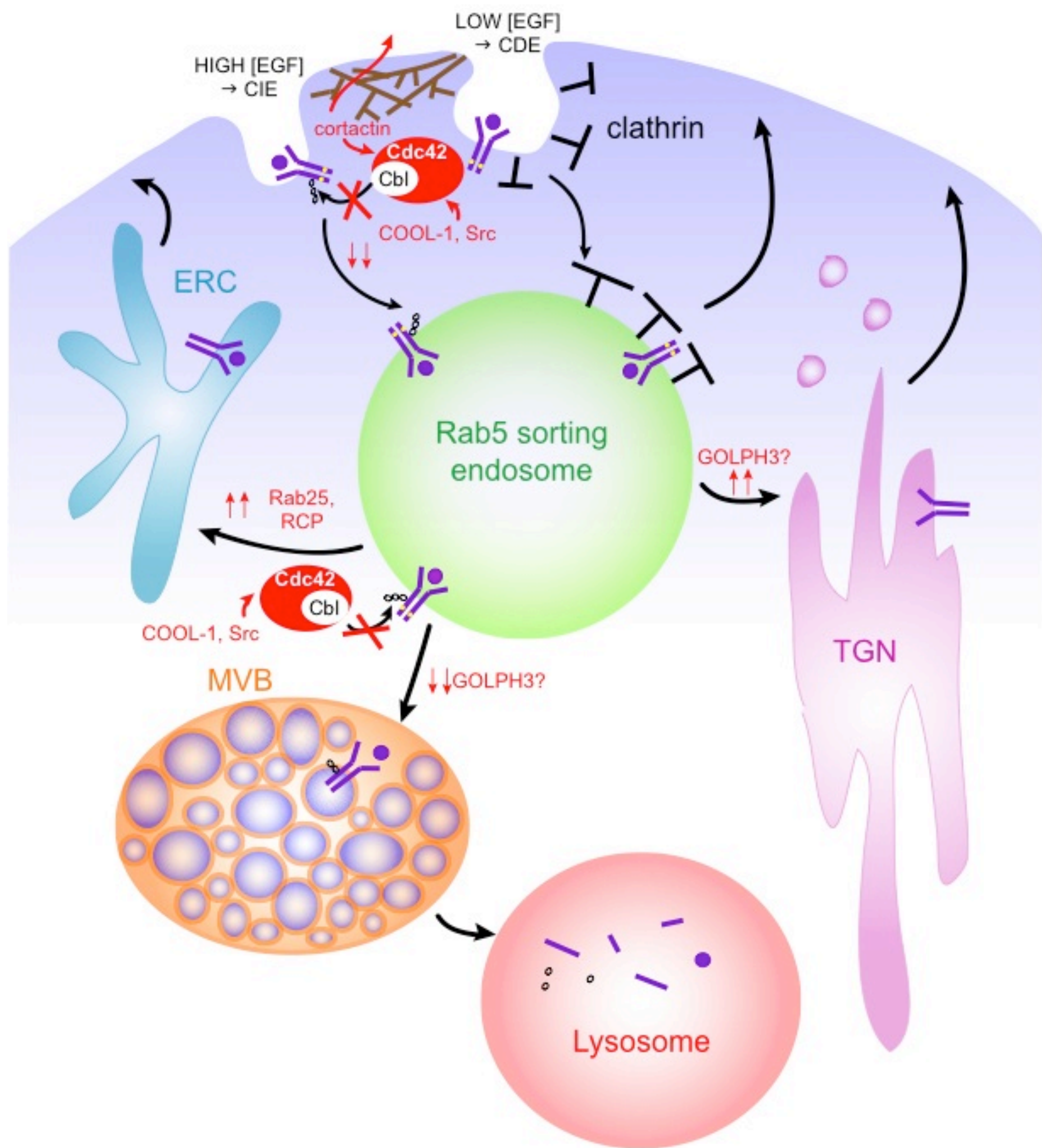


Figure 1.3 Dysregulated RTK Endocytosis in Cancer. Oncoproteins that manipulate the endocytic pathway lead to increased recycling and decreased degradation of tumorigenic receptors.

1.5 HIP1 Interaction with Receptor Tyrosine Kinases in Tumorigenesis

Similar to the proteins listed previously, HIP1 demonstrates oncogenic function in RTK endocytosis. Like Eps15, HIP1 is able to induce anchorage independent growth in NIH 3T3 cells (Rao et al., 2003). Furthermore, HIP1 transformed NIH 3T3 cells demonstrate increased expression of EGFR and another RTK, FGFR4 (Rao et al., 2003). More recent, a direct interaction between HIP1 and both of these receptors has been detected (Bradley et al., 2007a; Wang et al., 2008). Little is known, however, about how this interaction is regulated, and how this interaction may lead to RTK upregulation and subsequent tumorigenesis.

Transient transfection of HIP1 and HIP1r was also found to stabilize EGFR and PDGFβR for hours following their stimulation with ligand (Hyun et al., 2004b). In addition to HIP1's ability to slow the degradation of receptors *in vitro*, HIP1 overexpression is associated with the *in vivo* overexpression of RTKs in tumors. In breast cancer, HIP1 was strongly associated with the expression of EGFR (Rao et al., 2003). This association with EGFR was also demonstrated in brain cancer, where an additional correlation was observed between HIP1 expression and PDGFβR expression (Bradley et al., 2007a). Interestingly, the HIP1 family member HIP1r has not been observed to express aberrantly in cancer. Therefore, HIP1r likely lacks many of the characteristic through which HIP1 promotes tumorigenesis.

The effect of HIP1 on RTK signaling is more ambiguous. Stable HIP1 overexpressing cell lines demonstrate increased signaling in the MAP kinase pathway (Rao et al., 2003). HIP1 knockout mice show impaired growth and failure to thrive,

however no gross alterations in RTK signals have been observed (Bradley et al., 2007c). These knockout mice also demonstrate a resistance to cancer when crossed with known cancer models, including the TRAMP prostate cancer mouse and the MMTV-myc breast cancer model (Bradley et al., 2005; Graves et al., 2008). This indicates that although HIP1 is not necessary for normal RTK signaling, it can augment signaling and is manipulated for growth in several cancer models.

HIP1 also has been identified as a potent antigen in cancer that provokes production of antibodies against this protein in patients with HIP1 expressing tumors such as prostate, lymphoid, and brain cancers (Bradley et al., 2007a; Bradley et al., 2005; Bradley et al., 2007d). This indicates that in cancer, HIP1 is in greater contact with the immune system, perhaps due to dying HIP1 overexpressing cancer cells. In this case, the body may also be targeting cancer itself as a means of suppressing tumor cell growth by killing cells expressing abnormally high HIP1 levels. For example, in B-cell lymphoma, improved survival is associated with the production of anti-HIP1 antibodies (Bradley et al., 2007d). In this case, antibody production against HIP1 antigens could be complimented by a cell-mediated response, which could allow T cells to attack tumors cells expression high levels of HIP1. These data also show that HIP1 immunotherapy may be possible in targeting this oncoprotein in cancer.

Still unknown is the mechanism for HIP1 upregulation in cancer. Transcriptional upregulation of HIP1 has been observed in prostate cancer (Wang et al., 2008), but to date no genomic amplifications of HIP1 have been observed. The means for HIP1 transcriptional regulation, however, are also unknown (Graves, 2009). There also remains a possibility of HIP1 upregulation through translational control or protein

stabilization. For example, the HIP1 transcript contains a large 3' untranslated region (UTR), of 6 kbp, which may make it susceptible to regulation by complementary small ribonucleic acids (RNAs) such as microRNAs (Graves, 2009). Altered HIP1 translational regulation, therefore, could contribute to HIP1 overexpression.

The mechanism through which increased HIP1 expression stabilizes RTKs in cancer also remains an important unknown. Based on its association with the clathrin-coated vesicle and direct association with cargo, there are several ways in which HIP1 could slow receptor degradation or increase recycling. For one, HIP1 could be a limiting agent in the process of clathrin-dependent endocytosis and recycling. Therefore, an increase in the expression of HIP1 could increase the concentration of EGF required for EGFR receptor degradation through the clathrin-independent endocytosis pathway. Additionally, if localized to the sorting endosome, HIP1 could influence the decision to recycle or degrade receptors or slow transitions from neutral signaling environments to acidic, non-signaling environments. Through its interactions with the cytoskeleton, HIP1 could also promote cellular invasion. In order to differentiate between these possibilities, further characterization of HIP1 localization, regulation, and affect on its binding partners needs to be determined.

1.6 Conclusions

Cancer therapy has long been an indiscriminate battle against rapidly dividing cells, leading to significant toxic side effects in the patient. Recently, targeted therapy against oncogenes has shown new progress with the advent of small molecule inhibitors, such as imatinib, and targeted antibodies, such as trastuzumab, against activated RTKs (Collins and Workman, 2006). Though these drugs have had great success, they are also

subject to resistance within cancers, which utilize other RTKs or develop active-site point mutants to allow growth (Collins and Workman, 2006). For this reason, targeting oncogenes within the endocytic system would be ideal because it would increase the degradation of several types of oncogenic receptors at the same time. One difficulty in this quest is that with endosomal oncogenes such as HIP1, the targetable "active site" that allows stabilization of receptors is not as obvious as it is for receptor tyrosine kinases. For this reason, extensive research is necessary to identify structural elements within these proteins that are necessary to stabilize receptors, increase signaling, and transform cells. The following work examines HIP1 interaction with receptor tyrosine kinases in depth to help achieve this aim.

Chapter 2:
Huntingtin Interacting Protein 1: a New Merkel Cell Carcinoma Marker That Interacts with c-Kit

2.1 Abstract

Merkel cell carcinoma (MCC) is a neoplasm thought to originate from the neuroendocrine Merkel cells of the skin. Although the prevalence of MCC has been increasing, treatments for this disease remain limited because of a paucity of information regarding MCC biology. We have found that the endocytic oncoprotein Huntingtin interacting protein 1 (HIP1) is expressed at high levels in 90% of MCC tumors and serves as a more reliable histological cytoplasmic stain than the gold standard, cytokeratin 20. Furthermore, high anti-HIP1 antibody reactivity in the sera of a cohort of MCC patients predicts the presence of metastases. Another protein that is frequently expressed at high levels in MCC tumors is the stem cell factor (SCF) receptor tyrosine kinase, c-Kit. In working toward an understanding of how HIP1 might contribute to MCC tumorigenesis, we have discovered that HIP1 interacts with SCF-activated c-Kit. These data not only identify HIP1 as a molecular marker for management of MCC patients but also show that HIP1 interacts with and slows the degradation of c-Kit. HIP1, therefore, is a putative oncoprotein in Merkel Cell tumorigenesis that could potentially be targeted to treat MCC.

2.2 Introduction

Merkel cell carcinoma (MCC) is an aggressive skin cancer thought to be derived from the sensory Merkel cells of the skin (Bichakjian et al., 2007). Although the overall incidence of this disease is low and affects 1400 patients per year in the United States, this cancer is associated with a poor prognosis, and most patients with metastatic disease do not survive more than 5 years (Bichakjian et al., 2007). Clinical management of patients with MCC is limited because of a lack of prognostic markers and effective therapies. These limitations stem from a lack of understanding of the biology of MCC's initiation, maintenance, and progression to the metastatic stage. Currently, surgical excision of the primary tumor and radiation remain the main therapeutics for MCC (Bichakjian et al., 2007).

Although receptor tyrosine kinases (RTKs), such as c-Kit, have been shown to be increased in expression in MCC, convincing clinical data regarding the effectiveness of specific c-Kit inhibitors, such as imatinib, on patient survival are not yet available (Lemos and Nghiem, 2007). Recently, a virus designated as Merkel cell polyoma virus has been found in tumors from MCC patients but not their normal skin tissue (Feng et al., 2008). In contrast, its use as a serum biomarker is not clear because of limited specificity. Antibodies against the virus have been found in a large number of tumor-free individuals as well (Carter et al., 2009). Furthermore, its role in initiating MCC remains hypothetical (Carter et al., 2009). A better understanding of how c-Kit or Merkel cell polyoma virus contributes to the induction, maintenance, and progression of MCC will facilitate the development of effective therapies.

Huntingtin-interacting protein 1 (HIP1) is a highly conserved protein that interacts with components of the endocytic machinery, including 3-phosphoinositides, clathrin, and AP2 (Engqvist-Goldstein et al., 1999; Engqvist-Goldstein et al., 2001; Hyun and Ross, 2004; Metzler et al., 2001; Mishra et al., 2001; Rao et al., 2001; Waelter et al., 2001). All of these molecules are involved in the clathrin-mediated internalization of surface receptors. Unlike AP2 (Mitsunari et al., 2005), HIP1 is not necessary for embryogenesis or early postnatal development, but young adult mice deficient for HIP1 do develop a degenerative phenotype (Oravecz-Wilson et al., 2004). In addition, HIP1 overexpression transforms fibroblasts (Rao et al., 2003) and prostate epithelial cells (Wang et al., 2008), and HIP1 transgenic mice develop plasma cell neoplasms (Bradley et al., 2007d).

In addition to its transforming activity, high HIP1 expression is associated with a variety of human cancers, including prostate, colon, breast, brain, and lymphoid cancers (Bradley et al., 2007a; Bradley et al., 2007d; Rao et al., 2003; Rao et al., 2002). HIP1 expression in prostate tumors is associated with a poor prognosis, suggesting that HIP1 overexpression may functionally promote tumorigenesis (Bradley et al., 2005). Anti-HIP1 antibodies have been detected in the sera of patients with prostate, lymphoid, and brain cancers more frequently than in the sera of cancer-free individuals (Bradley et al., 2007a; Bradley et al., 2005; Bradley et al., 2007d). These findings indicate that testing for anti-HIP1 antibodies in serum may serve as a useful noninvasive test to detect the presence, recurrence, or progression of some human tumors.

Further investigation into the role of HIP1 in tumorigenesis has demonstrated an association between the presence of this oncoprotein and enhanced RTK expression

(Bradley et al., 2007a; Rao et al., 2003). These findings are not unexpected because of the association of HIP1 with components of the clathrin-mediated endocytosis pathway, which is a mechanism for modulation of receptor levels. Indeed, not only is HIP1 overexpression in tumors associated with the overexpression of RTKs, but HIP1-mediated transformation also can be blocked with tyrosine kinase inhibitors (Rao et al., 2003). We have found that co-expression of HIP1 with EGFR increases the half-life of EGFR upon EGF stimulation (Hyun et al., 2004b) and that HIP1 physically associates with EGFR (Bradley et al., 2007a). Others have found that HIP1 also stabilizes and associates with the fibroblast growth factor receptor 4 (Wang et al., 2008). These interactions together with HIP1's overexpression in multiple cancers suggest that HIP1-mediated transformation may occur via concomitant increases in multiple RTK signals.

The potential of HIP1 as a clinical marker for several types of tumors, along with its high expression in neural crest derived peripheral neurons (Rao et al., 2002), led us to examine the possibility that HIP1 could serve as a marker for MCC. We evaluated a large series of MCC tissue samples and found vastly elevated HIP1 protein levels compared with normal surrounding skin tissue. We also detected high levels of anti-HIP1 antibodies in sera from a separate cohort of MCC patients. Some of the patients with metastatic MCC exhibited higher levels of anti-HIP1 antibodies compared with MCC patients with localized disease. Furthermore, in an effort to discover how HIP1 expression may functionally contribute to MCC biology, we discovered that HIP1 physically associates with and stabilizes c-Kit, a RTK specifically expressed at high levels in MCC (Sattler and Salgia, 2004; Su et al., 2002).

2.3 Results

MCC specimens demonstrate high levels of HIP1 staining

To evaluate HIP1 as a possible MCC marker, paraffin-embedded tissue samples from MCC tumors and the similar ‘‘round blue cell’’ neuroendocrine tumors, small-cell lung cancer (SCLC), were immunostained for HIP1 expression (Figure 2.1a). These tumors were evaluated for both HIP1 expression level and cellular localization. HIP1 is not expressed at high levels in normal skin with the exception of vascular endothelium (Rao et al., 2002). Tumor tissue was visually scored for HIP1 expression on a scale of 0–3, in which a score of 3 represented the highest HIP1 staining and a score of 0 indicated a lack of staining (Bradley et al., 2007a).

In all, 89% (n=25/28) of MCC tissue samples in our first MCC patient cohort exhibited high HIP1 expression (Table 2.1). MCC tissue samples displayed both diffuse cytoplasmic staining as well as perinuclear dot patterns of staining. HIP1 expression occurred much more frequently in MCC tumors than in SCLC tumors (n.5/12 for SCLC; 42%). In particular, frequency of HIP1 overexpression was significantly higher in metastatic MCC as compared with metastatic SCLC (Table 2.1). This finding is clinically significant, as MCC and SCLC are often difficult to distinguish from one another in the metastatic setting.

Additionally, as one would predict with increased HIP1 protein expression in MCC tumors, using mRNA microarrays and a distinct cohort of patients, a 6-fold average increase in HIP1 message was detected in MCC tumors. A cohort of 30 patients was studied and 29 of them displayed an elevated HIP1 message compared with squamous cell carcinomas. High HIP1 levels have not been observed in squamous cell carcinomas

(Rao et al., 2002). In contrast to HIP1, HIP1 related, the only known mammalian relative of HIP1, was not elevated at the message level (personal communication, Paul Harms, University of Michigan).

We also compared HIP1 tumor staining with the known MCC markers, cytokeratin 20 (CK20) and c-Kit (Figure 2.1b). A separate cohort of 14 MCC tumors from archived samples from MCC patients (all of whose diagnoses were based on immunophenotyping and clinicopathological correlations at the University of Michigan) was used for generation of a tissue microarray (TMA) to compare HIP1, CK20, and c-Kit. Each tumor was represented by three different spots on the TMA slide for purposes of better tumor coverage. As is evident in the top row, HIP1 staining for MCC was very strong, diffuse, and sensitive (100% positive; n.14/14). In comparison, CK20 staining was less reliable and positive in only 64% of the tumor samples (third row versus the top row; n = 9/14). This frequency of CK20 staining is lower than previous reports where it has been found to be positive in 80% of MCC tumors. This also does not reflect the original pathological assessment for each of the tumors from which they were derived, as 13 of the 14 were reported as positive for CK20 staining. This difference is likely due to the use of a TMA rather than the entire slide for testing each tumor. As CK20 staining is not as uniformly distributed in the tumor cells as HIP1 staining is, it is possible that a positive tumor could test falsely negative for CK20 because of there being less tissues represented on a TMA. These data nevertheless indicate that the chance of misdiagnosing a MCC when staining for CK20 is greater than when staining for HIP1. As the HIP1 antibody stained every tumor, the HIP1 test was positive in all tumors that tested positive

for CK20 (third row versus top row) and another important MCC marker c-Kit (second row versus top row).

Table 2.1 HIP1 immunostaining of paraffin-fixed MCC and SCLC tissue.

Tumor type		3+	2+	1+	No staining	HIP1 positivity (%)
MCC	Total	3	8	14	3	89**
	Primary	2	4	9	1	94
	Metastatic	1	4	5	2	83*
SCLC	Total	0	1	4	7	42
	Primary	0	0	3	2	60
	Metastatic	0	1	1	5	29

**Significant difference compared with patients with SCLC ($p < 0.0025$, χ^2 analysis).
*Significant difference compared with patients with metastatic SCLC ($p < 0.025$, χ^2 analysis).

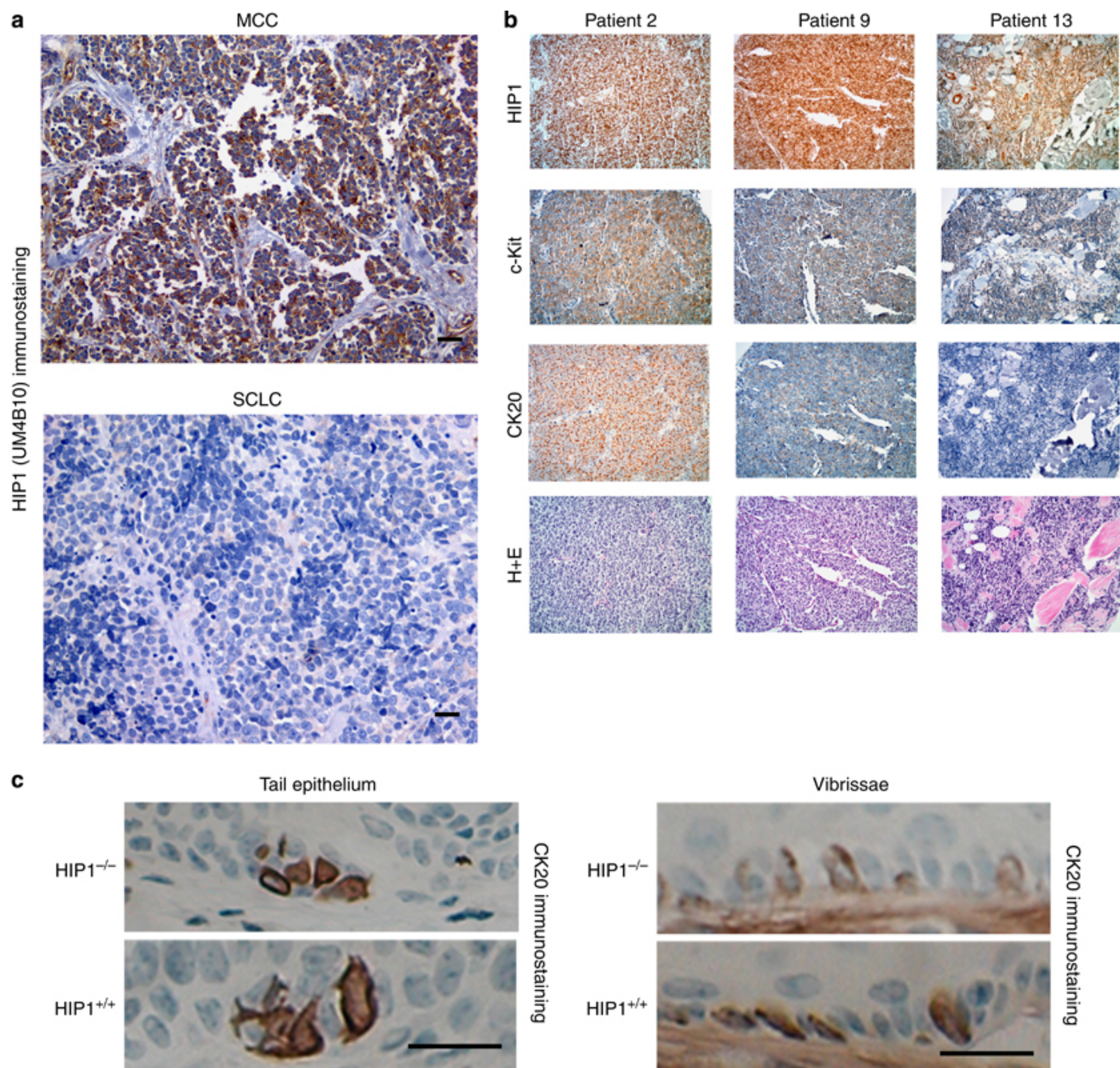


Figure 2.1 Huntingtin-interacting protein 1 (HIP1) is expressed at high levels in primary Merkel cell carcinoma (MCC) but not in small-cell lung cancer (SCLC), and is not required for normal Merkel cells. (a) Example of HIP1 staining in MCC compared with SCLC tumors. Scale bar represents 50 μm. (b) Three representative patient tumors co-immunostained for HIP1 (UM4B10), c-Kit, cytokeratin 20 (CK20), and hematoxylin and eosin (H+E). These tumors were selected from a MCC tissue microarray that contained 42 MCC spots from 14 patients. (c) CK20 staining of Merkel cells in wild-type and Hip1null/null (Oravecz-Wilson et al., 2004)mouse tail skin and vibrissae. Scale bar represents 50 μm.

