REGULATION OF EPIDERMAL GROWTH FACTOR RECEPTOR SIGNALING BY CLATHRIN-COATED MEMBRANE MICRODOMAINS

By

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Abstract

REGULATION OF EPIDERMAL GROWTH FACTOR RECEPTOR SIGNALING BY CLATHRIN-COATED MEMBRANE MICRODOMAINS

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Master of Science, Molecular Science, Ryerson University. Toronto, Ontario 2015

The phosphatidylinositol-3-kinase (PI3K)-Akt signaling axis controls cell survival, proliferation and metabolism, and is activated by receptor tyrosine kinases (RTKs) such as the epidermal growth factor (EGF) receptor (EGFR). In addition to activation of PI3K-Akt signaling, the binding of EGF to its receptor results in rapid recruitment of EGFR to clathrin-coated pits (CCPs) followed by eventual EGFR internalization. Hence, receptor-proximal activation of signaling intermediates occurs while EGFR resides within CCPs; however, whether CCPs are required for EGFR signaling remains poorly understood. Using a combination of pharmacological inhibition and siRNA gene silencing of clathrin, we have examined how clathrin controls EGF-stimulated activation of Akt. We find that perturbation of clathrin, but not of EGFR endocytosis by perturbation of dynamin leads to disruption of EGF-stimulated Akt phosphorylation. This indicates that clathrin acts in a function separate from its role in endocytosis to regulate EGFR signaling at the plasma membrane. The EGF-stimulated phosphorylation of the signaling intermediate Gab1, but not that of EGFR itself, was also abrogated upon disruption of clathrin. We then utilized total internal reflection fluorescence microscopy (TIRF-M) to examine the hierarchy of recruitment of EGFR signaling components to CCPs. Collectively, these findings suggest a role for clathrin as a central regulator of EGFR signaling leading to Gab1 and Akt phosphorylation.

Acknowledgements

I would like to first thank my supervisor, Costin Antonescu. Being a student under his supervision has been nothing short of wonderful, exciting and extremely rewarding. Our relationship could have potentially started off on the wrong foot due to a poor choice of words regarding how I really felt about cell signaling, however it did not, in fact I believe those words are one of those memories that will bond us forever. His dedication to the field of molecular science, and research students, both at the graduate and undergraduate levels is unparalleled at Ryerson University. As one of his first of four graduate students, I had the esteemed pleasure to see his commitment and dedication to the program in collaboration with fellow faculty members (Dr. Roberto Botelho, and Dr. Bryan Koivisto) be extremely fruitful, from hosting the annual Ryerson Science Symposium, to the acquisition of a deconvolution and TIRF microscope for the department. His commitment remained in the back of my mind throughout my term at Ryerson, and made me want to do more and better, turning 9 hr days in the lab, into 14hr days in the lab, eventually graduating into overnights in the lab. For this extra push I am eternally grateful.

I would also like to thank Roberto Botelho who was the bad cop to Costin's good cop. Together they create a dynamic and engaging learning environment that has a "take no prisoners" feel. Whether group meetings were held in a classroom over treats or in a bar over drinks they were always stimulating and enjoyable to say the least. Roberto is not shy when it comes to putting someone on the spot or speaking his mind about how we are not at the

level he expects us to be. However, it was obvious that it was never malicious and always from the kindness of his heart. Further, I do have to thank Roberto for planting the signaling seed within me, as he is the person responsible for sparking my interest in cell signaling, and eventually recommended me to Costin kick-starting my graduate career. Lastly, I am thankful for their combined efforts in helping craft my scientific thinking and also my ability to effectively communicate science.

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INTRODUCTION

Epidermal growth factor receptor (EGFR) is one of 58 receptor tyrosine kinases (RTK) and is part of the ErbB subgroup of RTKs. A wide variety of signal transducers that induce cell proliferation and cell survival are activated by EGFR including the MAPK/ERK and PI3K-AKT signaling pathways. Thus, EGFR activity is necessary to sustain cellular function as well as a hallmark for many cancers that exhibit aberrant EGFR signaling. EGFR signaling is highly regulated by multiple methods including dephosphorylation of the kinase domain by phosphatases as well as direct removal from the plasma membrane by endocytosis, thus implicating endocytosis in EGFR signaling. Further, there are differences in EGFR signaling from the plasma membrane versus endosomes following endocytosis of the receptor, suggesting that EGFR signaling is contingent on its transit to different microdomains.

1.1 Cancer and EGFR

The United States of America in partnership with The American Cancer Society estimated that there would be approximately 1.7 million Americans diagnosed with cancer in 2014 alone. The Canadian government in partnership with Canadian Cancer society estimated that 1 in 4 Canadians would die from cancer, making cancer the leading cause of death in Canada, responsible for 30% of deaths. Although it is well documented that women are more likely to acquire certain types of cancers such as breast cancer in comparison men, the number of estimated new cases and deaths are shared equally between both

sexes. Further, the National Institute of Health (NIH) has estimated that cancer has a major impact on the economy, costing $216.6 billion in direct and indirect healthcare costs. But what is cancer and why is cancer biology important? In simple terms, cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells, which can result in death if not controlled. Uncontrolled growth has been further characterized and is represented by 6 hallmarks: proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan & Weinberg, 2011). However, in order to fully understand cancer biology an emphasis has to be put on 'tumor microenvironments', this includes the recruitment of normal stromal cells and their active participation. Therefore it is important to not only understand the biological hallmarks of cancer in cancer cells but in non-cancer cells as well.

As a result cancer biology is a vast and growing discipline in the natural sciences. Great strides have been made, and many key players responsible for tumor development have been identified, a central player being EGFR. A general schematic of RTK activity resulting in cell proliferation and cell survival Figure 1.