HIP1 does not affect the development or maintenance of normal Merkel cells

To determine whether HIP1 is necessary for the development of normal Merkel cells, we analyzed the skin of wild-type and HIP1-null mice (Oravecz-Wilson et al., 2004). Anti-CK20 antibodies were used to identify mature Merkel cells in the mouse tail skin and vibrissae, locations where Merkel cells generally congregate around hair follicles. No visible changes in the abundance of mature Merkel cells were observed in the HIP1-null mouse skin as compared with wild-type littermate skin (Figure 2.1c). These data indicate that HIP1 is not required for the development or maintenance of normal Merkel cells.

MCC patients harbor anti-HIP1 autoantibodies in their blood

To detect the presence of anti-HIP1 antibodies in MCC patient blood, patient sera were tested for immune reactivity to HIP1 antigens as described previously (Bradley et al., 2005). Initially, serum samples were screened against the previously described carboxyl-terminal HIP1 recombinant antigen (Bradley et al., 2007a; Bradley et al., 2005; Bradley et al., 2007d). In all, 85% of MCC patients (n=34/40) displayed the presence of autoantibodies (Figures 2.2 and 2.3). This frequency was similar to that previously found in patients with glioblastoma multiforme (Bradley et al., 2007a). This high antibody prevalence and the elderly nature of this population (Table 2.2) raised the question of whether the humoral response was the result of coexistence of other types of tumors with elevated HIP1 levels. We found that there were many coexisting basal and squamous cell carcinomas in this patient cohort but very few other tumors. There was no correlation of a humoral response with previous cancer diagnoses (Table 2.3). The high degree of HIP1

seropositivity to this antigen also made intergroup comparisons difficult; hence, in the interest of improving the specificity of the test, patient sera were also tested for reactivity against a different HIP1 recombinant antigen that encoded the amino terminus. This antigen contains the lipid-binding (ANTH), clathrin-binding, and activator protein-2-binding domains (Figure 2.2C). Only 30% of MCC patients (n=12/40) harbored antibodies against the HIP1 N-terminal antigen (Figure 2.2A and 2.3B). This frequency was low enough to make possible clinical correlations between patients that were positive or negative for these antibodies.

Sera from patients with metastatic MCC exhibited high antibody reactivity to the HIP1 N terminus.

To examine the possibility that anti-N-terminal HIP1 antibodies in MCC patient sera could correlate with a biological outcome, the humoral response to the N terminus of HIP1 in MCC patients were compared with a number of clinical parameters. These parameters included tumor size, disease status at time of blood draw, presence of metastasis at time of blood draw, past (or concurrent) other cancer diagnoses (Table 2.3), survival of 2.5 years after blood draw, age, and gender. Of these parameters, only the presence of metastasis and female gender displayed a significant association with high serum reactivity to HIP1.

Patients with metastatic MCC tested positive for elevated anti-N-terminal HIP1 antibody reactivity much more frequently than patients with localized primary tumors (Table 2.2; Figure 3; 46% vs. 0%; $p<0.005$; Pearson's χ^2 analysis). This test in this cohort was 100% specific. No patient with localized disease tested positive for anti-HIP1 antibodies in their serum. Hence, a positive test for anti-N-terminal HIP1 antibody

reactivity marked the presence of metastatic disease. Additionally, a non-significant trend was also observed (Table 2.2) between autoantibody presence and extensive metastatic disease compared with microscopic and local lymph node metastases (Table 2.2). In contrast, the frequent presence of antibodies against the C-terminal HIP1 antigen in patient sera (85% of patients) did not significantly associate with metastasis as 62% of patients with localized disease also tested positive for antibodies against the HIP1 C-terminal antigen.

As might be expected, survival after a positive test for the anti-N-terminal HIP1 antibodies was poor because the patients were of advanced stage (metastatic disease). In total, 5 out of 10 patients (50%) with metastatic disease and high antibody reactivity against the HIP1 N terminus (Figure 2.4; group 1) were deceased from disease after a 2.5-year observation period. This contrasts with the fact that 25% (3/12) of the patients with metastatic disease and negative anti-N-terminal HIP1 antibody tests were dead at 2.5 years (below the line in Figure 2.4; group 2). This was not a significant survival difference. In contrast to the 25–50% death rate in the patients with metastatic disease was a 100% survival of those patients with localized disease and a low anti-N terminal HIP1 antibody reactivity (right hand side of Figure 2.4; group 3). Two of the original patients in cohorts 1 and 2 were lost to follow-up, explaining the decrease in patient numbers between cohorts 1 and 2 in the metastasis (Table 2.2) and subsequent 2.5-year survival data described above.

A disproportionate number of female patients had elevated anti-HIP1 antibody reactivity in their blood. Approximately 45% of female patients had high anti-N-terminal HIP1 antibody reactivity, whereas only 11% of male patients had high anti-N-terminal

HIP1 antibody reactivity (data not shown). The positive association between anti-HIP1 antibodies and female gender was significant (p=0.05; Pearson's χ^2 analysis). In fact, when males were excluded, the association between metastasis and the presence of N-terminal antibodies tightened (p=0.001). In addition, no association between the presence of anti-N-terminal HIP1 antibodies and age or tumor size was identified in this elderly patient population (Table 2.2).

Table 2.2 Frequency of positive anti-HIP1 (N-terminal) antibody blood test in metastatic MCC.

Patient status	Positive	Negative	Frequency	Age (years) ±SD	%Male
All MCC patients (n=40)	13[#]	27	0.33	69 ± 12	45
No metastases (n=13)	0	13	0.00	71 ± 12	54
Metastatic disease (n=26)	12	14	0.46**	67 ± 11	42
Extensive Metastases	6	3	0.67	72 ± 12	33
Local Metastases	3	4	0.38	63 ± 10	50
Micrometastases	3	6	0.33	66 ± 9	44

**Significant difference compared with patients without metastatic disease ($p < 0.005$, χ^2 analysis)
[#] One patient was lost to follow-up and metastatic status could not be determined

Table 2.3: Prior cancer diagnoses in MCC patients tested for anti-HIP1 antibodies.

	No. of Patients	anti-N-terminal HIP1	anti-C-terminal HIP1
Basal Cell Carcinoma (BCC)	1	1	1
Squamous Cell Carcinoma (SCC)	2	0	2
SCC and BCC	6	2	6
Lymphoma	2	0	2
BCC and Lymphoma	1	0	0
Breast Cancer	1	0	0
BCC and Breast Cancer	1	0	1
Melanoma	1	0	1
Polycythemia vera	1	0	1
Oral SCC +BCC	1	0	1
Active Disease at Blood Draw*	5	1	5
No other cancers^	23	9	19

*Patients with active MCC disease at the time of blood draw are listed below the other diagnoses.

^HIP1 antibody findings for MCC patients carrying no other cancer diagnoses are also listed for comparison, as well.

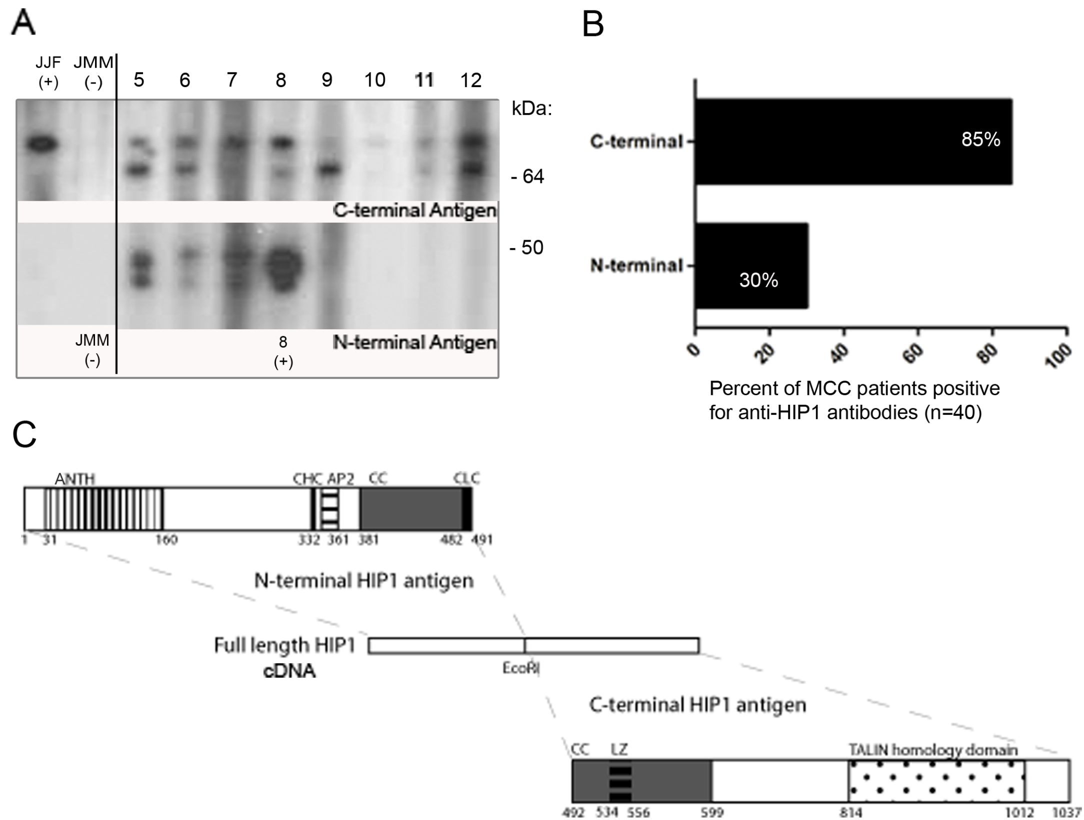
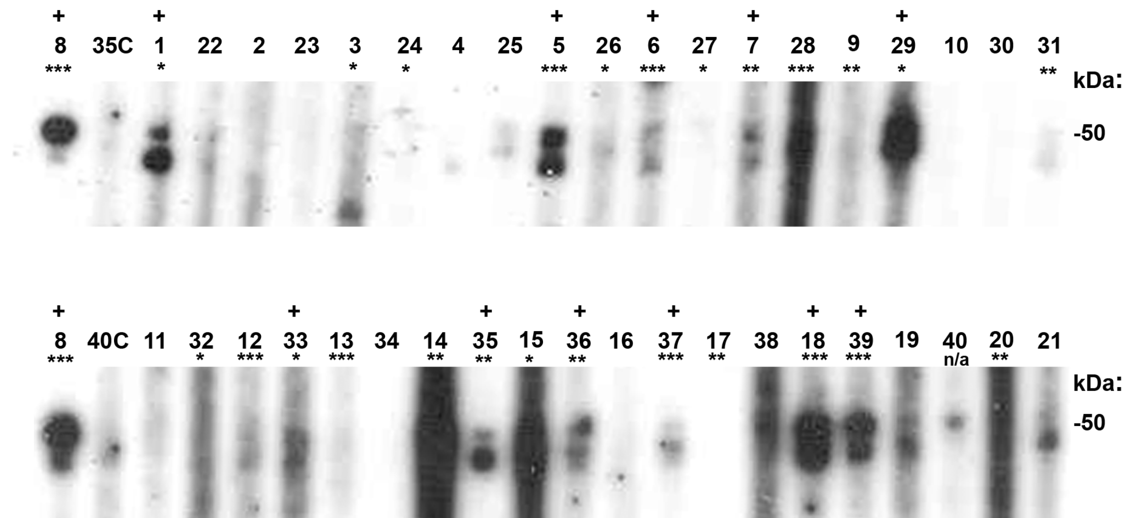


Figure 2.2 Anti-HIP1 antibody testing in MCC patient samples using an additional newly designed antigen. *A*, Representative serum immunoblots from individual MCC patients (numbers 5-12) for anti HIP1 autoantibodies against the new N-terminal and the old C-terminal antigens. Positive and negative control samples for the C-terminal antigen are from patients JJF and JMM, respectively. These two patients were from our previous lymphoma study (Bradley et al., 2007d). Patient 8 from the N-terminal antigen blot (*) was used as a positive control for the remaining samples that were analyzed for N-terminal anti-HIP1 antibodies. *B*, Percent of MCC patients with anti-HIP1 antibodies specific to the N-terminal or C-terminal antigens. Auto-antibodies against a HIP1 antigen were considered present if the optical density of the western blot reading was equal to or greater than 20% of an internal positive control. *C*, Diagram of the functional domains of the HIP1 protein that are contained within the C-terminal and N-terminal antigens. ENTH= Epsin N-terminal homology domain. CHC=clathrin heavy chain-binding domain. AP2 = AP-2 adaptor protein-binding region. CC = coiled-coil domain. CLC = clathrin light chain-binding domain. LZ=leucine zipper. Talin Homology domain = actin-binding region.

N-terminal antigen



+ = >20% of positive control
* = micrometastatic disease
** = local metastatic disease
*** = extensive metastatic disease

C-terminal antigen

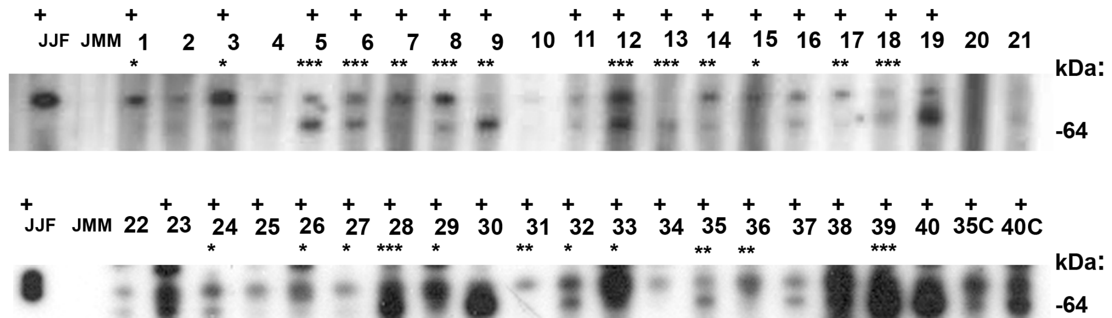


Figure 2.3 Serum blots for N-terminal and C-terminal HIP1 autoantibodies from MCC patients. Blots are shown which were used to calculate serum antibody titer values used in Figure 3 and Table 2. Individual lanes are marked with a patient identification number, a + signs to mark positive tests, and stars to indicate disease stage, as listed in the figure legend. JJF and JMM (6) served as positive and negative controls for C-terminal antigen blots, respectively. Patient 8 serum and 35C or 40C (companion controls) were used as positive and negative controls for N-terminal antigen blots, respectively.

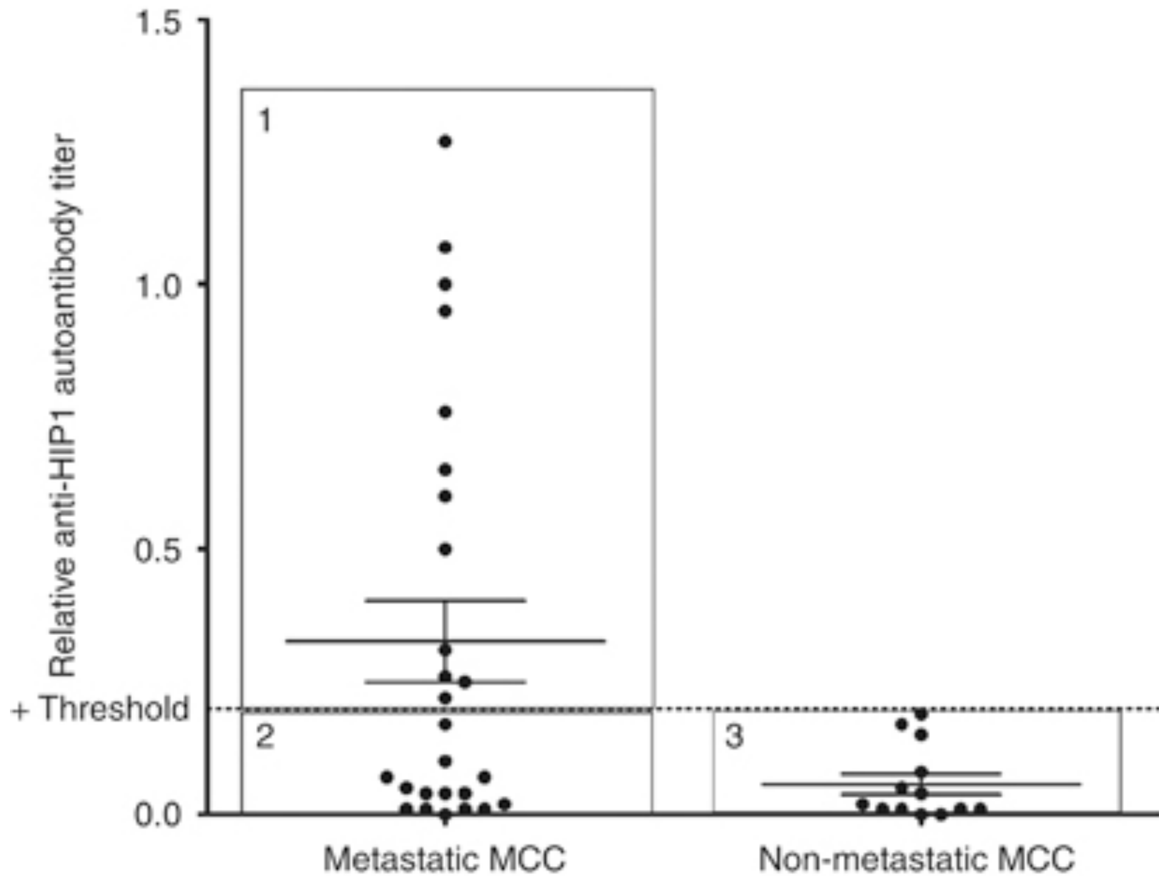


Figure 2.4 Antibody reactivity against the N-terminal antigen is elevated in metastatic MCC patients. Individual dots represent relative antibody titers from patients with either metastatic MCC or localized MCC. Members of cohorts 1-3 were defined by test result and whether or not their disease was metastatic are enclosed by the boxes. Mean and standard error of the mean for each data set are indicated by horizontal and vertical lines, respectively.

HIP1 interacts physically and functionally with the c-Kit RTK

MCC tumors express significant levels of several RTKs (Brunner et al., 2008); however, expression of the receptors previously reported to interact with HIP1, including EGFR (Bradley et al., 2007a) and fibroblast growth factor receptor 4 (Wang et al., 2008), were not among those found to be increased in MCC tissues. Thus, we tested the ability of HIP1 to physically interact with c-Kit, an RTK that is frequently evaluated in these patients because it is expressed at high levels in MCC tumors (Figure 2.1c)(Brunner et al., 2008). As c-Kit is rarely expressed in normal adult tissues other than progenitors of the hematopoietic system such as rare hematopoietic stem cells (Bernex et al., 1996), we coexpressed V5 tagged complementary DNA for c-Kit along with myc tagged complementary DNA for HIP1 in human embryonic kidney 293T cells to obtain enough material for analysis. HIP1 was immunoprecipitated from the whole-cell lysate using rabbit anti-myc sepharose beads. Western blot analysis of the immunoprecipitate showed that c-Kit was specifically co-immunoprecipitated with HIP1. In the absence of overexpressed HIP1, there was no co-immunoprecipitation (Figure 2.5a, top panel, lane 1 vs. 2). In addition, the reverse immunoprecipitation with anti-V5 sepharose beads also demonstrated co-immunoprecipitation with HIP1 (Figure 2.5a, bottom panel, lane 4 vs. 5).

We hypothesized that if this interaction was functionally related to endocytosis, it may be dependent on activation of the receptor. Addition of stem cell factor (SCF), the c-Kit ligand, 1 hour before cell collection for immunoprecipitation did indeed enhance the detected interaction between c-Kit and HIP1 (Figure 2.5a, lanes 3 and 6). Western blot analysis of whole-cell lysates from these cells showed no differences in c-Kit

expression in those cells treated with SCF as compared with untreated cells. We also observed an interaction between endogenous HIP1 and c-Kit in a MCC cell line (MCC565) when SCF was added to the cell media 1 hour before cell harvest. This interaction was not observed in the absence of SCF (Figure 2.5b). We also examined whether HIP1 overexpression has the ability to inhibit the degradation of the c-Kit receptor similar to the effect of HIP1 on EGFR and fibroblast growth factor receptor 4 levels. Indeed, HIP1 stabilized c-Kit following SCF stimulation of starved and cycloheximide-treated cells. The receptor levels were significantly higher 1 and 2 hours after stimulation when HIP1 was overexpressed with c-Kit (Figure 2.5c). These data together suggest that the interaction of HIP1 with c-Kit is functionally important.

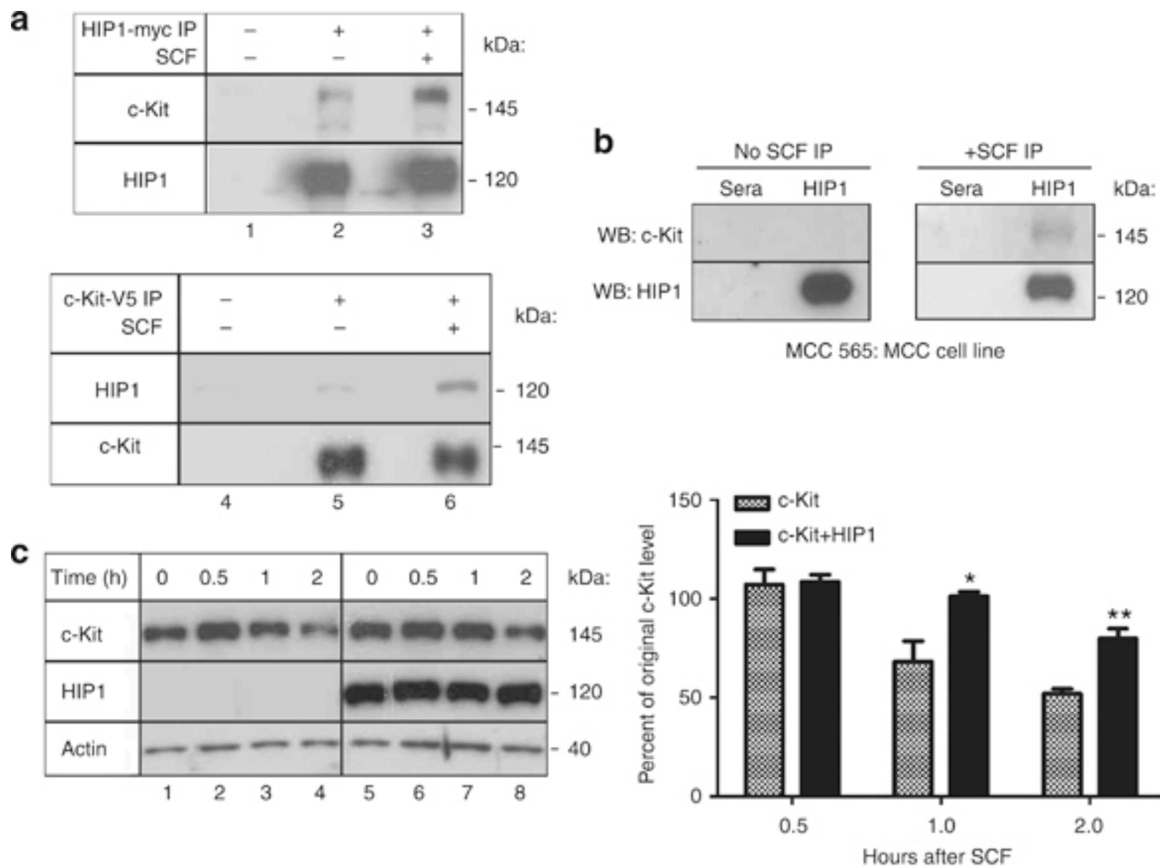


Figure 2.5 HIP1 interacts with c-Kit a RTK that is expressed at high levels in MCC. *A,* Association of HIP1 with c-Kit in HEK 293T cells was detected by co-immunoprecipitation. This interaction was enhanced by stimulation with the c-kit ligand SCF (lanes 3 and 6). *B,* HIP1 associates with c-Kit in a SCF stimulated MCC cell line. The MCC565 cell line was, or was not treated with SCF for one hour prior to collection. HIP1 was precipitated from the cell lysates (9mg) using the rabbit polyclonal antibody UM410 and blotted for human HIP1 or c-Kit. *C,* Prolongation of c-Kit's half life by HIP1 was observed in three independent experiments. On the left is a representative western blot demonstrating the stabilization of c-Kit by HIP1 compared to vector transfected cells after treatment of cells with SCF. * $p < 0.05$, ** $p < 0.01$, Two-tailed t-test.

2.4 Discussion

MCC is a rare cancer, for which investigation of the molecular mechanisms of its cause and maintenance, to guide the development of better treatment regimens, has only recently received significant attention. Patients with MCC have a poor prognosis similar to patients with other neuroendocrine tumors such as SCLC. In contrast to SCLC patients

(Socinski and Bogart, 2007), MCC patients suffer from a lack of therapies and prognostic markers (Bichakjian et al., 2007). In this study, we demonstrate not only that HIP1 is a useful immunohistochemical marker for MCC but also that autoantibodies against the N terminus of HIP1 in patient sera predict the presence of metastatic disease. The discovery of high HIP1 levels in the tumors of MCC patients has led to the hypothesis that HIP1, which acts as an oncoprotein when expressed at high levels, contributes to the mechanism(s) of MCC development, maintenance, or progression. Our data suggest that overexpression of HIP1 leads to elevated RTK levels through its prevention of receptor degradation and therefore may increase pro-growth signals, leading to transformation of Merkel cells.

The high levels of HIP1 or fragments of HIP1 released from the cytoplasm of necrotic or dying MCC tumor cells likely serve as immunogens in MCC patients, leading to the cancer specificity of a positive anti-HIP1 antibody test (Bradley et al., 2005). Here, we employed both a different N-terminal HIP1 test antigen and the previously described C-terminal HIP1 test antigen for anti-HIP1 antibody analysis (Bradley et al., 2005) and found that antibodies against the N terminus of HIP1 are present more frequently in patients with metastatic MCC than in patients with localized MCC. This association was not found for the C-terminal antigen, as many more patients had a positive test result, making correlation with prognostic factors insignificant. Notably, although anti-HIP1-N-terminal antibodies were associated with metastasis, no other clinical parameter, such as tumor size, was associated with antibody test results. As locally treated MCC is often recurrent and can unpredictably metastasize and become unmanageable (Bichakjian et al., 2007), future prospective studies of this upgraded anti-HIP1 blood test using both

antigens will be important to execute. These clinical trials will also help to determine whether the presence of these antibodies serves as a marker of existing metastatic disease alone or whether the antibodies are predictive of tumor metastatic potential. If the presence of HIP1 autoantibodies can serve as a predictive marker in early disease stages, then positive blood test results could indicate the need for more aggressive early adjuvant therapy.

The prognostic results associated with this improved HIP1 N-terminal antigen blood test support re-evaluation of patients with other metastatic and localized epithelial cancers for antibodies against the N-terminal HIP1 antigen compared with patients with localized disease. For example, as HIP1 overexpression in prostate cancer tumors is associated with poor prognosis (Rao et al., 2002), it is possible that relapsing patients will also have increased anti-N-terminal HIP1 antibody reactivity like metastatic MCC patients. As the decision to surgically resect prostate tumors is often difficult because of potential urological side effects, the value of a blood test to predict which tumors are likely to metastasize would be useful. Moreover, a combination test for both HIP1 N- and C-terminal antibodies may be more sensitive and specific than either test alone.

The gender-specific association of metastases with the N-terminal anti-HIP1 antibodies that was found mainly in the female patients was intriguing. This observation is similar to the known increased frequency of autoimmune diseases in women compared with men (Lockshin, 2006). In addition, a recent report found that in MCC patients female gender correlated with better survival (Kaae et al., 2010). Perhaps, the ability to immunologically respond to high HIP1 levels improves prognosis. For example, in B-cell lymphoid malignancies, anti-HIP1 reactivity correlated with good outcome (Bradley

et al., 2007d). Of course, future tests of the prognostic value of antibodies to the HIP1 N-terminal antigen will still include men, as this study examined too few men with metastases (n=10) to be conclusive. Future prospective trials will be important to either confirm or refute these initial gender-specific results. The mechanisms of how HIP1 transforms cells remain a subject of investigation. The prevailing hypothesis is that HIP1 inhibits the degradation of active RTKs during the process of receptor-mediated endocytosis (Hyun and Ross, 2004) because of the data showing that its overexpression stabilizes RTKs following receptor activation (Hyun et al., 2004b). For example, cells transformed by HIP1 overexpression have elevated EGFR levels, and specific EGFR inhibitors inhibit the transformed phenotype, suggesting that this receptor stabilization is an essential element of HIP1- mediated transformation (Rao et al., 2003). Previous reports have also shown that HIP1 physically interacts with EGFR (Bradley et al., 2007a) and fibroblast growth factor receptor 4 (Wang et al., 2008). Neither of these receptors is known to be expressed in MCC, suggesting that the tumorigenic function of HIP1 in MCC may be mediated through modulation of a different RTK.

For example, the c-Kit RTK is often overexpressed in MCC and other tumor types, such as breast tumors, SCLC, colorectal cancers, and gastrointestinal stromal tumors, where it is a pharmacological target of imatinib (Sattler and Salgia, 2004; Su et al., 2002). A recent report has linked c-Kit overexpression to poor prognosis in MCC (Andea et al., 2010). The finding here of HIP1's ability to interact physically with c-Kit and to increase c-Kit levels provides a plausible mechanism for how HIP1 might promote tumorigenesis in MCC. It is less clear, although important to determine, how high HIP1 levels in MCC might relate to Merkel cell polyomavirus infection of Merkel cells. If

these two abnormalities are mechanistically linked, i.e., if HIP1 is upregulated by Merkel cell polyoma virus or vice versa, then targeting the regulator would be expected to affect the other. This possibility could be tested *in vitro* with knockdown of HIP1 or large T antigen to determine the effect on each other's expression.

In summary, we report that HIP1 is to our knowledge a previously unreported marker of MCC, a neuroendocrine tumor of the skin, and that a blood test for anti-HIP1 antibodies may provide prognostic information. The original assay (Bradley et al., 2005) was supplemented by use of a different recombinant HIP1 N-terminal antigen. The findings with this test will necessitate future studies to determine whether the distinct anti-HIP1 antibodies are reflective of metastatic potential of other tumors (prostate, lymphoid, and brain) expressing high levels of HIP1. Prospective trials that include a larger number of patients and serial blood samples will be needed to validate these results to facilitate improved management of MCC patients. Finally, we report that HIP1 physically interacts with and stabilizes the c-Kit RTK and that this interaction is modulated by the c-Kit ligand, SCF. Results of future studies that elucidate all of the RTKs that interact with HIP1 in MCC, the domains through which these interactions are mediated, and the effects of these interactions on transformation and signaling will be enlightening. Designing drugs for specific inhibition of the interactions between HIP1 and RTKs may prove therapeutic to many cancer patients including those afflicted with MCC.

2.5 MATERIALS AND METHODS

MCC and SCLC tissue samples

Archived formalin-fixed and paraffin-embedded MCC and SCLC tissue samples were obtained from the Pathology Department at the University of Michigan Medical Center. Diagnoses were determined by CK20, thyroid transcription factor-1, synaptophysin, chromogranin A, morphology, and the site of the primary tumor. TMAs were generated from 14 of the MCC patient tumors as described previously (Perrone et al., 2000) and cores were spotted in triplicate. These patient samples were not linked to clinical data or other identifying information.

Immunohistochemical staining

Immunohistochemical staining for HIP1 was performed as described previously (Bradley et al., 2007a) with appropriate negative (no primary antibody) and positive (glioblastoma) controls. Staining for Merkel cells in mouse skin was performed using the mouse monoclonal antibody Ks20.8 (Thermo Scientific). Photomicrographs of the immunohistochemical staining were taken with a Olympus BX41 microscope. Patients were analyzed for serum antibodies against HIP1. The study of patients with MCC and serum levels of anti-HIP1 antibodies was approved by the University of Michigan Internal Review Board, where written and informed patient consent and adherence to the Declaration of Helsinki Principles was confirmed. Serum from 40 clinicopathologically confirmed MCC patients was collected at the University of Michigan Merkel Cell Carcinoma clinic in a period of 6 months, ranging from 2007 to 2008. Serum was aliquoted into 20 μl portions for single use to avoid freeze–thaw cycles and stored at -80°C. The ages, genders, and tumor stages of these patients are displayed in Table 2.

Preparation of HIP1 antigen

Glutathione S-transferase HIP1 (3' and 5') fusion complementary DNAs were used to generate C- and N-terminal recombinant antigens, respectively. The C-terminal antigen has been previously described (Bradley et al., 2005). The N-terminal antigen was generated by subcloning an in-frame glutathione S-transferase fusion protein to the 5' end of the region of HIP1 that terminates at the internal EcoRI site in the HIP1 nucleotide sequence. The antigen was produced in bacteria and purified as previously described for the C-terminal antigen (Bradley et al., 2005).

Test for anti-HIP1 antibodies in MCC patient serum.

Immunoblot of patient serum was performed as previously described (Bradley et al., 2005). The optical density reflective of serum antibodies bound to the HIP1 antigen was measured using the ImageJ program, and measurements were made after subtracting the background density above and below the HIP1 antigen in each lane. Lanes that had an optical density of at least 20% of the internal positive control (JMM, a patient with acute lymphoblastic leukemia from Bradley et al. (Bradley et al., 2007d), was tested for the C-terminal antigen and MCC8, a patient with MCC in this cohort, was tested for the N-terminal antigen) were considered positive. From previous studies of mice and humans with prostate (Bradley et al., 2005) and lymphoid cancers (Bradley et al., 2007d), a cutoff of more than 20% of a strong standard (internal positive control) was used to determine if the sera was negative (<20%) or positive (>20%) for reactivity.

Statistics

Data were analyzed using GraphPad Prism 5 statistical software and ImageJ densitometry analysis software. Statistical significant values for Tables 1 and 2 were

calculated using Pearson χ^2 analysis. Statistical significance values in Figure 3 were calculated using the Student's t-test.

Immunoprecipitation.

HEK 293T cells were grown to 70% confluence and transfected with 20 μg of full-length HIP1 and c-Kit (Origene) cDNA in pcDNA3 and pCMV6, respectively. After 24 hours, transfected cells were lysed using an all-purpose non-denaturing lysis buffer (50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 5 mmol/L EGTA, 10% glycerol, complete EDTA-free protease inhibitor tablets (Roche), 30 mmol/L sodium pyrophosphate, 50 mmol/L NaF, and 100 umol/L sodium orthovandate). To ensure maximum protein extraction, cells were manually ground 50 times with plastic pestles and then incubated for 1 hour at 4°C with rotation. The mixture was centrifuged for 15 minutes at 4°C at 13,200 rpm, and the supernatant stored at -80°C until use. Biorad's Protein Assay (cat# 500-0006) was used to determine the protein concentrations of the lysates.

One milligram of protein lysate was pre-cleared by incubating with a 50:50 slurry of the Protein G sepharose beads in lysis buffer (20 μL) for 30 minutes at 4°C with rotation. After centrifugation, the cleared supernatant was incubated with 20 μL of rabbit non-immune serum or the appropriate polyclonal antibodies overnight at 4°C. One hundred μl of a 50:50 slurry of beads were added to the protein-antibody mixture and incubated with rotation for 1 hour at room temperature. These beads were then precipitated and washed three times with lysis buffer. The pellet was then dissolved in 20 μL of 6X Laemmli buffer, boiled for 5 minutes, and centrifuged. The supernatant was used for western blot analysis.

To test if c-Kit immunoprecipitation co-precipitated HIP1, it was necessary to first construct a cDNA that expresses a C-terminal V5 tagged c-Kit receptor. To do this, the full-length c-Kit cDNA was PCR amplified with primers that were compatible for ligation with a HindIII and XhoI digested pcDNA3.1-V5-His vector. The resultant pcDNA3.1/c-Kit-V5-His construct was co-expressed with the pcDNA3.1/HIP1-myc construct in 293T cells. One mg of protein lysate was pre-cleared with Protein G sepharose beads as above and c-Kit-V5 was immunoprecipitated by incubating the lysate with 10 μL of anti-V5 conjugated beads (Sigma) and prepared for western blot as described above. Protein lysates from cells expressing HIP1-myc, but not c-Kit-V5, served as a negative control.

c-Kit receptor stabilization assay.

HEK-293T cells were grown to 70% confluence in 6-well plates and then transfected with 2 μg of c-Kit cDNA in pCMV6 and either 2 μg of full-length HIP1 cDNA in pcDNA3 or 2 μg of empty pcDNA3 vector (total 4 μg DNA). Twenty-four hours after transfection, cells were starved for 16 h in serum-free media. Cells were then treated with 100 mg/mL cycloheximide for 30 minutes followed by the addition of 150 ng/mL c-Kit ligand SCF. Cells were collected at 0, 0.5, 1, and 2 hours post SCF addition in RIPA lysis buffer (150 mM NaCl; 50 mM Tris, pH 8.0; 1% NP-40; 0.5% DOC (deoxycholic acid); 0.1% SDS; complete EDTA-free protease inhibitor tablets (Roche); 30 mmol/L sodium pyrophosphate; 50 mmol/L NaF; and 100 umol/L sodium orthovandate).

Western blot

Whole cell lysates and immunoprecipitated pellets were separated with 6% or 10% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked (TBST with 5% non-fat dry milk), incubated with the appropriate primary antibody overnight at 4°C, washed with TBST, incubated with secondary antibody for 1 hour at room temperature, washed with TBST and developed (ECL, Thermo Scientific). Antibodies for western blot included HIP1/4B10 (anti-human HIP1 mouse monoclonal), HIP1/UM354 (anti-HIP1 rabbit polyclonal-recognizes mouse and human HIP1), anti-c-Kit (Cell Signaling, mouse monoclonal), anti-myc (Upstate Biotechnology, rabbit polyclonal), anti-V5 (Invitrogen, mouse monoclonal) and anti-actin (Sigma, mouse monoclonal).

Chapter 3:
Huntingtin Interacting Protein 1 Binds to and is Tyrosine Phosphorylated by Full-length and N-terminally Truncated Forms of the EGFR kinase

3.1 Abstract

EGFR signaling is responsible for regulating the activity of many proteins, including those involved in its own endocytosis and degradation. Here we have identified a novel EGFR kinase substrate, Huntingtin interacting protein 1 (HIP1). HIP1 is involved in clathrin-mediated endocytosis of EGFR and HIP1 expression is elevated in multiple tumor types. Furthermore, HIP1 transforms fibroblasts and prostate epithelial cells when overexpressed and is hypothesized to promote transformation by slowing EGFR degradation. In addition to being tyrosine phosphorylated by EGFR, we show here that HIP1 preferentially binds a smaller form of EGFR that is the result of an N-terminal cleavage. This truncated EGFR structurally resembles the oncogenic EGFRvIII, with which we also observed interaction as well as phosphorylation. HIP1 also binds, but is not readily phosphorylated by the oncogenic receptors IGF-1R and ErbB2. Using structure/function analysis, we found that HIP1 interacts with the EGFR kinase domain and that EGFR requires the HIP1 UpStream Helix domain to phosphorylate HIP1. Additionally, HIP1 localization is affected by EGFR activation. HIP1 is recruited to the cell periphery following EGF stimulation, colocalizes with EGFR within clathrin-coated pits, and is subsequently recruited to Rab5 positive early endosomes. These data suggest

an EGF regulated role for the oncogene HIP1 in the early endosomal processing of EGFR and its oncogenic mutant EGFRvIII.

3.2 Introduction:

Activity mediated endocytosis of the receptor tyrosine kinase (RTK) epidermal growth factor receptor (EGFR) is a tightly regulated process that results in intracellular signaling, receptor recycling, or degradation. Although EGFR is one of many proteins in the RTK family, its mechanism of endocytosis is the best understood due to a wealth of reagents for its study and a strong foundation of prior research (Sorkin and Goh, 2008). EGFR endocytosis is triggered by activation of the EGFR kinase through binding to its ligand, epidermal growth factor (EGF), which leads to dimerization of the receptor and activation of its tyrosine kinase. EGFR then phosphorylates itself and other proteins to initiate downstream signaling and begin the process of endocytosis (Sorkin and Goh, 2008). EGFR self-activation is critical for recruiting growth factor receptor-bound protein 2 (Grb2) to its C-terminal domain through the phosphorylated tyrosine Y1068 (Huang and Sorkin, 2005). Also recruited to the C-terminal domain are adaptor protein 2 (AP2) (Nesterov et al., 1995), and Cbl, an E3 ubiquitin ligase responsible for targeting EGFR to late endosomes and lysosomes through interactions with ubiquitin interacting proteins Epsin 1, Eps15 and Hrs (Huang and Sorkin, 2005; Sorkin and Goh, 2008). The process of EGFR endocytosis is often dysregulated in cancer to enable increased pro-growth and pro-survival signaling through stabilization of EGFR (Mosesson et al., 2008).