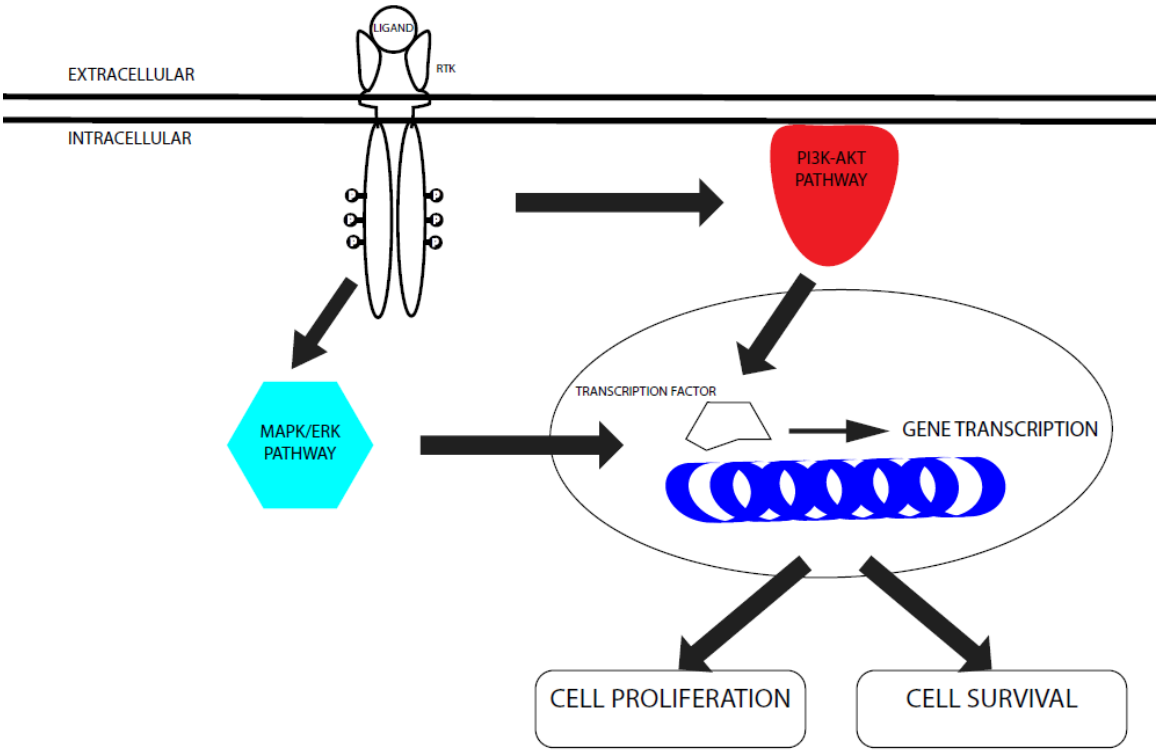

Figure 1. Schematic representation of general RTK signaling. Ligand binding of RTK results in the activation of the intrinsic kinase on the cytosolic subunit. RTK signaling induces the activation of cytosolic kinases that indirectly induce gene transcription. Activation of transcription factors increases the transcription of genes associated with cell proliferation and cell survival.

EGFR has been well documented as an agonist for tumor growth through unregulated cell division (Sharma, Bell, Settleman, & Haber, 2007; Tomas, Futter, & Eden, 2014). Cancers that exploit EGFR activity to further cell proliferation can result from one or a combination of perturbations: abnormal expression of EGFR, mutations to EGFR, or over activation of EGFR (Lafky, Wilken, Baron, & Maihle, 2008; Lee et al., 2006; Paez et al., 2004). There are two main types of mutant EGFRs responsible for the majority of tumors with constitutively active EGFR:

truncated EGFR mutants, and those with mutations in the kinase domain (Boerner, 2003). Over activation of EGFR results in the upregulation in the activity of MAPK/ERK and AKT pathways involved in cell proliferation and growth, therefore EGFR has been the target of many preclinical trials for over a decade now (Ciardiello & Tortora, 2001). However, cancer cells have been shown to exhibit aberrant activation by acquiring resistance to EGFR-targeted treatments through direct mutations on EGFR, or inhibition of negative regulators of EGFR, such as the phosphatase PTEN (Ji et al., 2006; Soria et al., 2002). Acquired resistance can be seen as early as 6-12 months following initiation of treatment (Pao et al., 2005; Sequist et al., 2011) This acquired resistance led to the idea of alternative EGFR-therapies such as irreversible inhibitors that form covalent bonds with EGFR, which have been shown to overcome the T790M mutant EGFR found in many cancers (Kwak et al., 2005). However, EGFR has many physiological roles as well, EGFR has been shown to be involved in facilitating IL-13 mucin secretion by airway cells necessary for innate immunity (Burgel & Nadel, 2008), alter neutrophil and mast cell infiltration and enhance angiogenesis during wound healing (Repertinger & Campagnaro, 2004), and lastly has been shown to reverse ischemic renal failure of the kidneys (Humes, Cieslinski, Coimbra, Messana, & Galvao, 1989). Therefore it is imperative that we turn to basic science to define the network of interactions and signaling axis regulated by EGFR in order to be better equipped and develop viable therapeutics that target specific aspects of EGFR signaling, not inhibit global EGFR signaling. However investigating all signaling pathways implicated by EGFR is beyond the

scope of my thesis and I will focus on EGFR activation of cell proliferation and survival signals.

1.2 EGFR activation

EGFR signaling is complex, with many positive and negative feedback interactions of activated signals, and encompasses signals that induce transcription, protein synthesis and cell proliferation, cell survival and inhibition of apoptosis through the JAK/STAT, MAPK/ERK, PLC/PKC and PI3K/AKT signaling pathways outlined in Figure B. This ability to affect so many downstream signals is partially due to the structure of EGFR itself.

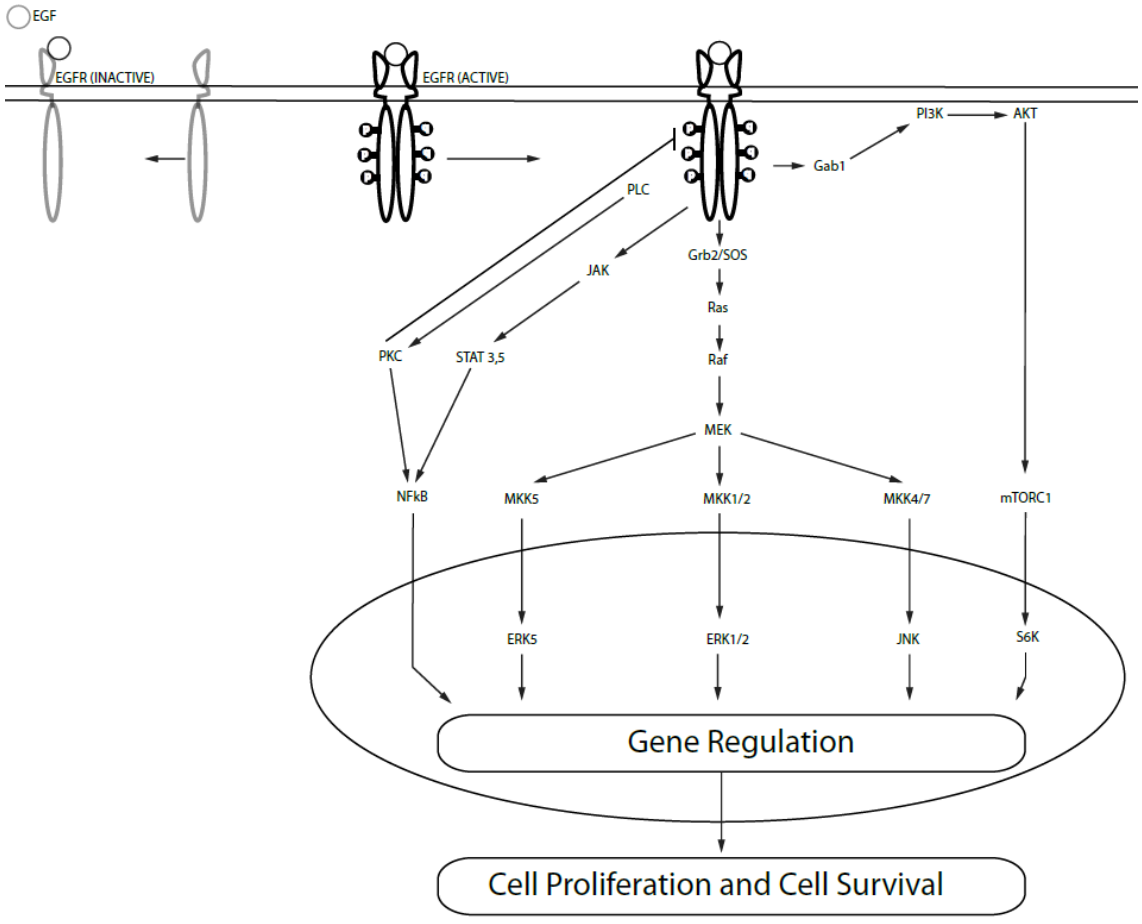

Figure 2. Schematic representation of EGFR signaling from the plasma membrane. EGF binding of EGFR results in rapid dimerization and autotphosphorylation of the cytosolic domain. EGFR activation results in the activation of many signaling pathways including PKC, JAK/STAT, MAPK/ERK, and PI3K/AKT. Activation of signaling transducers induces gene regulation followed by a key hallmark in cancer biology, cell proliferation/survival.

Like most RTKs, EGFR is composed of an extracellular domain, followed by a single pass transmembrane domain, and a cytosolic tail containing a conserved protein tyrosine kinase core, flanked by regulatory sequences (Schlessinger, 2002). The extracellular region consists of 4 domains: I, II, III, and IV, or L1, S1, L2, S2, respectively. It has been proposed that EGF ligand binds to domains I and III, and domains II and IV serve to mediate dimerization and interactions with other membrane proteins (Lax, Bellot, Howk, Givol, & Schlessinger, 1989). Mediation of dimerization and interactions by domains II and IV is accomplished through cysteine-rich regions on the respective domains. Specifically, it has been shown that domain IV regulates ligand-binding affinity through Intramolecular interactions, and domain II is responsible for acting as a scaffold that positions domains I and III in an orientation that generates a ligand binding pocket (Abe et al., 1998; Ogiso et al., 2002). Upon ligand binding a conformational change is induced that leads to activation of an intrinsic kinase activity of EGFR, the cytosolic domain becomes autophosphorylated and serves as a docking site for SH2 and PTB domains of signaling proteins (Pawson & Schlessinger, 1993). The active form of EGFR requires asymmetric dimerization where one member of the dimer is required to be bound to ligand.

EGFR has the ability to form homo- and hetero-dimers with members of the ErbB family of receptors: ErbB2, ErbB3, and ErbB4 (Graus-porta, Beerli, Daly,

& Hynes, 1997). In fact, the same study was able to show that EGFR preferred to make hetero-dimers with ErbB2 then with any other ErbB family member including homo-dimers. Interestingly, there are no known ligands that bind to ErbB2, so it cannot respond to ligand stimulation in the form of an ErbB2 homo-dimer (Brennan, Kumogai, Berezov, Murali, & Greene, 2000). Thus allowing functional hetero-dimerization with ErbB family members in the presence of ligand and functional homo-dimerization of ErbB2 in the absence of ligand. Hetero-dimerization would in turn serve as a means to modulate receptor-signaling abilities, as it has been shown that hetero-dimerization of ErbB receptors results in the recruitment and activation of a wide variety of signaling intermediates. Specifically, EGFR:ErbB3 can bypass the requirement of Gab1 in EGF-stimulated phosphorylation of AKT, necessary for EGFR:EGFR homo-dimer signaling (Mattoon, Lamothe, Lax, & Schlessinger, 2004). This is supported by work done by Prigent & Gullick (1994), which demonstrated that ErbB3 has 6 binding sites for PI3K, suggesting that EGFR:ErbB3 heterodimers may have the means to bypass the requirement for Gab1 in EGFR:EGFR homodimers signaling (Prigent & Gullick, 1994). Differences in signaling have also been shown for EGFR:ErbB2 hetero-dimers, EGFR homo-dimers were shown to indirectly activate PI3K (Hsieh & Moasser, 2007), whereas EGFR:ErbB2 hetero-dimers were shown to directly bind the P85 regulatory subunit of PI3K (Olayioye et al., 1998). Following dimerization, the activation loop within the cytoplasmic domain region of EGFR adopts an active configuration enabling ATP and substrate binding resulting in enhanced protein tyrosine kinase activity, in turn