Huntingtin interacting protein 1 (HIP1) is a largely uncharacterized EGFR adaptor protein. HIP1 was first linked to RTK signaling when it was identified in a leukemia patient as part of a chromosomal translocation linking the majority of the *HIP1* gene to

the C-terminal transmembrane and split tyrosine kinase domain-s of the platelet derived growth factor β receptor (PDGFβR) (Ross et al., 1998). This translocation led to the formation of a constitutively active RTK that could transform the Baf3 cell line to IL-3 independent growth (Ross and Gilliland, 1999). It was later determined that HIP1, when overexpressed alone, can transform NIH-3T3 cells as well as prostate epithelial cells (Rao et al., 2003; Wang et al., 2008). This transformation ability, however, remained linked to RTKs, as these transformed cells demonstrated increased expression of EGFR and fibroblast growth factor 4 (FGFR4) (Rao et al., 2003). Additionally, treatment of these cells with an EGFR inhibitor reversed the transformational ability of HIP1 (Rao et al., 2003). High levels of HIP1 protein have also been correlated with RTK overexpression in a series of tumors from brain and breast cancer patients (Bradley et al., 2007a; Rao et al., 2003). One possible cause of EGFR overexpression in these cancers is HIP1-dependent stabilization of the EGFR via inhibition of the endocytic degradation pathway. This inhibition of EGFR degradation is an activity of HIP1 that has been observed in EGF stimulated cells (Hyun et al., 2004b).

Proteins known to interact with HIP1 during receptor-mediated endocytosis include clathrin and the AP2 adaptor complex (Metzler et al., 2001). HIP1 also demonstrates limited actin binding through its TALIN homology domain (Legendre-Guillemin et al., 2002). Studies in yeast, using the homologous protein Sla2p, have led to a model in which HIP1 is recruited to newly forming endosomes, which leads to formation of an actin network, followed by clathrin assembly to form the endosome (Newpher et al., 2006). When clathrin binds HIP1, a conformational change occurs in the UpStream Helix (USH) domain that disrupts the binding of HIP1, and thus the endosome,

to surface actin, freeing it for internalization (Wilbur et al., 2008). More recently, HIP1 also has been shown to bind endosomal cargo, including EGFR (Bradley et al., 2007a). Beyond these steps, very little is known about the mechanics of HIP1 regulation of endocytosis. For example, the role of HIP1's association with internalized clathrin-coated vesicles and its mechanism of stabilizing receptors remain significant gaps in knowledge.

Here we have identified HIP1 as a novel substrate for the EGFR tyrosine kinase that binds EGFR within its kinase domain. We have also demonstrated that HIP1 preferentially binds to a truncated form of EGFR that has not been previously characterized, but is structurally similar to the EGFR oncogenic mutant EGFRvIII. HIP1 also displayed prolonged association with EGF within clathrin-coated vesicles and EGFR stimulation was sufficient to recruit HIP1 to early Rab5-positive endosomes. These data improve our understanding of HIP1's role in EGFR trafficking following endocytosis and may lead to a means for disrupting HIP1's oncogenic stabilization of RTKs during endocytosis.

3.3 Results:

HIP1 interaction with the EGF receptor depends on the presence of the kinase domain

We previously observed an interaction between HIP1 and the RTK EGFR (Bradley et al., 2007b). To further characterize the interaction between HIP1 and the EGF receptor, we decided to identify the structural elements within EGFR that are necessary for HIP1 binding. First, we tested the ability of HIP1 to interact with several different C-terminal truncation mutants of EGFR (Macdonald-Obermann and Pike,

2009). These mutants contain deletions that span the entire length of EGFR's cytoplasmic domain (Figure 3.1A). The EGFR mutants were sub-cloned into pcDNA3.1.V5.His with a C-terminal V5 tag to facilitate equal detection of all the mutants analyzed using the same V5 antibody.

As expected, HIP1 bound full-length EGFR (Figure 3.1B). HIP1 also interacted with the C-terminal truncation mutant c'973 (Figure 3.1C), but not c'698, an EGFR deletion mutant that also lacks the EGFR kinase domain (Figure 3.1D). Also as expected, HIP1 did not bind EGFR lacking the entire EGFR cytoplasmic domain (Figure 3.2). Sequences C-terminal to amino acid 973 are known to be involved in endocytic regulatory processes. Notably, the AP2 complex, which can bind both HIP1 and EGFR, binds EGFR in this domain (Sorkin and Goh, 2009) indicating that AP2 is not necessary to bridge the interaction between HIP1 and EGFR. The region C-terminal to amino acid 973 is also where Grb2 binds along with its binding partner, the E3 ubiquitin ligase Cbl, allowing receptor ubiquitination to occur (Sorkin and Goh, 2009). Most importantly, amino acid Y1045 is necessary for Cbl recruitment to EGFR and EGFR ubiquitination (Sorkin and Goh, 2009). Because HIP1 interacts with this deletion mutant that is known to have poor ubiquitination ((Sorkin and Goh, 2008)), receptor ubiquitination is also dispensable for HIP1 interaction with EGFR. To summarize, we found that the EGFR kinase domain but not the C-terminal, Cbl binding domain, is necessary for HIP1 association with EGFR.

Although ubiquitination was not necessary for HIP1 interaction with EGFR, we also hypothesized that ubiquitination may disrupt binding of HIP1 to the EGFR kinase domain, because this is the primary site for ubiquitin linkages. Immunoprecipitation of

HIP1 with EGF-stimulated EGFR demonstrated that HIP1 binding to EGFR is intact even in the presence of ubiquitin linkages (Figure 3.1E). HIP1 was also able to bind tyrosine-phosphorylated EGFR and may show a preference for binding this form (Figure 3.1F). Though we observed an interaction between HIP1 and a kinase inactivating point mutant of EGFR, K721A, this interaction was weak compared with the strong interaction between HIP1 and wild-type EGFR (Figure 3.1G). It should be noted that the levels of the EGFR K721A mutant compared to wild type EGFR were consistently lower confounding our ability to accurately quantitate the difference in affinity. Interestingly, a spontaneously generated point mutant of HIP1 that we have used in previous studies, F182S (Bradley et al., 2007a; Bradley et al., 2005; Bradley et al., 2007d; Hyun et al., 2004b; Rao et al., 2003; Rao et al., 2001; Rao et al., 2002), could not bind to the EGFR K721A kinase-dead mutant (Figure 3.1H). The phenylalanine residue at position 182 of HIP1 is conserved in HIP1r, the only known mammalian relative of HIP1, as well as in lower organisms such as drosophila and yeast, but is not part of a defined HIP1/HIP1r domain. In summary, among EGFR posttranslational modifications, phosphorylation of EGFR may increase HIP1 binding but ubiquitination is dispensible.

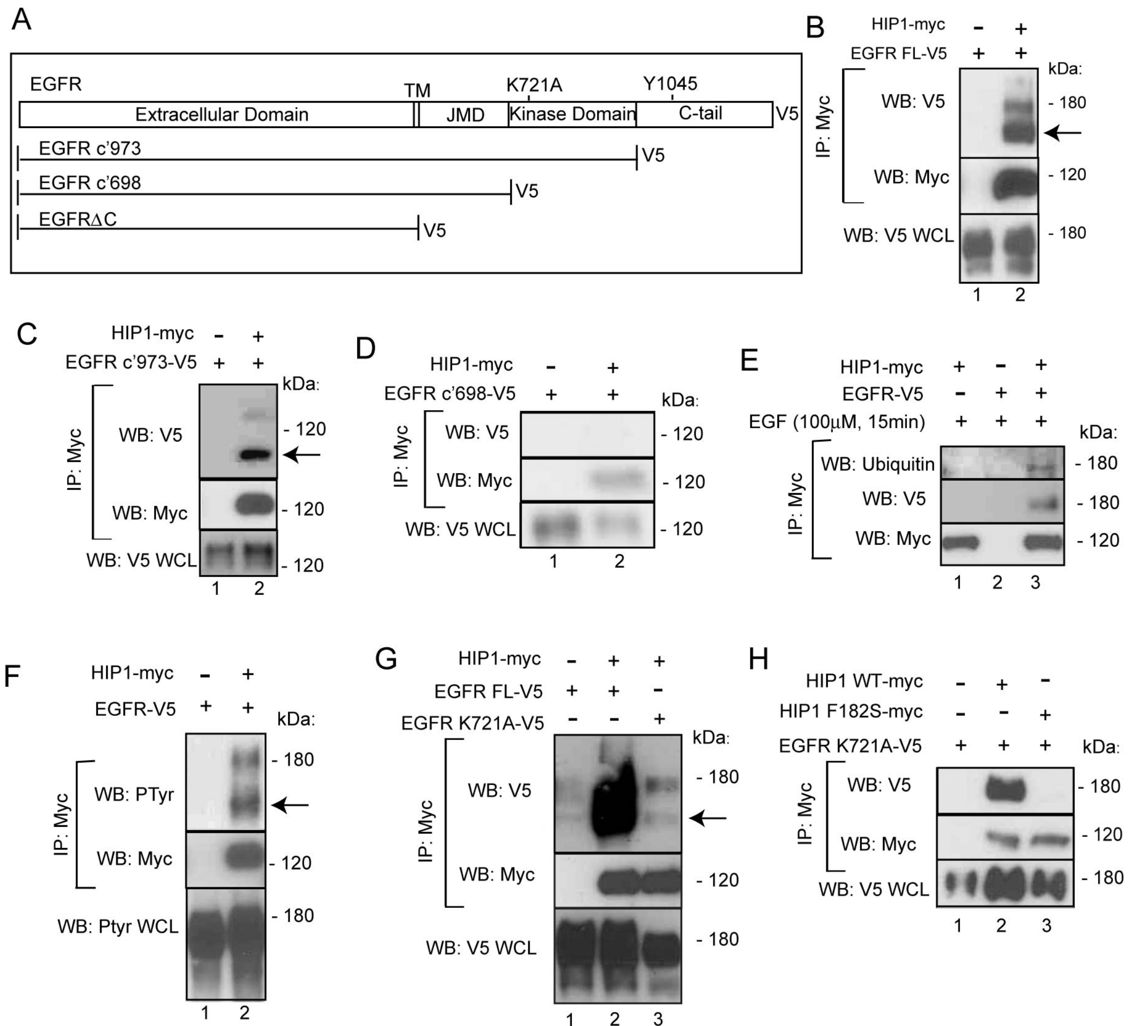


Figure 3.1 HIP1 immunoprecipitation with EGFR deletion and point mutants. *A,* Diagram of C-terminally truncated EGFR mutants (gift of Linda Pike) that were inserted into the pcDNA3.1-V5 expression vector for HIP1 association analysis. *B-D,* Immunoprecipitation of HIP1-myc in 293T cells transfected with HIP1-myc and EGFR FL-V5 (B), EGFR c'973-V5(C), EGFR c'698-V5 (D). *E,* HIP1 interaction with ubiquitinated EGFR. *F*, Interaction of HIP1 with phosphorylated EGFR. *G,* HIP1 interaction with EGFR K721A-V5 and compared to EGFR FL-V5. *H.* HIP1 F182S-myc and HIP1-myc immunoprecipitation with EGFR K721A-V5 in transfected HeLa cells.

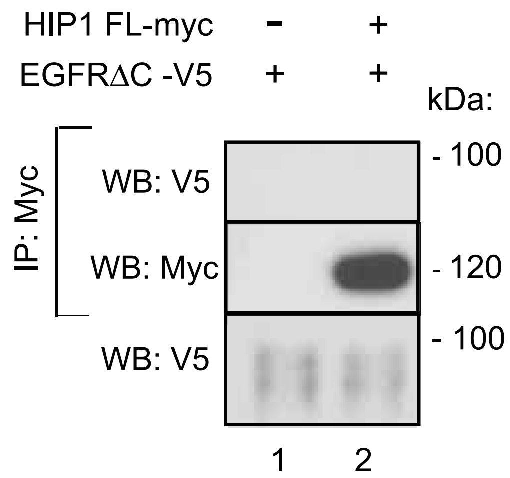


Figure 3.2 Lack of HIP1 interaction with EGFRΔC. HIP1-myc was immunoprecipitated from 293T cells that were co-transfected with EGFRΔC-V5. Pelleted lysates were probed for V5.

HIP1 binding to EGFRtr, a truncated form of EGFR

In addition to decreased interaction of HIP1 with the kinase-dead version of EGFR, we observed that HIP1 bound to a faster migrating form of EGFR that is particularly prominent in transfected HEK 293T cells (Figures 3.1B and 3.3A, arrows). This form will be subsequently referenced as EGFRtr. Because EGFRtr is recognized by the anti-V5 antibody, we concluded that its truncation must have occurred at the N-terminus. Although, EGFRtr was more difficult to detect in HeLa cells, we did observe a small amount of EGFRtr in the HIP1 wild type but not the F182S variant expressing cells (Figure 3.3B). Western blot analysis using the anti-phosphotyrosine antibody 4G10 indicated that EGFRtr was also strongly tyrosine phosphorylated (Figure 3.3A, arrow panel 2), demonstrating that this form of EGFR is mature and has been inserted into the plasma membrane for activation. EGFRtr did not interact with Eps15 and was not readily observed in whole cell lysates, suggesting that HIP1 has a specific and strong affinity for this EGFR form (Figure 3.3C, lane 3). Additionally, the HIP1 F182S variant could not interact with EGFRtr (Figure 3.3A+B). HIP1r was also tested for its ability to bind EGFRtr (Figure 3.3D), and we determined that HIP1r does not bind this EGFR form, although it retains the F182 conserved domain. The increased surface localization and actin binding of HIP1r compared with HIP1(Engqvist-Goldstein et al., 1999; Engqvist-Goldstein et al., 2001; Legendre-Guillemin et al., 2002) may explain why it does not bind EGFRtr.

HIP1's highly specific interaction with EGFRtr led us to hypothesize that HIP1 may also interact strongly with the N-terminally truncated oncogenic mutant of EGFR, EGFRvIII. This mutant, which is often expressed in tumors cells from patients with

EGFR amplified glioblastoma multiforme, has a 267 amino acid deletion at its N-terminus, which leads to constitutive activity and poor degradation (Grandal et al., 2007; Hatanpaa et al., 2010; Huang et al., 1997; Loew et al., 2009). Indeed, we found that HIP1 interacts with this mutated form of EGFR (Figure 3.3E, lanes 3 and 4). EGFRvIII is also approximately 10 kDa larger than EGFRtr, giving a guide for the point of possible EGFR cleavage that leads to EGFRtr formation. We hypothesize that possible EGFR cleavage will be just downstream of the N-terminus of EGFRvIII.

In order to determine the cause for the generation of EGFRtr, we tested a number of agents that could cause a difference in protein migration. First, to see if hyper-phosphorylation was causing accelerated migration, we treated lysates with calf intestine alkaline phosphatase (CIP) to determine if this would shift the migration of this band. Treatment with this phosphatase had no effect on band migration (Figure 3.4A). To test for protease-mediated cleavage through A Disintegrin and Metalloprotease (ADAM) enzymes or other extracellular metalloproteases, we treated cells with the metalloprotease inhibitor marimastat for 12 hours at 100μM. We found that marimastat had no effect on the formation of EGFRtr (Figure 3.4B), indicating that if EGFRtr is a cleaved EGFR, it is formed by another class of proteases.

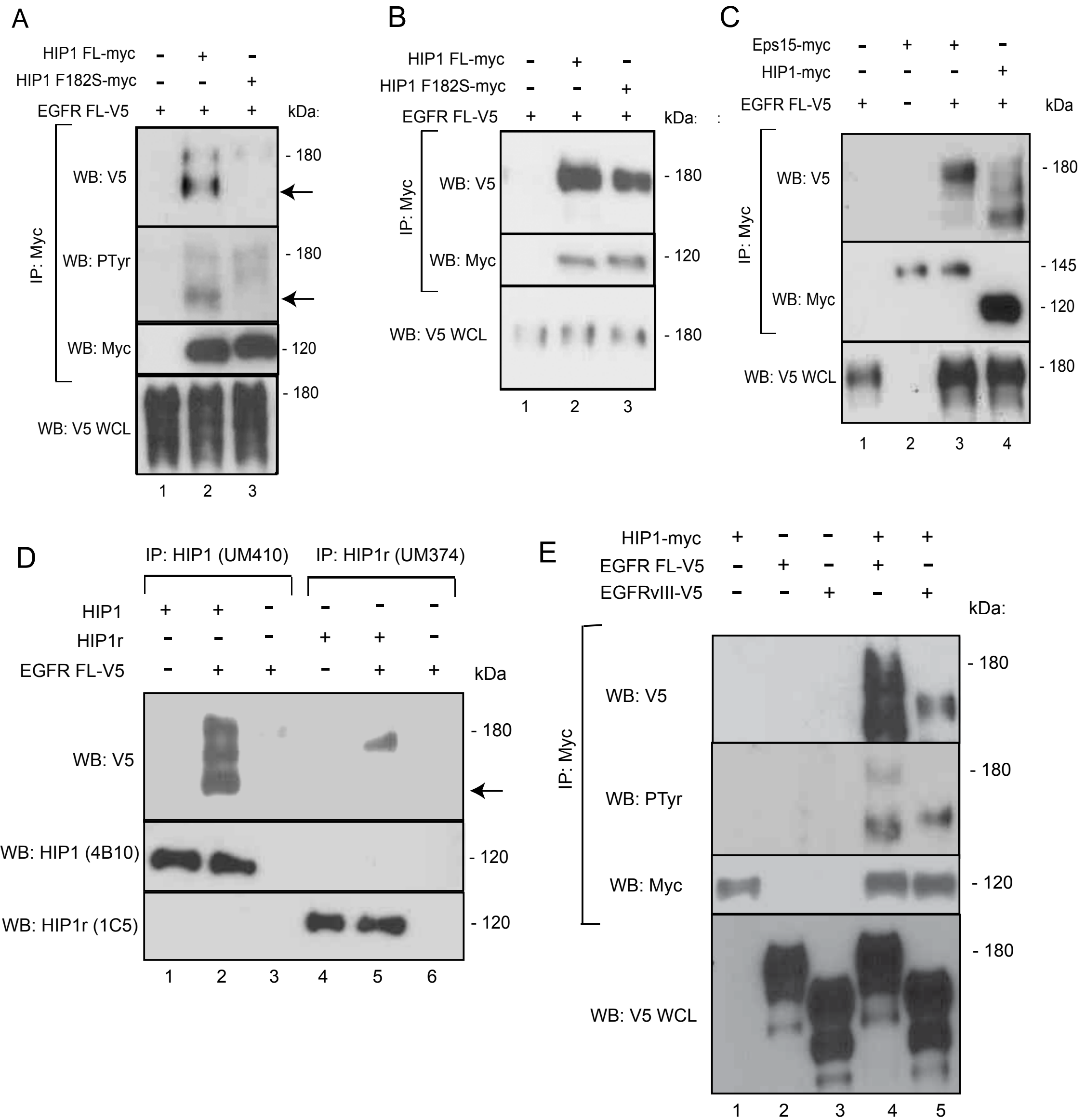


Figure 3.3 HIP1 interaction with a fast migrating form of EGFR. *A-B,* Comparison of EGFR-V5 forms co-immunoprecipitated with HIP1-myc and HIP1 F182S in transfected 293T cell lysate (a) or HeLa cell lysate (B). *C*, EGFR-V5 co-immunoprecipitated with HIP1-myc and Eps15-myc in transfected 293T cell lysate. *D*, Immunoprecipitation of HIP1 and HIP1r with EGFR-V5. UM410 and UM374 polyclonal antibodies (Hyun et al., 2004b) were used for HIP1 and HIP1r immunoprecipitation, respectively. Pellets were probed for the V5 antigen tag via Western blot. *E,* HIP1 interaction with the EGFRvIII EGFR mutant as compared to full-length EGFR.

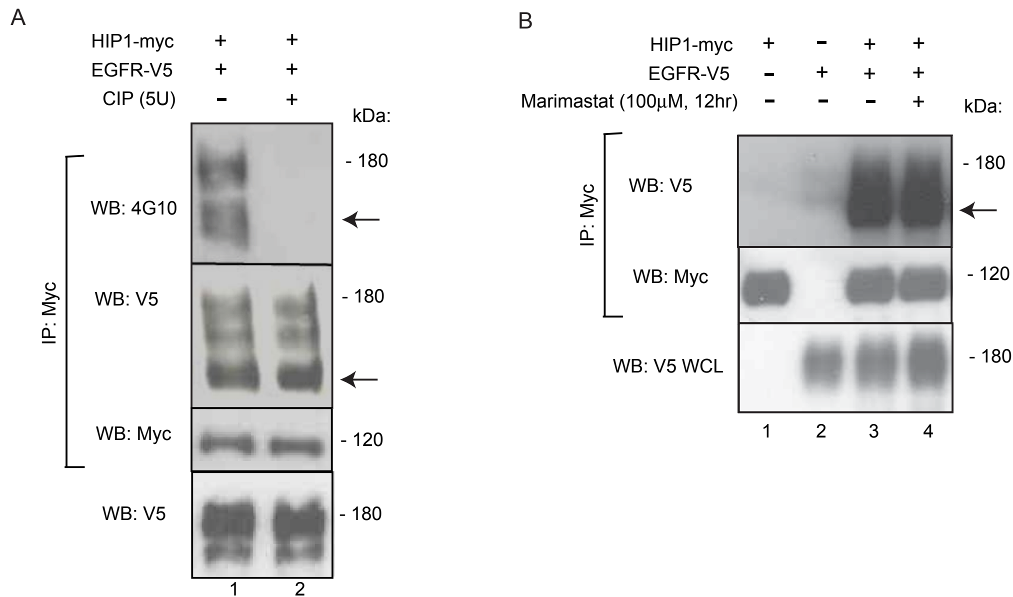


Figure 3.4 Treatment of EGFR with phosphatase and protease inhibitors. *A,* Calf intestine alkaline phosphate (CIP) treatment did not eliminate the EGFRtr band that interacts with HIP1 (arrow). *B,* Treatment of cells with the metalloprotease inhibitor marimastat did not eliminate EGFRtr (arrow).