resulting in receptor autophosphorylation on many residues (Abe et al., 1998; Ogiso et al., 2002). This is followed by accumulation of phosphorylation modifications on EGFR, and further activation of downstream signaling pathways such as MAPK/ERK and the AKT pathways (Schlessinger, Shechtert, Mark, & Pastant, 1978). However, EGF:EGFR interactions are more complicated then previously mentioned, EGF binds with two distinct dissociation constants, the minority (2-5%) binding with high affinity receptors, and the majority (95-98%) binding with low affinity receptors (Schlessinger, 2002). Further, ligand affinity is controlled by the extracellular domains I and III of EGFR, and has implications on ligand-induced tyrosine autophosphorylation of EGFR (Garrett et al., 2002). These differences in binding affinity could be a cause for signaling variability and/or regulation of signaling. However, doing an in-depth analysis of all EGFR ligands and mechanism of signaling for each ligand is beyond the scope of my thesis so I will focus primarily on EGF-stimulated activation of the PI3K-AKT signaling axis.

AKT signaling has been shown to regulate cell proliferation, cell survival, and inhibit apoptosis, it is also the major pathway that is up-regulated in a number of cancers that exhibit aberrant EGFR signaling. Thus it is necessary to fully understand the mechanisms for EGF-stimulated AKT activation. PI3K-AKT signaling axis can be fairly complicated so I will approach this topic in sections beginning with EGFR recruitment of the adaptor protein Grb2, Grb2-Gab1 interactions, Gab1-PI3K interactions, PI3K induced interaction of PDK1, mTORC2, and AKT, and finally AKT signaling Figure 3.

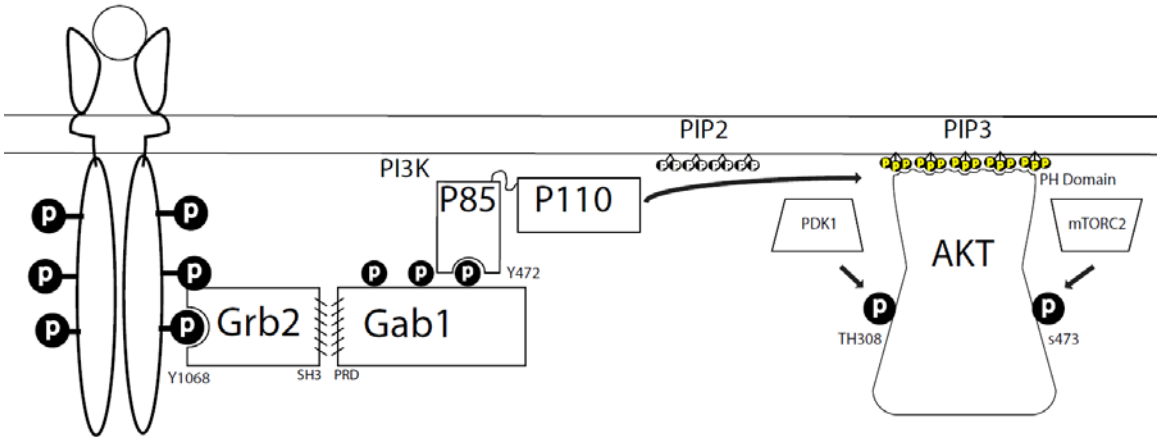

Figure 3. Schematic representation of EGF-stimulated activation of AKT. EGF binding of EGFR asymmetric dimerization followed by recruitment and binding of the adaptor protein Grb2 on Y1068 docking sites. Further, Gab1 is recruited to Grb2 via its proline-rich domain docking on the SH3 domain on Grb2. Gab1 is then phosphorylated and serves as a docking site for binding of the P85 regulatory subunit of phosphatidylinositol-3-kinase (PI3K). P85 binding of Gab1 relieves the inhibition of the P110 catalytic subunit of PI3K, which then converts PIP2 into PIP3. AKT is then recruited to the cell membrane via its PH-domain where it is phosphorylated by PDK1 and mTORC2 on T308, and S473, respectively.

1.3 EGFR recruitment of Grb2

EGFR activation of AKT pathway relies on the phosphorylation of principle tyrosine residues, specifically Y1068, which is phosphorylated primarily by the intrinsic kinase ability of EGFR and a minority is phosphorylated by Jak2 (Yamauchi, Ueki, & Tobe, 1997). Y1068 provides a docking site for the SH2 domain containing proteins, specifically the adaptor protein Grb2, a critical signaling intermediate necessary for EGF-stimulated activation of the MAPK/ERK and AKT pathways (Pawson & Schlessingert, 1993; Saito et al., 2004). To lesser extent Grb2 directly binds Y1086, and indirectly binds Y1173 and Y992 through another adaptor protein, Shc (Batzer, Rotin, Skolnik, & Schlessinger, 1994).

1.4 Grb2 recruitment of Gab1 via SH3 domains

Following Grb2-Y1068 interaction, Grb2-associated binding protein 1 (Gab1) binds to the carboxyl-terminal SH3 domain on Grb2 via a proline rich domain (PRD) (Fixman et al., 1997). Gab1 does not directly bind to EGFR; therefore Grb2 is an essential adaptor protein in mediating EGF-stimulated activation of downstream signaling cascades, specifically through the phosphorylation of 3 tyrosine residues on Gab1 Y446/472/589 (Mattoon et al., 2004). However, the interaction between Grb2 and Gab1 is considered an atypical SH3-PRD interaction, as it requires a proline and arginine motif (PXXXR), which is considerably different then the canonical PXXPXR motifs that bind SH3 domains (Lim, Richards, & Fox, 1994; Lock, Royal, Naujokas, & Park, 2000). The reasoning for this discrepancy in SH3 binding motifs is unknown, and is a continued area of research. Further, Gab1 negatively regulates EGFR signaling through the activation of the protein tyrosine phosphatase SHP2, shown to directly dock on Y627/659 on phosphorylated Gab1 (Cunnick, Mei, Doupnik, & Wu, 2001). Gab1 does not only recruit and activate SHP2, but is also one of its targets, thus serving as a negative regulator (Yart et al., 2001).

1.5 P85 regulatory subunit of PI3K binding of phospho-Gab1

As mentioned previously there are 3 principal binding sites on Gab1 that are essential in EGF-stimulated activation of the AKT pathway; they are Y446/472/589. These tyrosine residues are essential to cell proliferation because they serve as docking sites for the P85 regulatory subunit of the signaling intermediate, PI3K. Although PI3K has been shown to directly bind RTKs it is

accepted that Gab1 is the major docking site for the P85 subunit of PI3K (Mattoon et al., 2004). Once P85 domain binds Gab1, P85 is displaced from the catalytic subunit essentially freeing the P110 catalytic subunit of PI3K allowing it to specifically catalyze the addition of a phosphate group on the D-3 position of the inositol ring of phosphotidylinositol-4,5-bisphosphate [PI(4,5)P2] in response to EGF (Fruman, Meyers, & Cantley, 1998). This gives rise to phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3] from the plasma membrane lipid [PI(4,5)P2] (Cantley, 2002). This rush in D-3 phosphorylated lipids results in the recruitment of cytosolic proteins to the plasma membrane with a high affinity for [PI(3,4,5)P3] via a Pleckstrin homology domain (PH domain). These proteins vary from serine-threonine kinases and tyrosine kinases, to exchange factors that regulate G-proteins. Of specific interest is the serine-threonine kinase AKT and phosphoinositide dependent protein kinase-1 (PDK1). The recruitment and accumulation of these PH-domain containing proteins at the cell surface facilitates activation of AKT by PDK1 (Lawlor & Alessi, 2001).

1.6 AKT activation following recruitment to the membrane

Upon PI3K activation, AKT is rapidly recruited to the plasma membrane via its PH domain (Thomas, Deak, Alessi, & Aalten, 2002). AKT then provides a docking site for PDK1 in the form of a hydrophobic motif (HM) facilitating phosphorylation of the T308 site on AKT and serving as an allosteric regulator of catalytic activity (Antal et al., 2002; Yang et al., 2002). Following PDK1 phosphorylation of T308 on AKT, there is a charge-induced change in conformation allowing substrate binding and a substantial increase in catalytic

activity of AKT (Alessi et al., 1996). However, for full activation of AKT, it needs to be subsequently phosphorylated on S473 residue, which is carried out by mTORC2 (Sarbassov, Guertin, Ali, & Sabatini, 2005). There is also evidence to suggest AKT phosphorylation of S473 can be accomplished by autophosphorylation of the kinase (Toker, 2000). However, it is commonly accepted that mTORC2 is the kinase responsible for the majority of S473 phosphorylation. Phosphorylation of AKT on both T308 and S473 allows for full activation of AKT kinase activity, which allows for activation of downstream signaling pathways that prevent apoptosis and induce cell proliferation and growth. Activation of AKT leads to the inhibition of inhibitory signaling intermediates such as the TSC1/2 of the mTORC1 pathway. AKT thus serves to induce a wide variety of transcription factors such as FOXO, GSK3, and S6K all which are well known activators of cell survival (Burgering, 2003; Cross, Alessi, Cohen, Andjelkovich, & Hemmings, 1995; Mcmanus, Alessi, & Pkb, 2002). For example, AKT activation leads to the phosphorylation of the apoptotic Forkhead-related-transcription factor 1 (FKHR-L1), which creates a binding site for the 14-3-3 family of proteins, leading to the cytosolic sequestering of FKHR-L1 inhibiting apoptosis (Brunet, Datta, & Greenberg, 2001).

1.7 Attenuation of EGFR signaling from the plasma membrane

As mentioned earlier aberrant EGF-stimulated activation of AKT can lead to aberrant cell proliferation, and potentially cancer, therefore there are many pathways to regulate AKT activation. The phosphatases SHIP2 and PTEN are two very common pathways in the regulation of growth factor signaling. SHIP2

and PTEN regulate the product of PI3K activity, PI(3,4,5)P3 . Specifically SHIP2 dephosphorylates the D-5 position of PI(3,4,5)P3, and PTEN dephosphorylates the D-3 position of PI(3,4,5)P3, and the SHIP2 product PI(3,4)P2 (Clement et al., 2001; Maehama & Dixon, 1999). However, PTEN is the stronger regulator of AKT activity, because AKT has been shown to bind PI(3,4)P2, resulting in recruitment of AKT to the plasma membrane leading to moderate levels of AKT activation (Alessi et al., 1997; Franke, Kaplan, & Cantley, 1997; Klippel, Kavanaugh, Pot, & Williams, 1997). Another mode of AKT activity regulation is through endocytosis of EGFR. Over the timeframe of a couple of hours endocytosis of EGFR can attenuate the strength or duration of the signal by reducing the numbers of receptors on the plasma membrane, or by removing the receptor from necessary substrates (Sorkin & von Zastrow, 2009). In fact endocytosis of EGFR inhibits receptor activation of the PI3K-AKT pathway as endosomes lack their lipid substrate PI(4,5)P2 (Haugh & Meyer, 2002). Aside from the PI3K-AKT and MAPK/ERK pathways EGFR does serve as a network node for signaling of a number of signal transducers including PLCγ/PKC and JAK/STAT (Figure 2). In regards to JAK/STAT signaling, Janus kinase 1/2 (JAK) is recruited to the phosphorylated cytosolic domain of EGFR where it is activated and recruits STAT1/3 with SH2-binding motifs to EGFR where it can be phosphorylated directly by EGFR but primarily by JAK (Brooks et al., 2014). This signal activation is implicated in inducing cell migration as well as cell proliferation mediated by NFkB (Andl et al., 2004). However, negative regulation of JAK/STAT is not accomplished by endocytosis of EGFR, but by a number of