HIP1 interacts with the EGFR family member ErbB2 and the insulin-like growth factor-1 (IGF-1) receptor

Because of the tyrosine kinase domain of EGFR is necessary for HIP1 association, we tested HIP1 binding to other RTKs that have divergent functions but when hyperactive contribute to tumorigenesis in a variety of cancers. The oncoprotein ErbB2, also known as HER2/neu, is a member of the EGFR family. It lacks its own ligand binding capability but is activated when it dimerizes with itself or other, ligand-bound, EGFR family members. It retains an active kinase domain but is more slowly internalized by receptor-mediated endocytosis pathways compared with EGFR, a quality that has been thought to contribute to its increased signaling in breast cancer (Yarden, 2001). Our data show that HIP1 co-precipitates with both the ErbB2 kinase-active wild-type receptor and with the kinase-dead ErbB2 K753A (Figure 3.5A, lanes 4 and 5), although the kinase-dead mutant showed reduced co-precipitation with HIP1. Additionally, HIP1 bound the activated ErbB2 receptor as evidenced by the tyrosine phosphorylated state of the Her2/neu in the HIP1 co-immunoprecipitation experiment (Figure 3.5A).

The insulin-like growth factor 1 receptor (IGF-1R) is a widely expressed RTK that has two subunits joined by disulfide linkages. It contains a tyrosine kinase domain that is activated by IGF-1 binding. This binding leads to dimerization and downstream pro-growth signaling events. IGF-1R is also elevated in numerous cancer types that have increased HIP1 levels, including prostate cancer, colon cancer, breast cancer, and Merkel cell carcinoma (Ames et al., 2011; Frasca et al., 2008; Keehn et al., 2004; Rao et al., 2002). As expected, wild-type IGF-1R interacted with HIP1 (Figure 3.5B, lane 3).

Additionally, as observed with both EGFR and ErbB2, a kinase dead mutant of IGF-1R (K1003R) demonstrated diminished, although not absent binding to HIP1 compared with that of wild-type IGF-1R (Figure 3C, lane 3 vs 4). HIP1's interaction with these two RTKs, along with previous interactions observed with EGFR, FGFR4, and c-Kit (Ames et al., 2011; Bradley et al., 2007a; Wang et al., 2008), add to a growing list of receptors that interact with HIP1. Given the ability of HIP1 to transform cells and stabilize receptors from ligand-mediated degradation, HIP1 is an oncoprotein that could be targeted when multiple receptors are upregulated in the same tumor (Figure 3.5D).

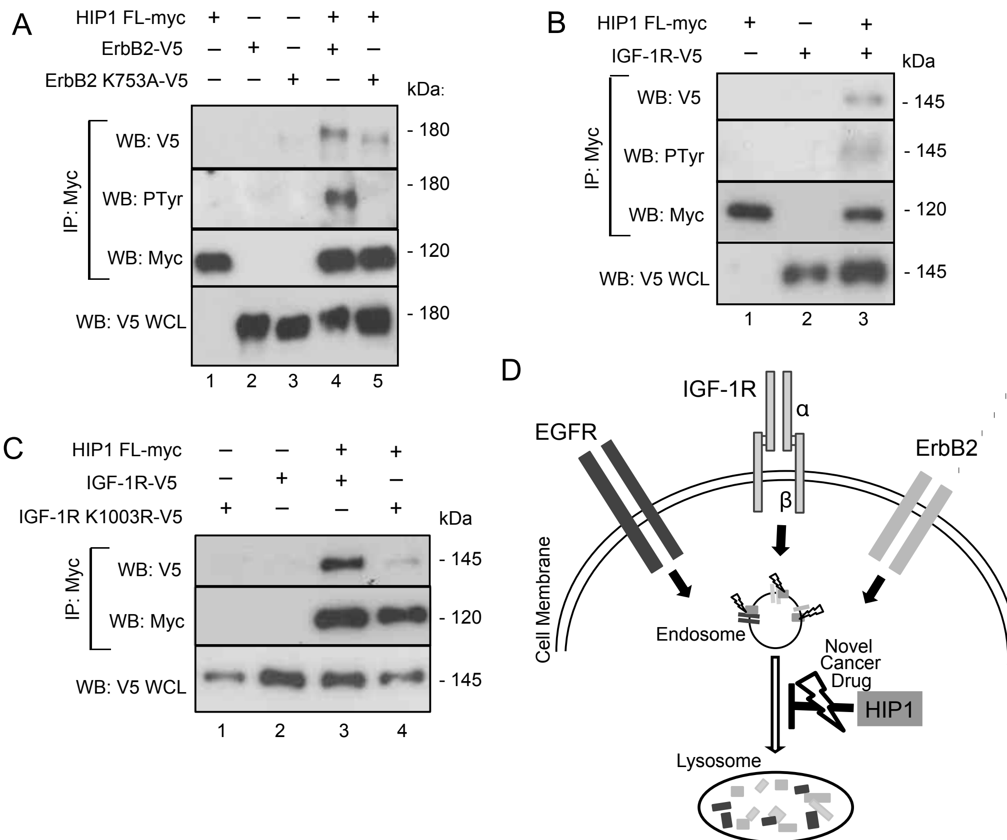


Figure 3.5 HIP1 immunoprecipitation with ErbB2 and IGF-1R. *A,* Immunoprecipitation of HIP1-myc co-transfected with ErbB2-V5 and ErbB2 K753A-V5 in 293T cells. *B,* Interaction of HIP1 with mature, phosphorylated IGF-1R. *C,* Immunoprecipitation of HIP1-myc co-transfected with IGF-1R-V5 and IGF-1R K1003R-V5 in 293T cells. *D,* Diagram of the potential of HIP1 targeted therapy to disinhibit the degradation of multiple RTKs.

HIP1 is tyrosine phosphorylated by EGFR

The binding of HIP1 to the kinase domain of EGFR led us to hypothesize that HIP1 may act as a substrate for this receptor. Furthermore, when we immunoprecipitated HIP1 from adipose tissue derived from a human HIP1 transgenic mouse and blotted for phosphotyrosine containing proteins, we observed the presence of a tyrosine-phosphorylated, HIP1-sized band (Figure 3.6A), supporting the possibility that HIP1 is tyrosine phosphorylated. To further test our hypothesis, we co-expressed HIP1-myc along with wild-type EGFR or with kinase-dead EGFR (K721A), then immunoprecipitated HIP1, and tested it for phosphotyrosine content using the anti-phosphotyrosine 4G10 antibody. HIP1 was indeed tyrosine phosphorylated in the presence of wild type EGFR, but not in the presence of its kinase dead form (Figure 3.6B, lane 3 vs 4). Additionally, we could detect tyrosine phosphorylation of HIP1 by endogenous EGFR in human HIP1-transformed NIH-3T3 fibroblasts (Rao et al., 2003) (Figure 3.6C, lane 2). This phosphorylation was inhibited when cells were treated with the potent EGFR inhibitor AG1478 (Figure 3.6C, lane 3). We also found that HIP1 was phosphorylated by EGFRvIII equally as well as by wild type EGFR (Figure 3.6D, lanes 4 and 5). IGF-1R and ErbB2, however, did not readily phosphorylate HIP1, even with ligand stimulation, or when the cell culture media was supplemented with the phosphatase inhibitor sodium orthovanadate for 1 hour prior to lysate collection (Figures 3.7A and 3.7B).

Because the kinase inactive form of EGFR, EGFR K721A, retains some but not all of its ability to bind HIP1, we decided to test if AG1478 would affect HIP1's interaction with EGFR. In this case, treatment of HeLa cells for 1 hour with 1μM

AG1478 was able to prevent HIP1 tyrosine phosphorylation by EGFR, but did not disrupt the interaction between HIP1 and EGFR (Figure 3.7C). Therefore, although HIP1 is phosphorylated by EGFR, its interaction with EGFR does not completely depend on EGFR-mediated phosphorylation of itself or HIP1.

To explore the chronology of HIP1 phosphorylation by EGFR after ligand stimulation, we turned to a timed, "cold load" ligand stimulation method (Parachoniak and Park, 2009) in HIP1 and EGFR-transfected HeLa cells (diagramed in Figure 3.6E). This method allows coordinated internalization of receptors following incubation of the receptors with ligand at temperatures that are not permissive to internalization. Cells were starved, and then EGFR was "loaded" with 100μM EGF for one hour at 4°C. The media was then exchanged for DMEM that had been heated to 37°C. This temperature change allows coordinated receptor internalization to proceed. In this experiment, no HIP1 phosphorylation was observed during starvation. In contrast, strong phosphorylation of HIP1 was observed during the EGF load at 4°C (Figure 3.6F, lane 1 vs 2). HIP1 phosphorylation remained high 2 minutes post increase of the media temperature to 37°C (the start of endocytosis), and then remained steady until the 10-minute time point. The HIP1 phosphotyrosine signal began to diminish at 15 minutes and was absent by 60 minutes (Figure 3.6F). This pattern was also observed in HEK 293T cells (Figure 3.7D). This temporal pattern of phosphorylation of HIP1 by EGFR indicates that this state of HIP1 is transient, occurring only during the early phases of RTK endocytosis.

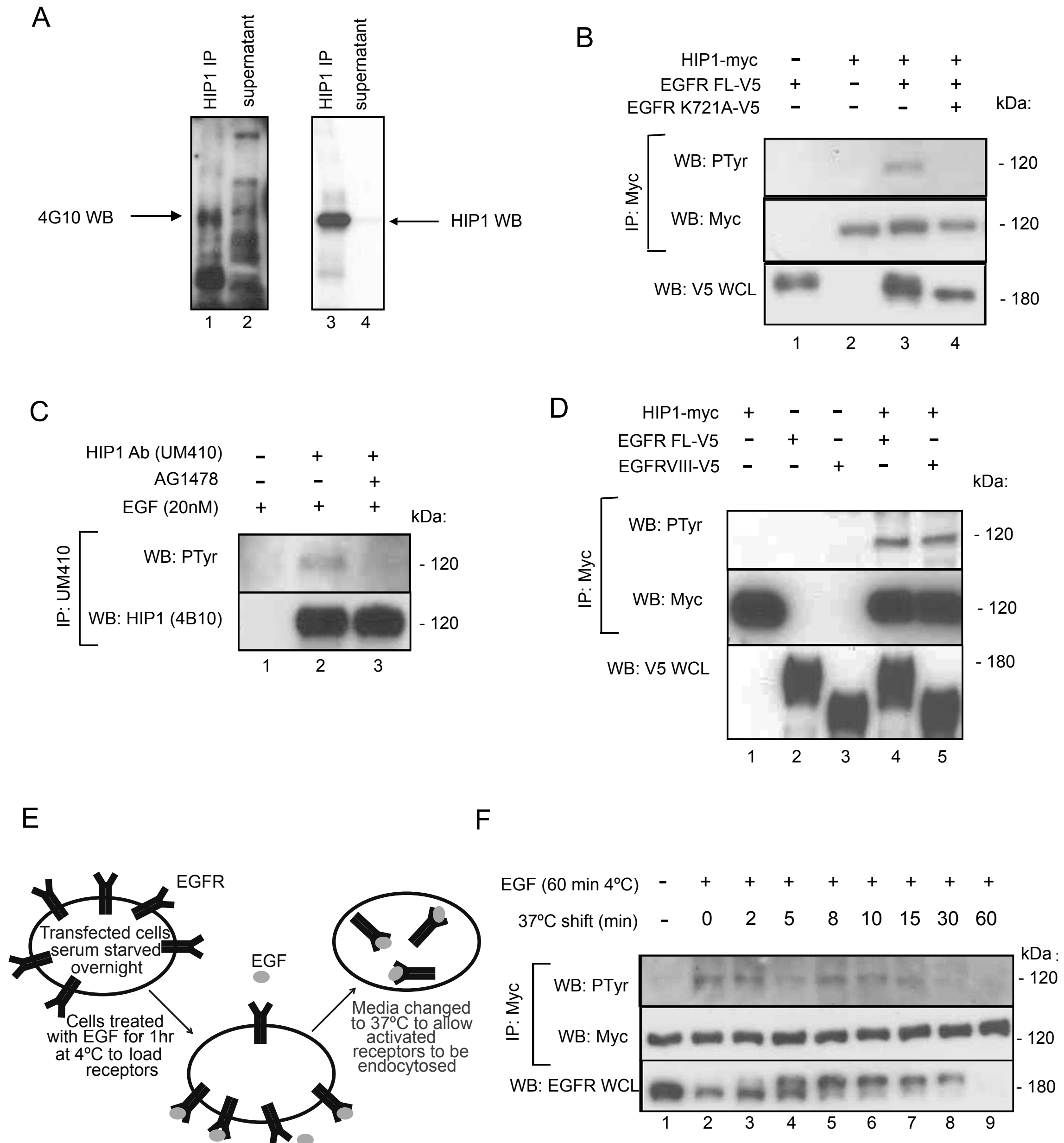


Figure 3.6 HIP1 tyrosine phosphorylation in the presence of EGFR. *A,* Immunoprecipitation of HIP1 (using polyclonal antibody UM410) from hHIP1hi adipose tissue revealed a HIP1 sized phosphotyrosine band when blotting the pellet with the anti-phosphotyrosine monoclonal antibody 4G10. *B,* Immunoprecipitation of HIP1 co-transfected into 293T cells with either EGFR-V5 or EGFR K721A-V5. Pelleted lysates were probed with 4G10. *C*, Tyrosine phosphorylated HIP1 was immunoprecipitated from NIH 3T3 cells that have been stably transformed by HIP1 and have been treated with 20nM EGF for 5 minutes. One group of cells was pre-treated with AG1478 to inhibit EGFR activation. *D*, HIP1 phosphorylation by the EGFR mutant EGFRvIII. *E,* Diagram illustrating cold-load stimulation – a strategy for precise temporal analysis of endocytic processes. *F,* Analysis of HIP1 tyrosine phosphorylation at several time points over 60 minutes after pre-treating cells with 100nM EGF at 4°C and then heating cell media to 37°C.

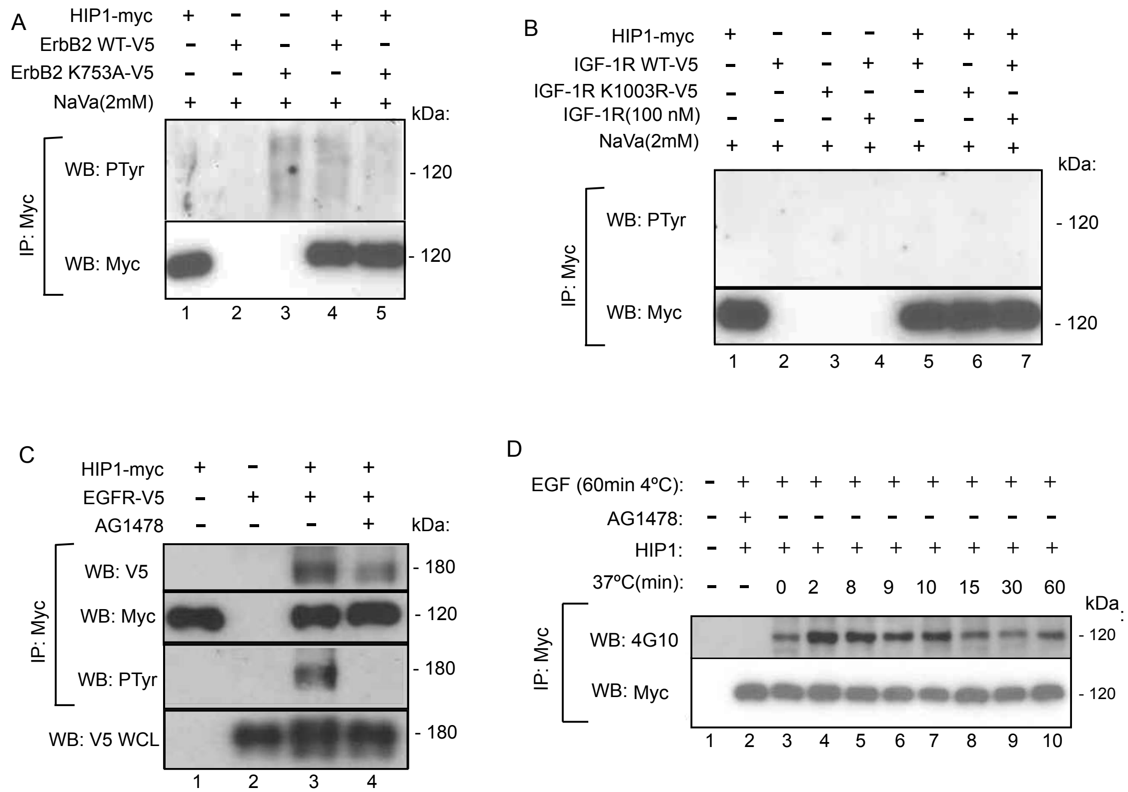


Figure 3.7 HIP1 tyrosine phosphorylation. *A,* Analysis of HIP1 phosphorylation by ErbB2. NaVa = sodium orthovanadate. *B,* Analysis of HIP1 phosphorylation by IGF-1R. *C,* HIP1-myc and EGFR-V5 transfected 293T cells were treated with 1μM AG1478 for 1 hour prior to lysate collection. HIP1-myc was then immunoprecipitated and pelleted lysates were probed with V5 and 4G10. *D,* HIP1-myc was co-transfected in 293T cells that were stimulated with EGF according to the cold load stimulation protocol and collected at several timepoints. HIP1 was then immunoprecipitated and probed for tyrosine phosphorylation.

HIP1 phosphorylation by EGFR requires the HIP1 UpStream Helix (USH) domain

To map the location of HIP1's phosphorylated tyrosine(s), we tested a series of HIP1 deletion mutants (Figure 3.8A) for their ability to serve as substrates for the EGFR kinase. We used the previously constructed HIP1 deletion mutants, HIP1ΔA and HIP1ΔT (Rao et al., 2002) and constructed the new mutants HIP1Δ184-400, HIP1Δ401-599 and HIP1Δ600-800 to generate deletions that spanned the entire length of HIP1 (Figure 3.8A). These myc-tagged mutants were then co-expressed with EGFR, immunoprecipitated with myc antibody-conjugated beads and probed with phosphotyrosine antibody 4G10 to determine which domain(s) were necessary for tyrosine phosphorylation. Tyrosine phosphorylation of HIP1ΔT, HIP1Δ184-400 and HIP1Δ401-600 mutants was intact (Figure 3.8B, lanes 3, 5, 6 and 8). In contrast, HIP1ΔA, and HIP1Δ600-800 were not phosphorylated (Figure 3.8B, lanes 4 and 7). Both of these mutants lack important HIP1 regulatory domains (Rao et al., 2002; Wilbur et al., 2008).

The HIP1Δ600-800 deletion region contains two domains of interest; a putative domain necessary for the transformative abilities of the HIP1-PDGFβR oncoprotein (Ross and Gilliland, 1999), and an UpStream Helix (USH) domain necessary for a conformational shift in the presence of clathrin that prevents actin binding (Senetar et al., 2004). To further explore the area between amino acids 600 and 800, we constructed deletion mutants HIP1Δ690-752 and HIPΔ752-800 to disrupt these two domains, respectively. HIP1Δ600-700 was constructed to delete the remaining area of this region. These mutants were tested for their ability to be phosphorylated by and interact with

EGFR. The HIP1Δ752-800 mutant that lacks the USH domain was not phosphorylated by EGFR whereas the other two mutants were phosphorylated (Figure 3.8C, Lane 6). Although all of these mutants bound EGFR, they demonstrated variable interaction with the faster migrating band of EGFR. The HIP1Δ600-700 mutant bound only weakly to full length EGFR and did not show significant binding to EGFRtr (Figure 3.8D, lane 3, Figure 3.9A, lane 4), and both HIP1Δ690-752 and HIPΔ752-800 showed slightly greater binding to EGFRtr than did wild-type HIP1 in HeLa cells (Figure 3.8D, lanes 4 and 5). The interaction of HIP1Δ752-800 with EGFR, with a lack of phosphorylation, was surprising as this sequence is devoid of tyrosine residues. HIP1Δ752-800 also retained strong binding to the EGFR c'973 mutant, which is unable to bind the HIP1 binding partner AP2 (Figure 3.9B, lane 6). The USH domain may therefore facilitate HIP1 phosphorylation or inhibit its dephosphorylation. Because the conformation of the HIP1 USH domain is altered upon clathrin binding, we hypothesized that clathrin binding may affect HIP1 phosphorylation. To test this hypothesis, we constructed a clathrin non-binding point mutant (L486A) that prevents light chain binding (Ybe et al., 2007). Although this mutation abolished HIP1 binding to clathrin as expected, no effect on HIP1 tyrosine phosphorylation was observed (Figure 3.9C, lane 4). HIP1Δ752-800 also interacted normally with clathrin (Figure 3.9D, lane 3). These findings indicate that the HIP1 USH domain modulates tyrosine phosphorylation in a manner independent of clathrin.

The HIP1ΔA mutant, which lacks the phospholipid binding ANTH domain, demonstrates poor protein expression due to its pro-apoptotic activity (Hyun et al., 2004b) (Figure 3.8B, lane 4). To thoroughly evaluate the ability of this mutant to be

phosphorylated, we repeated the immunoprecipitation experiment with 15 times the volume of lysate used for the positivecontrol, HIP1 wild-type, immunoprecipitation. Even under this condition the HIP1ΔA mutant was not phosphorylated, although binding to EGFR was retained (Figure 3.9E, lane 3 vs. 4). To determine which tyrosine(s) within this domain were phosphorylated, a series of tyrosine to phenylalanine point mutants were constructed (Y117F, Y135F, Y142F and Y152F) by individually disrupting each of the four tyrosines in this region. Additionally, one mutant was generated in which all four tyrosines were mutated to phenylalanines using PCR with the remaining three primer sets. These mutants were then co-expressed with EGFR and evaluated for tyrosine phosphorylation (Figure 3.8E). All of the above listed tyrosine-to-phenylalanine mutants retained their phosphorylation by EGFR, indicating that HIP1ΔA's loss of tyrosine phosphorylation is not due to its deleted tyrosines, but may instead be due to its pro-apoptotic phenotype. In sum, we have identified two different mutants of HIP1, the HIP1Δ752-800 and the HIP1ΔA mutants, which are not phosphorylated by the EGFR tyrosine kinase. However, neither of these mutants contains a single tyrosine that is the only substrate for the EGFR tyrosine kinase. This indicates that it is likely that multiple tyrosine residues in different domains of HIP1 are phosphorylated by the EGFR tyrosine kinase.