pathways including the phosphatases PTP1B, PTP-BL, and SHP as well as ubiquitin-mediated degradation by the cytokine signaling suppressor SOCS3 (Hatakeyama, 2012). Further, PLCγ is a signaling transducer associated with the activation of EGFR, as it is known to mediate intermolecular interactions in RTK signaling due to its multivalency. By having several domains (2-SH2 domains, 1-SH3 domain, 2-PH domains, and 1-C2 domain) this aids in directing the formation of signaling complexes (Lemmon & Schlessinger, 2010). PLCγ directly cleaves PI(4,5)P2 into the product diacylglycerol (DAG) that remains bound to the membrane and the cytosolic product inositol 1,4,5-triphosphate (IP_3). IP_3 then binds to calcium channels on the ER causing an efflux of calcium into the cytosol, this calcium then interacts with DAG and Protein Kinase C (PKC) to induce cell proliferation mediated by NF-kB. However, what makes PLCγ interesting is that the mediation of PKC activation also serves as a negative regulator of EGFR activation (Lemmon & Schlessinger, 2010). PKC phosphorylates T654 in the juxtamembrane domain of EGFR, eliminating binding of EGF to plasma membrane associated EGFR, thus attenuating EGFR signaling (Schlessinger & Ullrich, 1990). Thus providing another means for attenuation of EGFR signaling from the plasma membrane.

Endocytosis of EGFR

2.1 Clathrin-mediated Endocytosis of EGFR

Clathrin-mediated endocytosis (CME) is the primary method in which eukaryotic cells internalize cell surface receptors. The defining characteristic of CME is the recruitment and enrichment of soluble cytosolic clathrin to the plasma

membrane, which form triskelia by combining three clathrin heavy chains (CHC) together via their CHC proximal domains; each CHC is bound to two clathrin light chains (CLC) (von Kleist et al., 2011).

The progression of CME is initiated by interactions between PI(4,5)P2, clathrin, and adaptor protein-2 (AP2) (Antonescu, Aguet, Danuser, & Schmid, 2011). These three components form an endocytic hub responsible for binding the cargo, and inducing the inward invagination of plasma membrane, resulting in the formation of a clathrin-coated pit (CCP). At this stage, over 50 different accessory proteins have the ability to be recruited to the N-terminal domain of the clathrin forming CCPs. The majority of these signaling proteins are poorly understood and further characterization and role identification is necessary, however a subset is known to have signaling properties. Further, many of these proteins function in the maturation stage of CCP formation by inducing membrane curvature through ENTH, ANTH, and BAR domains, such as Eps15 and AP180/CALM, which also play a role in cargo selection (Conner & Schmid, 2003). Following membrane invagination and a poorly understood maturation process, the GTPase dynamin is eventually recruited to the neck of the CCP by multivalent interactions, involving its PH-domain, the GTPase effector and middle domains (GED) and its proline/arginine rich domains (PRD) (Conner & Schmid, 2003). At this stage, dynamin is thought to self-assemble into a coil around the neck of the CCP, followed by vesicle fission catalyzed by the hydrolysis of GTP, releasing a clathrin-coated vesicle into the cytoplasm. Following complete vesicle internalization, the clathrin coat is removed and the vesicle fuses with an early

endosome, where the cargo is either marked for recycling back to the plasma membrane or for degradation (McMahon & Boucrot, 2011). CME occurs constitutively in mammalian cells, and carries out nutrient uptake such as iron bound to the transferrin receptor (TfnR). Internalization of transferrin receptor is exclusive to CME and therefore commonly used as a tool to monitor how effective endocytic deficient cells can inhibit internalization of cargo via CME. CME is also regulated by spatial orientation; organized at endocytic hotspots held in place by the actin cytoskeleton and a number of accessory proteins that create protein-protein interactions to scaffold the endocytic machinery to the actin cytoskeleton (Conner & Schmid, 2003; Grossier, Xouri, Goud, & Schauer, 2014; Nunez et al., 2011)

There are two subpopulations of endocytic structures, short-lived ‘abortive’ and long-lived ‘productive’ structures. Interestingly, more than 60% of initiated endocytic events fall under the category of abortive structures (Aguet, Antonescu, Mettlen, Schmid, & Danuser, 2013) Figure 4. High frequency of abortive clathrin structures suggests that they may have other functions then simply failing at endocytosis. The expenditure of energy necessary to actively initiate and abort 60% of endocytic events seems unlikely to be just a waste. EGFR recruitment to clathrin structures occurs within seconds of activation, internalization on the other hand takes anywhere from 60-200 seconds. This begs the question, are these abortive clathrin structures necessary for EGFR signaling from the plasma membrane?

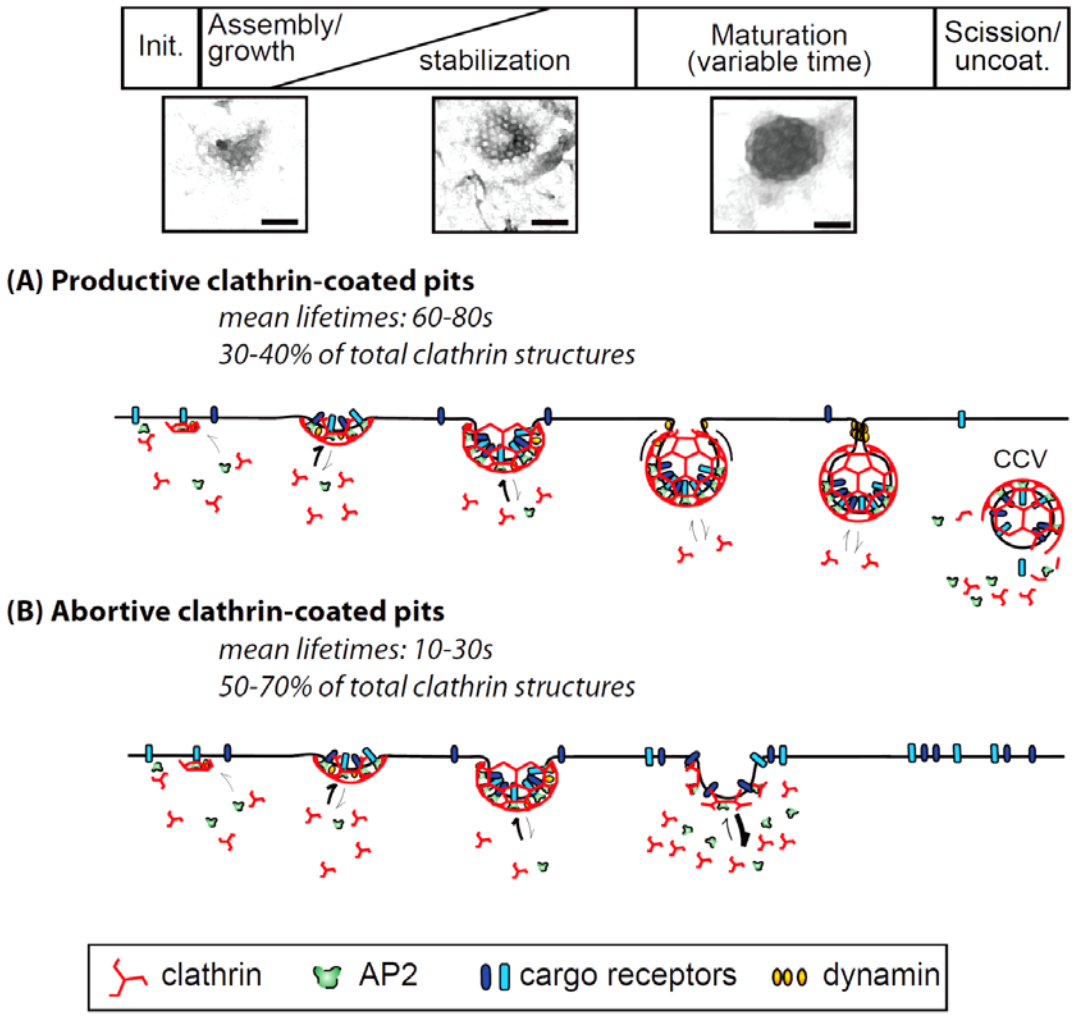

Figure 4. Schematic representation of clathrin-mediated endocytosis. Clathrin-mediated endocytosis leads to the formation of A) productive clathrin-coated pits that become internalized and form clathrin-coated vesicles, or B) abortive clathrin-coated pits that actively disassemble and never become internalized. Both processes begin with an initiation phase which involves PI(4,5)P2, AP-2, clathrin, and the cargo. This is followed by assembly/growth and a maturation phase. Productive CCPs undergo scission by the GTPase dynamin that creates a collar at the neck of the CCP and pinches it off resulting in the formation of CCV. Lifetime of productive pits is typically 60-200s. ***Importantly EGFR is recruited to either productive or abortive CCPs.***

2.2 Non-Clathrin Endocytosis of EGFR

Non-clathrin endocytosis (NCE) is an endocytic process that occurs independently of clathrin. In the absence of clathrin, actin, actin-associated proteins, and sorting nexin 9 become important players in vesicle formation. In

regards to internalization of specific cargo, it has proven to be more complex than predicted due to the various forms of regulation and the numerous proteins that direct vesicular uptake. A few modes of NCE are, RhoA-dependent and Cdc42-dependent uptake, Flotillin-dependent endocytosis, Caveolar endocytosis, Arf6-associated uptake, and micropinocytosis (Sandvig, Pust, Skotland, & van Deurs, 2011). NCE presents a problem when attempting to characterize EGFR signaling in a CME dependent manner as it offers another route for the internalization of EGFR, which can effect EGFR signaling. However, there is extensive literature describing how to ensure EGFR internalization is mediated through CME, completely bypassing NCE. A commonly implemented method is the use of low concentrations of EGF. It has been shown that by stimulation of cells with concentrations of EGF ranging from 0.1-20 ng/ml, EGFR internalization is exclusive to CME (Sigismund et al., 2005, 2008). Not only does the use of low EGF concentrations successfully limit the convolution of endocytosis by NCE, it also makes this research physiologically relevant. By using EGF concentrations of 5-20ng/ml during our experiments we are well within the physiological levels of EGF which have been reported to vary from 1-2ng/ml in serum, to 10-100ng/ml in several other biological fluids (Sigismund et al., 2005). By implementing physiological levels of EGF we add a component to our research that could potentially facilitate research further down the road, perhaps with a greater focus on EGFR signaling in cancer cell lines.