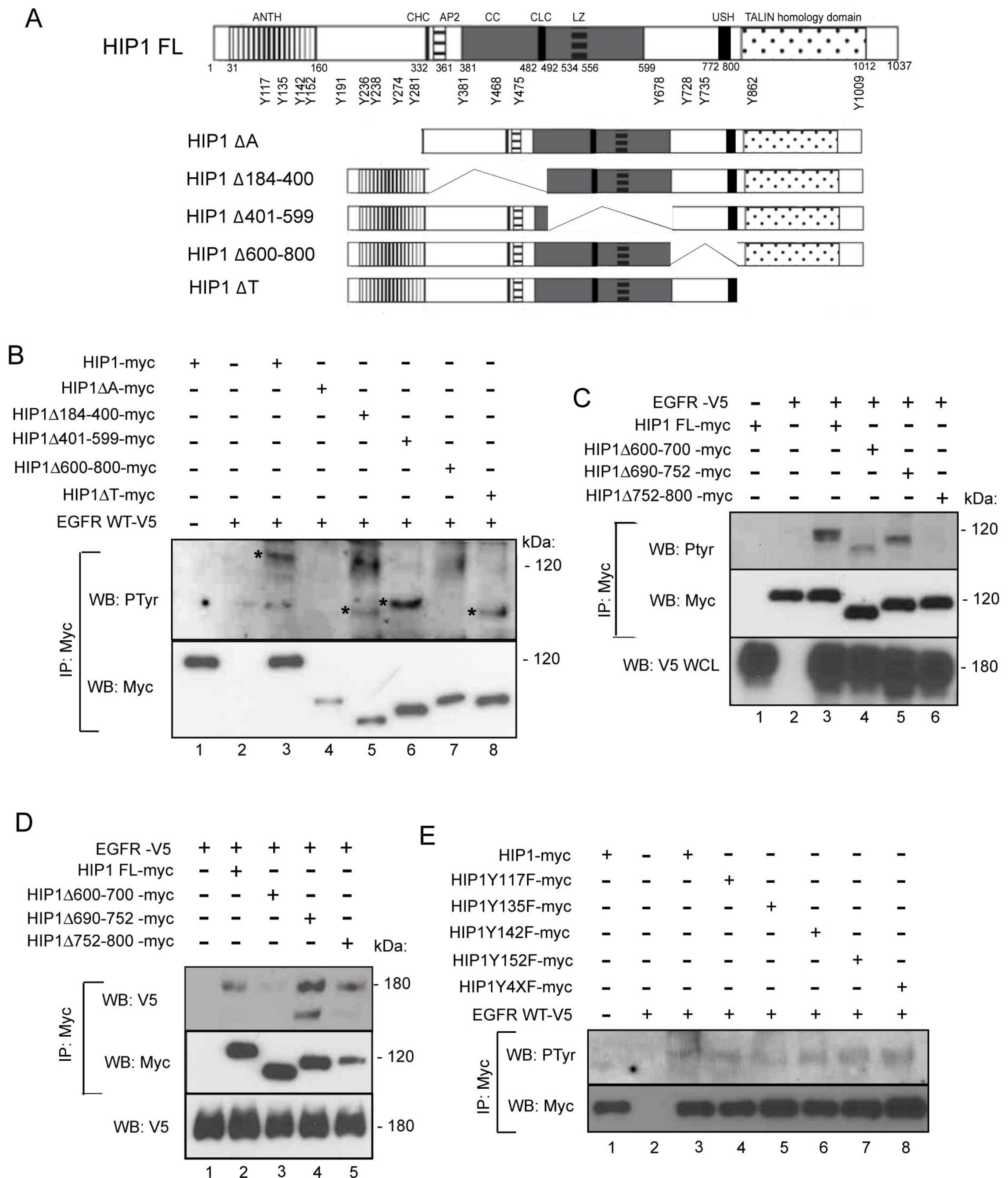


Figure 3.8 HIP1 domains regulating tyrosine phosphorylation and interaction with EGFR. *A,* Schematic diagram of HIP1 domain deletion mutants. HIP1ΔA and HIP1ΔT have been previously described (Rao et al., 2002). HIP1Δ184-400, HIP1Δ401-599, and HIP1Δ600-800 were generated using site specific mutagenesis in order to create deletions spanning the entirety of the HIP1 sequence. *B,* Immunoprecipitation of the described mutants co-expressed with EGFR. Pelleted lysates were probed via Western blot using the antibody 4G10. *C,* HIP1Δ600-700, HIP1Δ690-752, HIP1Δ752-800 were analyzed for phosphorylation by EGFR. *D,* Interaction of HIP1Δ600-700, HIP1Δ690-752, HIP1Δ752-800 with EGFRtr in HeLa cells. *E,* Phosphorylation HIP1 point mutants with tyrosine to phenylalanine mutations in the ANTH domain.

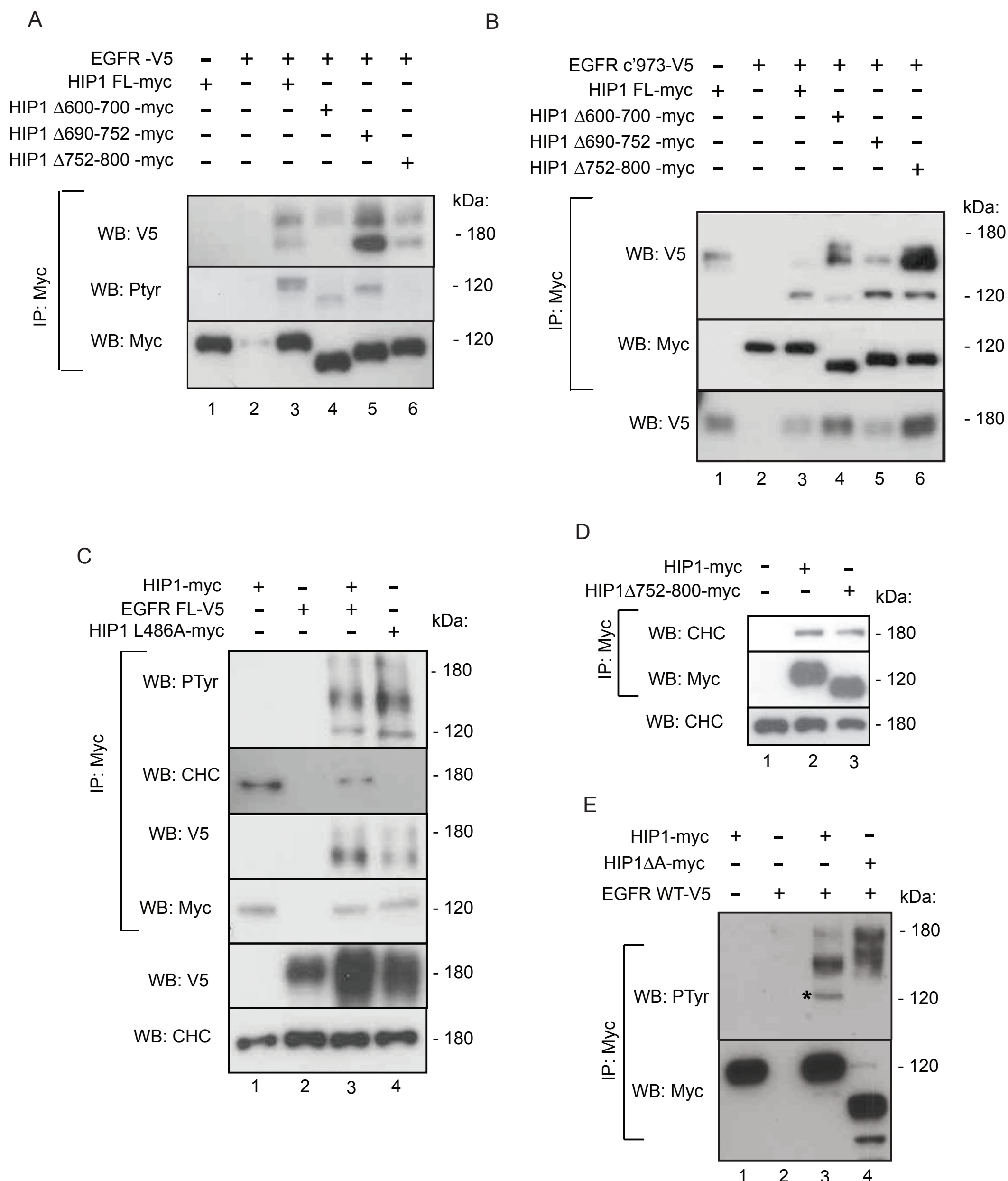


Figure 3.9 EGFR interaction with and tyrosine phosphorylation of HIP1 mutants *A,* HIP1Δ600-700-myc, HIP1Δ690-752-myc, and HIP1Δ752-800-myc were co-transfected with EGFR-V5 in 293T cells. Lysates were incubated with anti-myc sepharose beads, which were then pelleted through centrifugation. Pelleted lysates were then analyzed by western blot for V5, phosphotyrosine, and myc. *B,* HIP1Δ600-700-myc, HIP1Δ690-752-myc, and HIP1Δ752-800-myc interaction with EGFR c'973-V5 in 293T cells. *C,* HIP1-myc and HIP1 L486A-myc phosphorylation by EGFR. CHC=Clathrin Heavy Chain. *D,* Interaction of HIP1Δ752-800-myc with clathrin. *E,* Tyrosine phosphorylation of HIP1ΔA.

HIP1 demonstrates colocalization with EGF, clathrin coated vesicles, and Rab5 positive early endosomes

Although HIP1 is known to partially colocalize with clathrin-coated pits, its role in endocytic processes is poorly defined. We hypothesized that HIP1 localization may be affected by its EGFR interaction, allowing it to associate with further downstream vesicles in the endocytic pathway. To investigate this, we used the cold load stimulation protocol (Parachoniak and Park, 2009), described in Figure 3.6E, to allow HIP1 and EGF localization to be observed at several time points after EGF addition to the cells. Cells were fixed, stained, and imaged using confocal microscopy at different time points (Figure 3.10A). Clathrin staining was also performed in these experiments to serve as a marker for clathrin-coated vesicles. EGF localization was tracked using fluorescently tagged conjugate.

HIP1 and clathrin were recruited to the cell membrane in the early stages of EGF internalization. Both can be observed lining the internal portion of the plasma membrane, while EGF remains on the external portion on the surface of the cell (Figure 3.10A). As EGF is internalized with warming to 37°C, colocalization of HIP1 and clathrin with EGF containing vesicles increased to a peak at 15 to 30 minutes (Figure 3.10A, lower panels). Mander's co-efficient analysis of EGF colocalization with HIP1 indicated that there was a significant increase in the proportion of EGF associating with HIP1 over time (Figure 3.10B; $p<0.0001$, One-way ANOVA). Post-hoc analysis using the Newman-Keuls Multiple Comparison Test also showed that EGF association with HIP1 significantly increased between 0 minutes and 15 and 30 minutes (Figure 3.10B). This time period of 0 to 30 minutes approximately reflects the period when we observed

HIP1 tyrosine phosphorylation by EGFR (Figure 3.6E, Figure 3.7D). HIP1 and clathrin demonstrated similar patterns of colocalization with EGF as might be expected due to their stable, approximately 50%, colocalization with each other. Collectively, these data show that HIP1 is recruited to clathrin-coated pits as they form in response to EGFR activation and continues to associate with these structures as they internalize and become clathrin-coated vesicles.

Little is known regarding HIP1 localization to endosomes downstream of the clathrin-coated vesicle. Clathrin has been found to partially colocalize with Rab5 positive early endosomes (Raiborg et al., 2001), leading us to hypothesize that HIP1 also may be recruited to these endosomes. To more readily observe this colocalization, we transfected cells with a constitutively active form of Rab5, called Rab5Q79L, which produces enlarged, easily viewable, early endosomes. Although HIP1 was not observed in these endosomes after stimulation with EGF at 4°C, HIP1 clearly was recruited to enlarged Rab5Q79L-CFP endosomes after 20 minutes of incubation at 37°C (Figure 3.10C). This result is noteworthy because a major binding partner of HIP1, the AP2 adaptor protein, is specifically uncoated from endosomes via Rab5 activity (Semerdjieva et al., 2008). The HIP1 phosphorylation mutant HIP1Δ752-800 did not affect recruitment of HIP1 to Rab5 early endosomes (Figure 3.11A).

Rab5 endosomes contain two major Rab5 effectors that mark early and late stages of the endosomal pathway. These early and late effectors, APPL1 and EEA1, respectively, do not coexist in the same endosomes (Zoncu et al., 2009). We decided to examine if HIP1 was located exclusively with Rab5 endosomes that were early or late, as evidenced by the presence of APPL1 or EEA1. Interestingly, APPL1, along with HIP1,

was recruited to the cell edge at sites of bound EGF after holding EGF treated cells for 1hr at 4ºC (Figure 3.12A, 0 min). This colocalization between HIP1 and APPL1 continued for 8 to 10 minutes after elevating the cell culture temperature (Figure 3.12A, 10min). Though HIP1's colocalization with APPL1 was not as extensive as with clathrin, it occurred at EGF positive vesicles. Unlike APPL1, EEA1 was not recruited to cell edges upon stimulation. HIP1 and EEA1 did demonstrate a small amount of overlap in EGF-containing endosomes (Figure 3.12B, 15min). This was best observed at 13 to 15 minutes following elevation of the cell media to 37°C and occurred at an interface between the edge and center of the cell where HIP1 and EEA1 met. Association of HIP1 with the these two components of the Rab5 sorting endosome indicates that HIP1 oncogenic activity could be associated with prolonging endosomal signaling or targeting receptors for later recycling pathways.

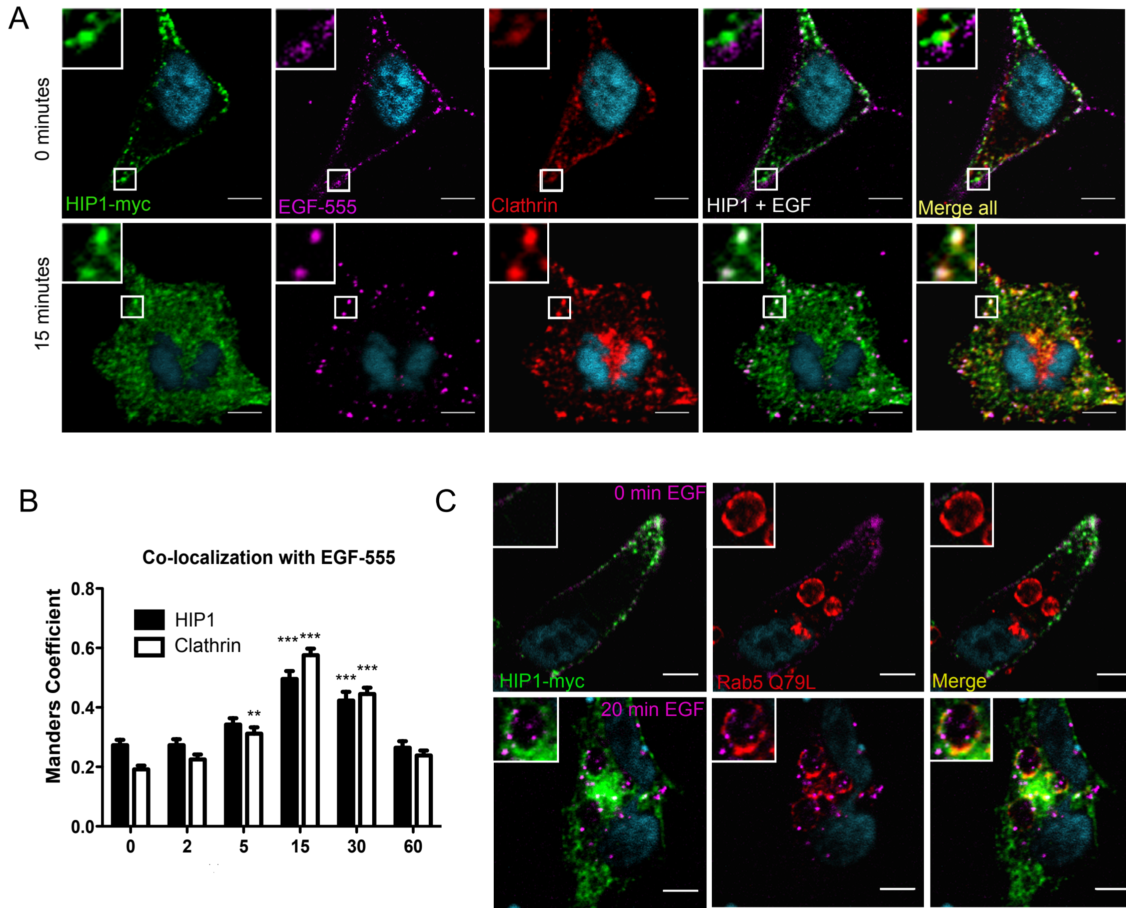


Figure 3.10 HIP1 localization to EGF positive endosomes. *A,* HIP1-myc transfected HeLa cells were treated with 1μM EGF-555 in the "cold-load stimulation" experimental paradigm. Cells were then fixed and stained for HIP1 (anti-Myc, Cell Signaling) and Clathrin (X22, AbCAM) and imaged using a 1μm slice thickness on a Zeiss confocal microscope. *B,* Quantification of EGF colocalization with HIP1 and clathrin over time. ***$p<0.0001$, **$p<0.001$ (n=37 cells per time point over 3 experiments). *C*, Confocal microscopy images of HeLa cells transfected with HIP1-myc and Rab5Q79L for 24 hours and then starved in DMEM for 16 hours overnight. Cells were then stimulated for either 0 minutes or 20 minutes using the cold-load stimulation protocol, fixed, and stained. Scale bar = 5μM.

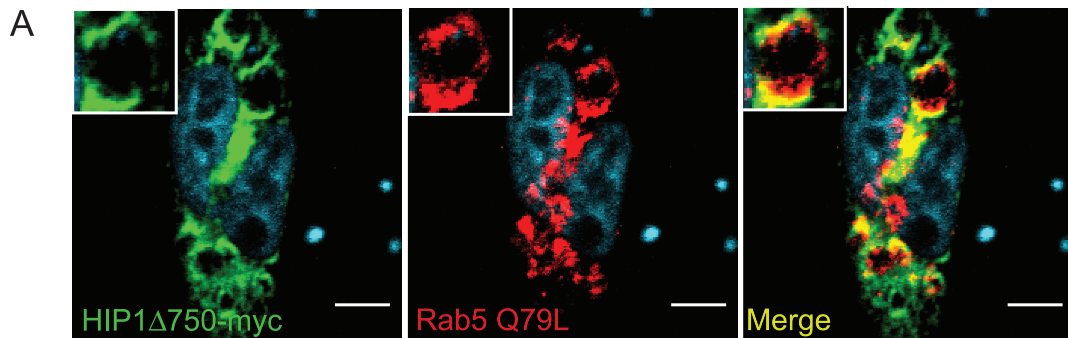


Figure 3.11 HIP1Δ752-800 recruitment to the Rab5 endosome. Confocal microscopy images of HIP1Δ752-800-myc transfected HeLa cells that were stimulated for 20 with 1μM EGF-555 using the cold load stimulation protocol for 20 minutes, then fixed, and stained for Myc. Scale bar = 5μm.

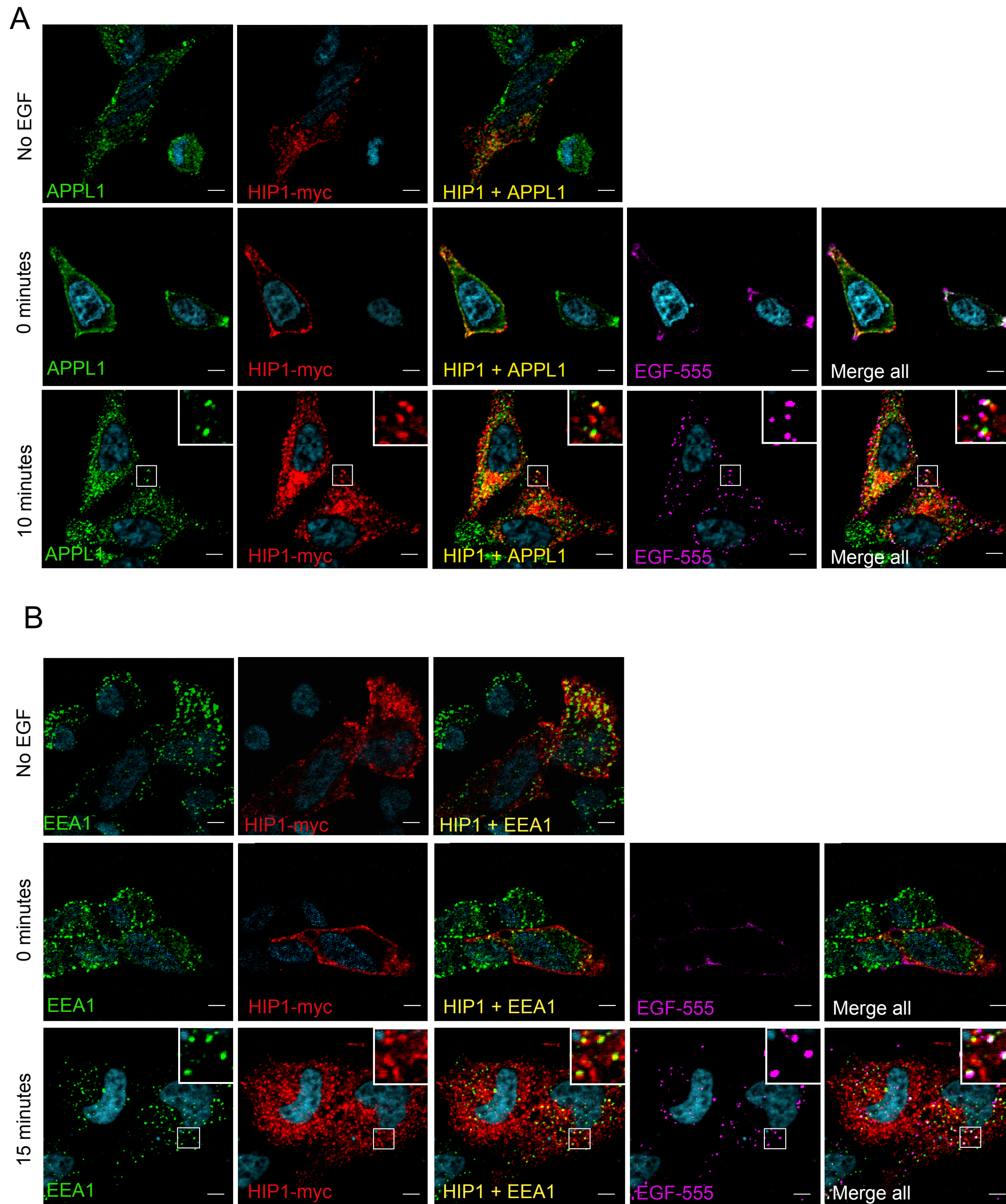


Figure 3.12 HIP1 localization to APPL1 and EEA1 positive early endosomes. *A,* Confocal images of HIP1 and APPL1 localization at steady state, after incubation with 1μM EGF-555 at 4°C for 1 hour, or after stimulating with EGF-555 at 37°C for 10 minutes. Scale bar = 5μm. *B,* Localization of HIP1 with EEA1 at steady-state, after incubation with 1μM EGF-555 at 4°C for 1 hour, or after stimulating at 37°C with EGF-555 for 15 minutes.

3.4 Discussion:

Although HIP1 has long been recognized to be present during the early stages of endocytosis and to slow degradation of RTKs, its mechanism of action has been elusive. Understanding the normal function of HIP1 is of particular interest as this information could provide clues for a better understanding of its role in tumorigenesis and potentially aid in design HIP1 inhibitors as anti-cancer agents. The results of our current study improve understanding of the interaction between HIP1 and EGFR by identifying the HIP1-interacting domain of EGFR and defining the cellular compartments at which the interaction occurs. We have also characterized a novel N-terminally truncated, EGFRvIII-like form of EGFR that shows preferential binding to HIP1 but not to HIP1r or the endocytic adaptor protein Eps15. Additionally, we have identified HIP1 as a novel substrate for the EGFR tyrosine kinase and its oncogenic form, EGFRvIII, and have identified critical regions of HIP1 required for phosphorylation.

The EGFR C-terminal region is the location for interaction with most of its binding partners, including the endocytic regulatory proteins Grb2, Cbl, and AP2. Surprisingly, the C-terminal domain was dispensable for HIP1 binding to EGFR, however the EGFR kinase domain was necessary for this association. Several endocytic adaptor proteins also associate with the EGFR kinase domain, but they are composed primarily of proteins that bind EGFR through ubiquitin linkages, such as Eps15, Epsin 1 and Hrs (Sorkin and Goh, 2008). Ubiquitination does not seem to affect HIP1 binding to EGFR, which makes HIP1 unique among these kinase domain-binding proteins. In addition to ubiquitination, several self-activated or trans-activated phosphorylation sites in the EGFR kinase domain may regulate HIP1 binding to EGFR. Although our work

has helped narrow these possible locations, mapping a more precise motif for HIP1 binding to RTKs is important to clarify how this binding is regulated.

Strong binding of HIP1 to the EGFRtr truncated form of EGFR was particularly surprising as previous studies analyzing the interaction between HIP1 and EGFR were performed using a spontaneously generated HIP1 point mutant (F182S) (Bradley et al., 2007a). Upon correction of this mutation, stronger binding between EGFR and HIP1 was observed as well as stronger binding of HIP1 to EGFRtr. Interestingly, the HIP1 family member HIP1r did not bind EGFRtr. The EGFR c'973 mutant primarily bound to HIP1 in its truncated state, indicating that this truncation may be more stable in the endocytosis- and degradation-deficient EGFR c'973 (Decker et al., 1992). EGFRtr was more readily observed in HEK 293T cells than in HeLa cells, possibly indicating less processing of EGFRtr in HeLa cells. EGFRtr was not absent from HeLa cells, however, as demonstrated by strong binding of HIP1Δ690-752 to EGFRtr. Comparison of protease expression in HeLa cells versus HEK 293T cells could therefore illuminate a possible cause for the differential levels of EGFRtr in these cell types and to evaluate the presence of EGFRtr in non-transfected primary cells. It would also be useful to determine if HIP1 contributes to the formation of EGFRtr through protease recruitment and if formation of EGFRtr contrubites to oncogenesis.

The 140kDa form of EGFR, EGFRtr, must represent N-terminal truncation, because the EGFRtr retained its C-terminal V5 tag. Not surprisingly, HIP1 also displayed binding to the oncogenic EGFR mutant EGFRvIII, which exhibits a 267-amino acid deletion at its N-terminus. EGFRvIII is often expressed in EGFR-amplified glioblastoma and has also been detected in breast, ovarian, and non-small cell lung

cancers (Hatanpaa et al., 2010; Loew et al., 2009). EGFRvIII was also able to tyrosine phosphorylate HIP1. Although constitutively active, the signaling strength of EGFRvIII is not considered to be particularly significant and its oncogenic ability is instead attributed to its prolonged stability that results from impaired degradation (Huang et al., 1997). This characteristic of prolonged RTK half-life as a mechanism tumorigenesis is consistent with the hypothesis that we have proposed previously upon observing HIP1 over-expression in cancers (Rao et al., 2003). It would be interesting, therefore, to determine if HIP1 is necessary for EGFRvIII-mediated tumorigenesis by knocking down HIP1 mRNA in EGFRvIII expressing tumor lines.

Tyrosine phosphorylation of HIP1 by EGFR and EGFRvIII underscores the likely functionality of the observed physical interaction. HIP1 phosphorylation was transient as it was lost when cells were treated with the EGFR inhibitor AG1478 and activated by EGF treatment, after which HIP1 tyrosine phosphorylation persisted for approximately 15 minutes. Attempts to map the tyrosine(s) phosphorylated by EGFR led to the identification of two domains that are essential for HIP1 tyrosine phosphorylation. The pro-apoptotic HIP1ΔA mutant could not be phosphorylated even though deletion of the tyrosines in this region did not disrupt phosphorylation. This indicates that the apoptotic phenotype is not due to loss of these tyrosines and that the apoptotic process induced by this mutant may prevent it from being phosphorylated.

More surprisingly, HIPΔ752-800, a sequence containing the HIP1 USH domain, is essential for HIP1 tyrosine phosphorylation, despite the absence of tyrosines in this region. Further, HIP1 phosphorylation was not dependent on activation of the USH domain by clathrin, suggesting a role for the USH domain in HIP1 tyrosine

phosphorylation that is independent of the clathrin-induced conformational change that inhibits HIP1-actin binding. Based on the inability of deletion mutants to identify a single phosphorylated tyrosine on HIP1, several combinations of phosphorylated tyrosines are possible throughout the HIP1 protein. Further analysis to identify which of the 17 tyrosines in HIP1 are phosphorylated and their role in HIP1-mediated transformation will be important.

The location of HIP1 during endocytosis has been under-investigated and remains somewhat controversial. While some work indicates a prolonged role for HIP1 in receptor trafficking during the early stages of endocytosis (Metzler et al., 2003), one total internal reflection fluorescence (TIRF) live microscopy study suggests that HIP1 and clathrin uncoat from the endosome seconds after internalization (Gottfried et al., 2009). Our results support the former findings, as we observed HIP1 colocalization with EGFR for 15-30 minutes following internalization, as well as recruitment of HIP1 to the Rab5 early endosome. Similarly to studies in neuronal endocytosis (Metzler et al., 2003), we observed HIP1 localization to the EEA1 positive endocytic vesicles, a later sub-compartment of the Rab5 positive vesicle. Additionally, the TIRF study imaged HIP1 using a C-terminal GFP tag (Gottfried et al., 2009), which, in our observations disrupts HIP1 binding to clathrin (Figure 3.13). Thus this tag may have disrupted the stability of the clathrin coat, leading to earlier uncoating in the TIRF study.

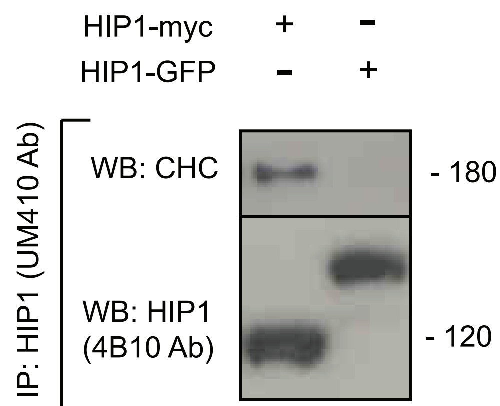


Figure 3.13 GFP bound HIP1 disrupts clathrin binding. HIP1-myc and HIP1-GFP immunoprecipitation with endogenous clathrin.

In addition to HIP1 colocalization with EEA1 after EGF treatment, we observed colocalization of HIP1 with the APPL endosome, which is logical for several reasons. First, the APPL endosome is enriched in PI(3,4)P_2 phosphoinositide lipids, for which the HIP1 ANTH domain shows strong affinity (Hyun et al., 2004b; Zoncu et al., 2009). Additionally, this endosome is the site of specific endosomal signaling through the Akt pathway (Schenck et al., 2008). The presence of an oncogenic protein to recruit RTKs here would likely increase cell growth in tumorigenesis. More surprising was the recruitment of APPL1 to the cell surface upon EGF stimulation. This ligand-initiated recruitment to the plasma membrane has not been previously observed.

In summary, HIP1 is an EGFR substrate and binding partner that is present throughout the early stages of endocytosis. While the precise mechanism of HIP1's action during these stages remains unknown, several hypotheses can be made based on the ability of HIP1 to stabilize receptors and on the endosomal trafficking choices that occur during this process. For example, HIP1 may stall progression to later endosomal stages by causing these vesicles to retain their clathrin coat and therefore prolong signaling within the APPL endosome. Clathrin-coated vesicles are also associated with recycling (Sigismund et al., 2005), and thus HIP1 might aid in targeting receptors to this path. Binding of HIP1 to the EGFR kinase domain could also displace binding of the kinase domain associated proteins Eps15 and Hrs, which target EGFR to the lysosome through binding to ubiquitin linkages. Binding of HIP1 to the weakly, yet constitutively phosphorylated EGFRvIII that is continuously recycled, indicates that HIP1 may play a stabilizing role in low-signaling conditions. All of these processes may be regulated

through EGFR tyrosine phosphorylation of HIP1. These data are a base for future work to identify why HIP1 is phosphorylated and to characterize EGFRtr to determine if these are regulatory mechanisms through which HIP1 promotes oncogenesis and/or normal cellular homeostasis.

3.5 Materials and Methods:

Cell culture:

Most experiments were performed in the human embryonic kidney 293T (HEK 293T) cell line. These cells were maintained at 37°C in Dulbecco's Minimal Essential Media (Gibco) supplemented with 10% fetal bovine serum, penicillin/streptomycin, and GlutaMAX (Gibco). The HeLa cervical cancer line was also used.

Constructs:

pcDNA3.1-V5-His constructs were generated by amplifying receptors from pBiTet contructs provided by Linda Pike (Washington University of St Louis)(Macdonald-Obermann and Pike, 2009). The amplifying primers were as follows:

EGFR FL (5'-TCGGTACCCGGGGATCCTCTAAGCTTCTGA-3', 5'-TGGCGACCGGTGGATCCCTCGAGCGCGG-3'),

EGFR K721A (5'-GCCCCCTGACTCCGTCCAAGCTTGATC-3', 5'-GGCTCATACTATCCTCCTCGAGCATGCTCCA-3'),

EGFR c'973 (5'- GGGATCCTCTAGTCAGCTGAAGCTTGCTAGC-3', 5'-GATCCGAGCTCGGTACCAACTCGAGAAGTTG-3'),

EGFR c'698 (5'- GATCCTCTAGTCAGCTGAAGCTTGCTAGCGTTTAA-3', 5'-ATCCGAGCTCGGTACCAACTCGAGGCAC-3'),

EGFRΔC (5'-GGATCCTCTAGTCAGCTGAAGCTTGCTAGCGT-3'),

ErbB2 (5'- CCCGGGGATCCTCTAGTCAGCTGAAGCTTGCTAG-3'). These amplified mutants were inserted into the pcDNA3.1.V5.His vector (Invitrogen) using the restriction enzymes *Hind*III and *Kpn*I or *Xho*I. An IGF-1R cDNA-containing pCR-XL-TOPO vector (NIH MGC 348) was purchased from Open Biosystems. The *KpnI* site in the IGF-1R cDNA was mutated via QuikChange mutagenesis with the following primer (5'- TCATCCCAAAGGTACCGAATGAAGTCTGGCTCC -3') and its compliment. IGF-1R was then amplified using the following primers, (5'-GAGAAAGGGGAGGTACCTCCCAAATAAAAGGAATGAAG-3', 5'-GCACAGATTCAGGATCCAATTCGAAGCAGGTCG-3') and inserted into pcDNA3.1-V5-His using the *KpnI* and *BstBI* restriction enzymes. EGFRvIII in the pRevTRE vector was obtained as a kind gift from Paul Mischel (UCLA). This EGFR mutant was amplified using the following primers (5'-CATAAACCAGTCCGTTCCCAAAAGG-3', 5'-GGGCTCATACTATCCTCCTCGAGCATGCTCC -3) and then inserted into pcDNA3.1-V5-His using the restriction enzymes *HindIII* and *XhoI*.

The ErbB2-K753A (5'- GTGAAAATTCCAGTCGCGATCATGGTGTTG AGGGAAAACACATCCCCC-3'),

IGF-1R K1003R (5'- CCAGAGTGGCCATTAGAACAGTGAACGAGGCCGC AAGTATGCGTGAGAGG-3'),

HIP1Δ184-400 (5'- GGCTGGAGAAAGTGACGTGAACAACTTTTTCCAG GTTGTGCTGCAGCTGAAGGGCCACGTC-3'),

HIP1Δ401-599 (5'- CACAGCTAGAAAACATGAAGACTGAGAGCCAGCGG GACACTCAGCTCAAACTGGCCAGCACAGAGG-3'),

HIP1Δ600-800 (5'- GGAGGAATTATCTGCTCTTCGGAAAGAACTGCAG AGAATAGAGGAGATGCTCAGCAAATCCCGAGCAGG-3'),
HIP1Δ600-700 (5'- GGAGGAATTATCTGCTCTTCGGAAAGAACTGCAG TGCCTCAGAGCCCCACCTGAGCCTGCC-3'),
HIP1Δ690-752 (5'- GGCCTGCCCAGAAGACATCAGTGGACTTCTC ATGAGGAACTGCCTGAGCAAGATCAAGGCCA-3'),
HIP1Δ752-800 (5'-GAAGCCTTGAGAATGCCGACAGCACAGCC AGAATAGAGGAGATGCTCAGCAAATCCCGAGCAGG-3'),
HIP1 L486A (5'- GGTTCAGAACCACGCTGACCTCGCGAGGAAGAATGCAG-3'),
HIP1 Y117F (5'- GGACTCTCTGAGATTCAGAAATGAGCTCAGTGACAT GAGCAGGATGTGG-3'),
HIP1 Y135F (5' CCTGAGTGAGGGGTTTGGCCAGCTGTGCAGCATCTACC-3'),
HIP1 Y142F (5'-CCAGCTGTGCAGCATCTTCCTGAAGCTTCTAAGAAC CAAGATGG-3'), and
HIP1 Y152F (5'-CCAAGATGGAGTTCCACACCAAAAATCCGCGGTTCCCAGGC-3') mutants were generated using a QuikChange site-directed mutagenesis kit (Stratagene). HIP1 mutants ΔA and ΔT have been previously described (Rao et al., 2002). pcDNA3.1 CFP-Rab5Q79L has also been previously described and was a generous gift from Kristen Verhey (University of Michigan) (Qian et al., 2009).

Antibodies:

The following antibodies were used in western blot experiments: mouse anti-Myc (1:5000; Cell Signaling), rabbit anti-myc (1:5000; Cell Signaling), mouse anti-V5 (1:5000; Invitrogen), 4G10 anti-phosphotyrosine (1:1000; Millipore), mouse anti-clathrin

(1:250; TD.1; AbCAM), IC5 anti-HIP1r (1:2000), and 4B10 anti-HIP1 (1:2000). IC5 and 4B10 have been previously described (Rao et al., 2002). For immunofluorescence experiments, the following antibodies were used: mouse anti-Myc (1:500; Cell Signaling), rabbit anti-Myc (1:500; Cell Signaling), rabbit anti-GFP (1:500; Santa Cruz Biotechnology), mouse anti-clathrin (1:500; X22; AbCAM), rabbit anti-EEA1 (1:500; AbCAM), and rabbit anti-APPL (1:500; AbCAM). AlexaFluor 488- and 647-conjuated secondary antibodies (Invitrogen) were used for visualization.

Co-immunoprecipitation:

Ten micrograms of HIP1-myc and EGFR-V5, EGFRVIII-V5, ErbB2-V5, or IGF-1R-V5 cDNA constructs in pcDNA3 were co-expressed in HEK 293T cells using the Superfect reagent (Qiagen). Twenty-four hours post-transfection, cells were lysed, cleared of excess membranes, and diluted to a concentration of 2 mg/mL. For immunoprecipitation, 500 μL of these lysates were incubated with 50 μL of anti-Myc or 10 μL anti-V5 beads (Sigma). These pellets were washed three times with lysis buffer, re-suspended in sodium dodecyl sulfate (SDS), and boiled for 5 minutes for western blot analysis. For immunoprecipitation of HIP1 and HIP1r without epitope tags, UM410 and UM374 antibodies were used, respectively (Rao et al., 2002).

Western blot:

Protein lysates in Laemmli buffer were separated on 6% or 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to nitrocellulose membranes. Membranes were probed with antibodies against HIP1-myc (mouse monoclonal 4B10, rabbit polyclonal UM354 and Myc rabbit monoclonal; Cell Signaling), IGF-1R (rabbit polyclonal; Santa Cruz Biotechnology), c-Kit-V5 (mouse

monoclonal [Cell Signaling]; V5 mouse monoclonal [Invitrogen]), or actin (rabbit polyclonal). Blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and developed using chemiluminescence (Pierce).

Immunofluorescence:

HeLa cells were cultured in 6-well plates with coverslips and transfected the following day with HIP1-myc, HIP1Δ752-800-myc and/or Rab5Q79L-CFP. Coverslips were stimulated with either 100 ng/mL EGF (Sigma) or 1 μg/mL EGF-555 (Invitrogen) at 4°C for 30 to 60 minutes and then media was changed to 37°C for the indicated times. Cells were fixed for 20 minutes in 4% paraformaldehyde at 4°C, blocked, and stained with the antibodies listed above.

Images were collected using a Zeiss LSM 510-META laser scanning confocal microscope with a 63× water immersion objective. Colocalization was calculated in ImageJ (National Institutes of Health) using the JACoP plug-in to calculate Manders' coefficients using automated thresholding. Statistical analyses were performed using GraphPad PRISM software.

Protease inhibitor and Phosphatase Treatment of Cell Cultures:

Prior to lysate collection, cells were treated with 100μM of marimastat (Sigma) for 12 hours to inhibit metalloprotease activity prior to lysate collection and immunoprecipitation. In experiments where cells were incubated with the phosphatase inhibitor sodium orthovanadate (NaVa), cells were treated with 2mM of NaVa in normal media for 2 hours before collection. For phosphatase treatment, 5U of calf intestine alkaline phosphatase (CIP, Promega) was added to collected lysates before immunoprecipitation.

Chapter 4:
Conclusion

The work presented in chapters 2 and 3 clarifies the role of the oncoprotein HIP1 in RTK endocytosis and supports the concept that it contributes to the pathogenesis of human cancer. We have discovered HIP1 overexpression in a deadly and poorly understood form of skin cancer called Merkel Cell carcinoma (MCC). HIP1 antibodies were detected in MCC patients, and a specific correlation was identified between N-terminal antibodies and patients with metastatic disease. Additionally, an interaction between HIP1 and c-Kit, a RTK expressed in MCC, was observed, leading to the hypothesis that HIP1 has broad functionality in RTK endocytosis.

We then further investigated the mechanics of the interaction between HIP1 and EGFR, the most studied RTK in receptor-mediated endocytosis research. This led to the identification of HIP1 as a novel EGFR substrate for tyrosine phosphorylation that binds the EGFR kinase domain. A truncated form of EGFR, termed EGFRtr, was discovered, which resembles the oncogenic EGFR mutant EGFRvIII. Like the full length EGF receptor, EGFRvIII also phosphorylates HIP1. Additionally, HIP1 colocalizes with activated EGFR in clathrin-coated vesicles and Rab5 early endosomes. This corresponded with localization to both early, APPL1, and late, EEA1, Rab5 positive endosomes.

4.1 HIP1 Antibodies as Markers of Metastatic Cancer

Previous work has shown that HIP1 auto-antibodies can serve as markers of cancer presence in prostate cancer, brain cancer and lymphoma patients (Bradley et al., 2007a; Bradley et al., 2007d). This effect is likely due to the high expression of HIP1 within these cells, which makes this protein more available as an antigen to the immune system as a result of dying tumor cells. Though these findings showed promise as an immunological blood test for cancer, their usefulness was limited by low specificity of the test, which involved detection of serum antibodies against the purified C-terminus of HIP1.

In the work described here, we have devised a new test in which patient serum was probed for antibodies against the N-terminus of HIP1. We evaluated this new test in patients with MCC and found that it specifically differentiated between patients with metastatic disease and those with localized disease. This was surprising, as both antigens were derived from the same protein. Antibodies against the C-terminus of HIP1 in these patients were prevalent, but showed no correlation with severity of disease. This new test is significant in that it may have prognostic value as an early indicator that someone will develop metastatic disease. This would be especially useful for cancer types where the benefits of aggressive intervention are often unclear, such as prostate cancer.

Another possible benefit of the development of these anti-HIP1 autoantibodies is an intrinsic immune resistance to tumors. Previous work has shown that patients with B cell lymphoma and anti-HIP1 antibodies have better clinical outcomes than those who lack antibodies against this oncoprotein, possibly due to a concurrent cell-mediated immune response (Bradley et al., 2007d). This indicates that vaccination against HIP1

may be a useful preventative measure or adjunct therapy for some types of cancer that overexpress HIP1.

4.2 HIP Interaction with Multiple RTKs

While HIP1 has been previously referenced as a cargo specific adaptor for ionotropic neurotransmitter receptors (Wieffer et al., 2009), the variety of RTKs that HIP1 binds and stabilizes suggests a much broader role for HIP1 in endocytosis. To date, HIP1 has been shown to interact with several types of cargo including AMPA receptors, NMDA receptors, androgen receptors and RTKs (Hyun and Ross, 2004). Among RTKs, HIP1 binds EGFR and FGFR4 and probably many other receptors in this category (Bradley et al., 2007a; Wang et al., 2008). The work in the previous chapters also identifies c-Kit, IGF-1R, and ErbB2 as RTK cargo bound by HIP1.

Although we have found that HIP1 binds the EGFR kinase domain, it is unknown if this is the domain through which HIP1 binds other RTKs. Binding to the EGFR kinase domain likely enables HIP1's tyrosine phosphorylation when this receptor is co-expressed. Since HIP1 tyrosine phosphorylation has not been observed in the presence of other RTKs, it is possible that HIP1 instead binds these receptors more indirectly, through AP2. Still, HIP1 is able to stabilize c-Kit, which from our observations thus far does not phosphorylate HIP1 (unpublished results, Sha Huang), so HIP1 phosphorylation may be dispensable for stabilizing receptors, but provides a different form of regulation in the presence of EGFR.

It is possible that HIP1 could affect the trafficking of some receptors differently than others. For example, in another murine HIP1 knock-out model, decreased AMPA receptor internalization was observed in cultured neurons, but the lack of HIP1 caused no

difference in transferrin internalization (Metzler et al., 2003). This indicates that although no effect on transferrin internalization or recycling was observed in our HIP1/HIP1r DKO MEFs, alterations in RTK recycling may still exist. Additionally, HIP1 overexpression may augment receptor recycling even if the loss of HIP1 does not cause recycling defects. In transformed NIH-3T3 cells that overexpress HIP1, for example, transferrin levels are increased. Meanwhile, when HIP1 is lost in knock-out mice, redundant pathways may allow transferrin recycling to persist normally. This phenomenon would echo the large amount of redundancy that is observed in receptor internalization (Goh et al., 2010). Future work, therefore, should examine the affect of HIP1 on RTK recycling in HIP1 overexpressing conditions, both transient and stable, to evaluate the ability of HIP1 to enhance the recycling of RTKs, particularly those involved in tumorigenesis.

4.3 HIP1 interaction with N-terminally truncated EGFR and EGFRvIII

Previous work in our laboratory has used a clone of HIP1 that has contained an amino acid variation from the established HIP1 sequence (Bradley et al., 2007a; Bradley et al., 2007c; Bradley et al., 2005; Bradley et al., 2007d; Graves et al., 2008; Hyun et al., 2004a; Hyun et al., 2004b; Rao et al., 2003; Rao et al., 2001; Rao et al., 2002). This mutation results in a serine substitution from phenylalanine at amino acid 182. When fixed, a truncated form of EGFR was observed to bind HIP1, termed EGFRtr. HIP1 bound EGFRtr more strongly than the full-length receptor, though the amount of binding to the full-length receptor remained at a similar strength as the F182S mutant. The amount of phosphorylation of HIP1 and HIP1 F182S was also similar when co-expressed

with EGFR. The retention of the C-terminal tag by EGFRtr also led us to infer that EGFRtr was formed through an N-terminal truncation.

EGFRvIII is an oncogenic mutant of EGFR that structurally resembles the N-terminally cleaved form of EGFR that we have observed interacting with HIP1. This receptor is present in 40% of glioblastomas that contain EGFR amplifications (Loew et al., 2009). We have also found that it is able to bind and phosphorylate HIP1. Though HIP1 is able to bind the N-terminally truncated EGFRvIII, this binding is not enhanced as compared to full-length EGFR, so increased affinity is not a likely cause for the enhanced binding of HIP1 to EGFRtr. This indicates that HIP1 recruits, or is recruited by, the protease that initiates EGFRtr formation. As the protease must be extracellular to cause the 140kDa fragment, it likely also spans the membrane to interact with HIP1 or interacts through another transmembrane protein. This could explain the inability of HIP1 F182S to bind EGFRtr, if it is in a critical domain for the recruitment of a transmembrane protease that cleaves EGFR. Amino acid 182 is just adjacent to the HIP1 ANTH domain, which would likely cause this region to abut the plasma membrane. Though this amino acid is conserved in HIP1r, phenylalanine 181 is not (this corresponds to HIP1r isoleucine 172), and could also disrupt protease binding and the formation of EGFRtr.

There is precedent in the literature suggesting that EGFR may undergo an N-terminal cleavage event. These observations date back to the early 1990s, where Decker *et al*., observed N-terminal cleavage of both full-length EGFR and EGFR c'973 mutant following EGF activation (Decker et al., 1992). Other studies have observed shedding of the extracellular domain of EGFR in both activation dependent and independent cases. In one study, metalloprotease-mediated cleavage was found to be activation independent,

while serine protease cleavage is dependent on protein kinase C activation (Perez-Torres et al., 2008). This study identified a form of EGFR that was cleaved at amino acid 625, resulting in a much larger truncation of EGFR than we have observed and concluded that this constitutive EGFR shedding seen in cells expressing high levels of EGFR was dependent on metalloprotease activity (Perez-Torres et al., 2008). The fragments formed by activity dependent serine protease cleavage were not identified.

Other enzymes, however, have been found to produce cleavage of EGFR that result in truncated EGFR similar in size to the form that we have observed. For example, there are a number of serine proteases shown to cleave, or induce the cleavage of, the EGFR N-terminus. These enzymes include matriptase and hepsin, which both directly enable EGFR cleavage (Chen et al., 2010). Matriptase activity in the presence of prostasin, another serine protease activated by matriptase, cleaves EGFR, resulting in 135kDa and 110kDa C-terminal fragments that are unable to bind EGF (Chen et al., 2008). Prostasin, which is also activated by hepsin, does not affect the ability of hepsin to cleave EGFR, which results in a 100kDa C-terminal EGFR fragment. Though the precise function and initiating factors for these forms of EGFR cleavage are unknown, it is possible that they could contribute to oncogenesis. For example, EGFRvIII, like the forms of EGFR produced by serine protease cleavage, is unable to bind EGF, but is still able to dimerize and is constitutively active at low levels (Huang et al., 1997). This oncogenic property is also present in matriptase-cleaved EGFR, though the hepsin-cleaved receptor lacks strong kinase activation (Chen et al., 2008).

As one might expect, due to its ability to create a constitutively active form of EGFR, matriptase is upregulated in a wide variety of epithelial tumors (Webb et al.,

2011). Matriptase is also thought to be oncogenic by contributing to invasion of the extracellular matrix. Meanwhile, the matriptase positive regulator prostasin has been identified as a tumor suppressor in prostate cancer, that when re-expressed reduces EGFR levels and downstream signaling (Chen et al., 2008). However, it is possible that the tumor suppressor role for prostasin is independent of its role in EGFR cleavage. Like matriptase, hepsin is overexpressed in cancer, specifically prostate and breast cancer (Webb et al., 2011).

Due to their role in tumorigenesis, serine protease inhibitors have been subjects of investigation in cancer research. These substances are especially useful for our future investigations of the role of EGFR cleavage in HIP1 endocytic and oncogenic function. 3,4 Dichloroisocoumarin is a small molecule serine protease inhibitor that is able to inhibit all enzymes with this form of active site, which makes it useful for inhibiting these enzymes but not very specific in its effects. Additionally, this drug provokes apoptotic cascades after treating cells for four hours (Hameed et al., 1998). More specific protease inhibitors have been developed targeting matriptase, however these are not currently commercially available. CVS-3983, for example, was a small molecule inhibitor of matriptase that was produced by the now defunct Corvas International biotech company and was able to slow prostate cancer cell line growth (Galkin et al., 2004). Hepatocyte growth factor activator inhibitor (HAI-1) is an endogenous inhibitor of matriptase (Webb et al., 2011). When co-expressed with matriptase in a transgenic mouse model, HAI-1 was able to suppress squamous cell carcinoma formation (Webb et al., 2011). This specific function may make HAI-1 a useful tool to investigate whether the cleaved form

of EGFR that strongly binds HIP1 is truncated through matriptase-mediated enzymatic activity.

4.4 Putative roles for HIP1 tyrosine phosphorylation by EGFR

Our work has identified HIP1 as a novel substrate for tyrosine phosphorylation in the presence of EGFR. Interestingly, although HIP1 is tyrosine phosphorylated by EGFR and its oncogenic mutant EGFRvIII, this has not been observed in the presence of other RTKs, even when stimulated by ligand or when phosphorylation was stabilized by treatment of cells with the phosphatase inhibitor sodium orthovanadate. Tyrosine phosphorylation of HIP1 has been previously observed, however, as a substrate of the cytoplasmic tyrosine kinase dual specificity tyrosine phosphorylation regulated kinase 1 (Dyrk1), which is activated in the presence of basic fibroblast growth factor (bFGF), but not EGF (Kang et al., 2005; Yang et al., 2001). It is unknown if HIP1 is phosphorylated at the same site through both of these mechanisms, because the site of tyrosine phosphorylation has not been identified in either of these conditions.

We have determined that HIP1 is phosphorylated at multiple sites within multiple domains. Additionally, two domains have been identified as functionally necessary for HIP1 tyrosine phosphorylation in the presence of EGFR, independently of the sites of phosphorylation. The fact that HIP1ΔA is not phosphorylated by EGFR is not particularly surprising, considering the strong, pro-apoptotic effect of this mutant and the fact that it does not seem to be exported to the cell surface (Hyun et al., 2004b). The lack of phosphorylation of HIP1Δ750-800 is more surprising as this deletion does not remove any tyrosines and this mutant expresses and localizes normally. Additionally, it shows normal recruitment to Rab5 early endosomes upon EGF stimulation and non-aberrant

binding to EGFR or clathrin. It is possible that this region, which contains the flexible USH domain, allows an additional surface for EGFR binding that helps mediate phosphorylation. Additionally, it may aid in the recruitment of a third factor that is necessary for HIP1 phosphorylation upon EGFR activation.

Though it is unknown what the role of HIP1 tyrosine phosphorylation is for its function in EGFR trafficking, numerous other endosomal proteins that are phosphorylated by EGFR have been characterized. Phosphorylation fulfills a variety of roles in these proteins. Tyrosine phosphorylation of endosomal proteins could cause them to dissociate from other adaptors or lipids within endosomes. For example, tyrosine phosphorylation of Hrs causes it to dissociate from the endosome (Urbe et al., 2000). Additionally, protein phosphorylation can allow binding to other proteins that would not be possible when the kinase substrate is not phosphorylated. Threonine phosphorylation of AP2, for example, increases its binding to lipids and to endosomal cargo proteins (Fingerhut et al., 2001). The role of tyrosine phosphorylation of AP2, however, remains unknown. The timing of HIP1 phosphorylation upon EGF stimulation places HIP1 in the early endosome at the point of dephosphorylation. This may indicate that HIP1 is involved in the regulation of another protein at this location and time point or that this is the point of HIP1 dissociation from EGFR.

4.5 HIP1 localization

HIP1 localization within the endosomal pathway has been a matter of debate with inconsistencies. For example, some groups only observe HIP1 association with the endosome during the process of internalization, after which it is quickly shed (Gottfried et al., 2009). Others have found HIP1 localizing to sorting endosomes (Metzler et al.,

2003). This distinction is important because it informs us of the stage of endocytosis in which HIP1 may promote tumorigenesis.

We found that HIP1 was recruited to Rab5 early endosomes upon EGF stimulation, which marks both early and late sorting endosomes that contain the Rab5 effectors APPL1 and EEA1, respectively. Recently APPL1 has emerged as a marker of intermediate endosomes forming the bridge between clathrin coated pits and EEA1 positive endosomes (Zoncu et al., 2009). Interestingly, these vesicles contain a unique array of phospholipids, including PI(3,4)P_2 (Zoncu et al., 2009). These endosomes are Rab5 positive, but do not endogenously colocalize with EEA1, though this colocalization can be induced within enlarged vesicles formed by constitutively active Rab5 (Q79L). HIP1 was also found to colocalize with EEA1 after EGFR stimulation, slightly after its colocalization with APPL1. This finding was consistent with previous observation in neuronal trafficking of the AMPA receptor, where colocalization with EEA1 was observed (Metzler et al., 2003).

HIP1 colocalization with the Rab5 endosome was surprising, however, as Rab5 helps mediate the uncoating of the HIP1 binding partner AP2. Clathrin, meanwhile, has been previously shown to localize to these endosomes in a manner regulated by the Hrs C-terminus. It is possible that Hrs is involved in the recruitment of HIP1 to the Rab5 endosome as well. Other areas of HIP1 localization following EGFR stimulation deserve examination as well, namely whether HIP1 localizes to Rab11, Rab35 or Rab4 recycling endosomes. It is also possible that HIP1 could be involved in recycling clathrin-coated endosomes directly back to the cell surface. This work would require extensive tracking of HIP1 association with surface labeled EGFR to determine their travel patterns. Lastly,

HIP1 could be involved in a clathrin-mediated recycling pathway from the TGN, which would require a similarly strict level of live imaging analysis for what would be a novel pathway.

This live tracking of HIP1-positive endosomes would be difficult, due to the destabilizing effect of the GFP fluorescent protein on HIP1 binding to clathrin. This effect is a possible cause for the rapid shedding of HIP1 from endosomes observed in an earlier TIRF study (Gottfried et al., 2009). It is possible that tagging HIP1 with a N-terminal fluorescent protein rather than a C-terminal could eliminate this effect. There are also newer methods involving smaller tags and fluorescent dyes that could also reduce tag interference with HIP1 function. One of these methods involves the use of a 6 amino acid tetracysteine tag (CCPGCC) that can be specifically stained using the cell-permeable biarsenical dyes FlAsH and ReAsH. The high level of background staining associated with this method, however, requires extensive washing of cells before imaging, which can be cytotoxic (Hinner and Johnsson, 2010). One recent breakthrough in small molecule fluorescent labeling has been the engineering of a recombinant lipoic acid ligase (LplA) that is able to conjugate the 7-hydroxycoumarin fluorophore to a 13 amino acid LAP2 (LplA acceptor peptide 2) tag (GFEIDKVWYDLDA) (Uttamapinant et al., 2010). When co-expressed in mammalian cells, this enzyme has shown ability to specifically stain several LAP2 conjugated recombinant proteins, including actin and vimentin (Uttamapinant et al., 2010). As HIP1 function does not seem to be significantly disturbed by the 10 amino acid Myc tag, LplA-mediated staining may be a viable option for imaging HIP1 to evaluate its association with EGFR in the process of recycling.

4.6 The Role of HIP1 in RTK stabilization: a potential cancer drug target

The data presented in the previous chapters present a number of mechanisms through which HIP1 could stabilize RTKs (Figure 4.1). These possibilities all need to be tested in order to determine the means through which the HIP1 oncoprotein could be targeted in cancer treatment. Along with the targeting of other oncoproteins in the endocytosis pathway, inhibiting HIP1 function has the potential to promote the degradation of many oncogenic receptors in many tumor types.

For one, HIP1 could stabilize oncogenic signaling in RTKs by prolonging the time that activated receptors remain in the APPL1 endosome, from which signaling through the Akt pathway is active. Secondly, HIP1 could promote rapid recycling of clathrin-coated vesicles if it directs receptors to the clathrin-mediated endocytosis pathway rather than the degradative clathrin-independent pathway. Additionally, HIP1 could promote RTK recycling simply by inhibiting its progression to the MVB through competitive binding with Hrs or Eps15b to RTKs. HIP1 could also have an oncogenic effect by increasing recycling of receptors through the trans Golgi network (TGN), as HIP1-transformed NIH-3T3 cells demonstrate redistribution of clathrin to the TGN (Rao et al., 2003). Lastly, HIP1 could participate in the formation of an inherently stable, constitutively active EGFR in the form of EGFRtr.

Differentiating between the above possibilities is no easy task, but may provide a large clinical benefit. It could also help us understand the function of other oncogenes with which HIP1 interacts, including EGFRvIII and possibly matriptase. This depth of knowledge is therefore invaluable to patients and we look forward to seeing our

understanding of HIP1-mediated oncogenesis improve and evolve to the point where this protein can be targeted in cancer therapy.

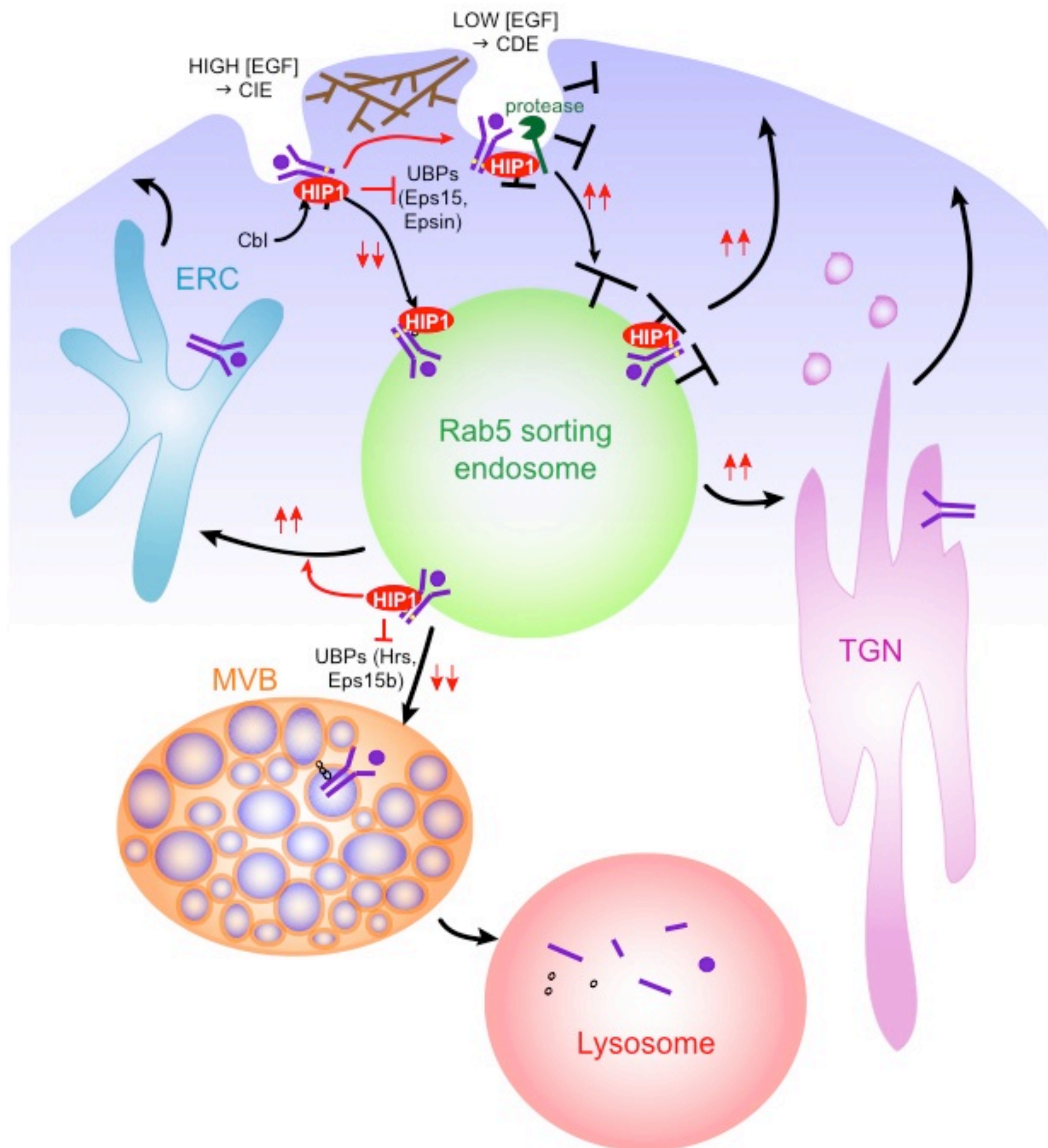


Figure 4.1 Putative Mechanisms for HIP1-mediated Oncogenesis. Due to its binding to the EGFR kinase domain and localization to the sorting endosome, HIP1 could stabilize EGFR through a number of mechanisms including inhibition of degradation, promotion of recycling, or a combination of both.

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