2.3 Endocytic sorting of EGFR

Upon internalization of EGFR following ligand binding, two major fates await EGFR: recycling of the receptor from early endosomes or progression to lysosomes for degradation. This serves as a means of regulating EGFR signaling. Degradation of EGFR is directed by high concentrations of EGF, and is mediated primarily by NCE, such that 80% of EGFR is degraded following NCE upon stimulation of cells with 100ng/ml EGF. Further, low concentrations of EGF favor recycling of EGFR, and is mediated primarily by CME, such that 70% EGFR is recycled back to the plasma membrane following CME following initial stimulation with 1.5ng/ml EGF (Sigismund et al., 2008). Thus, there are two distinct pathways with their own requirements for EGFR sorting and trafficking from early endosomes.

Degradation of EGFR begins with the ubiquitination of the receptor by the E3 ligases c-Cbl and Cbl-b. Although, c-Cbl and Cbl-b are each sufficient on their own to ubiquinated EGFR, a double knockdown was involved to inhibit EGFR downregulation, thus both are necessary in EGFR regulation (Pennock & Wang, 2008). Ubiquitinated EGFR is then sorted by ESCRT machinery into the intraluminal vesicles of endosomes, successfully removing the intrinsic kinase of EGFR from the cytosol and inhibiting EGFR signaling and targeting the receptor for lysosomal degradation (Tomas et al., 2014). Further, dephosphorylation of EGFR serves as a regulatory method, which is carried out by protein tyrosine phosphatases (PTPs) such as PTP1B that dephosphorylate endocytosed EGFR on contact sites between the endoplasmic reticulum and endosomes (Eden,

White, Tsapara, & Futter, 2010). Degradation of internalized EGFR is further directed by AKT phosphorylation of the phosphoinsotide-kinase PIKfyve, ensuring the trafficking of EGFR from the early endosome to lysosomes (Er, Mendoza, Mackey, Rameh, & Blenis, 2013).

Recycling of EGFR, which is primarily mediated by CME at low concentrations of EGF, is less dependent on ubiquitination of EGFR (Eden, Huang, Sorkin, & Futter, 2012), but regulated by Rab4- and Rab35-route to the plasma membrane or more commonly by the Rab-11 dependent route via endocytic recycling-compartments. Rab-11 dependent recycling of EGFR is directed by many substrates such as Eps15s, CAML, the adaptor protein Odin, loss of Hrs phosphorylation, and AMSH-mediated deubiquitination of EGFR (Tomas et al., 2014). Once targeted for recycling, EGFR bound to EGF is shuttled to the plasma membrane where it is free to undergo another round of signaling and endocytosis-directed receptor recycling or degradation. Lastly, endosome acidity serves as another method for the regulation of EGFR signaling. The low pH of endosomes causes a disruption in of receptor interaction with some EGFR ligands (e.g. amphiregulin, TGFa), attenuating signaling. However EGF itself retains strong interaction with EGFR, thus prolonging stimulation in endosomal compartments. .

Nonetheless, further analysis of the fate of EGFR internalization following stimulation is beyond the scope of my thesis, but remains an area of interests taken into account the complexity of networks involved in this process.

Differences in EGFR signaling at the Plasma Membrane and Endosome

In addition to the complexity in the activation and attenuation of EGFR signaling, there are substantial differences in EGFR signaling dependent on the spatial organization of EGFR. Differences in EGFR signaling has been documented for almost 20 years for its important in both normal and abnormal cell functions, with a great focus EGFR signaling from the plasma membrane versus EGFR signaling from an endosome. Initial studies carried out in 1996 by the Schmid lab demonstrated that endocytic defective cells enhanced EGF-dependent cell proliferation, but that ERK required normal endocytosis for full activation (Vieira & Schmid, 1996). In 2003, the Wang lab developed a technique to specifically activate endosome-associated EGFR (e-EGFR) in the absence of plasma membrane-associated EGFR (PM-EGFR) activity. This study demonstrated that activation of e-EGFR was sufficient to satisfy the requirements for S-phase entry, therefore inducing cell survival. However, e-EGFR signaling was unable to induce cell proliferation, unlike PM-EGFR (Pennock & Wang, 2003). The idea of signaling differences between e-EGFR and PM-EGFR is supported by earlier work done by various labs that were able to show that critical proteins in the MAPK/ERK and AKT pathways are found docked on endosomes, including Grb2, SHC, MEK1 and MEK2 (Guglielmol, Baass, Ou, Posner, & Bergeron, 1994; Jiang & Sorkin, 2002). As well it has been shown extensively by the Di Fiore lab that endocytosis of EGFR is necessary for full activation of MAPK/ERK signaling pathway (Sigismund et al., 2008), which may be in part be due to a MAPK scaffold complex found in late endosomes, which

may facilitate phosphorylation of ERK1/2 (Teis, Wunderlich, & Huber, 2002). Recent work has also shown that accumulation of EGFR in endosomes can induce apoptosis, therefore showing diversity in EGFR signaling from endosomes, which must be heavily regulated in order to achieve the desired outcome whether it is cell survival or cell death (Rush, Quinalty, Engelman, Sherry, & Ceresa, 2012). Therefore it seems that the MAPK/ERK pathway relies heavily on e-EGFR signaling as opposed to PM-EGFR signaling, creating a clear distinction between e-EGFR and PM-EGFR signaling.

To contrast e-EGFR signaling, PM-EGFR signaling has been shown to not only sustain but is necessary for AKT pathway activation. In a study done in 2012 by the Schlessinger lab they were able to demonstrate that in endocytic-deficient cells and at low concentrations of EGF there was a strong inhibition of EGFR internalization, a robust enhancement of EGFR autophosphorylation, and sustained AKT activation, therefore suggesting that it is primarily activated by PM-EGFR (Sousa et al., 2012). Sousa implemented low levels of ligand stimulation (1.5-5ng/ml EGF) and a Tamoxifen-inducible dynamin conditional knockout mouse fibroblast ($Dyn^{-/-}$). Thus not only exclusively internalizing EGFR via CME but also inhibiting the internalization of the receptor. Therefore, inducing conditional knockout of dynamin successfully traps EGFR in clathrin-coated structures (CCPs) during EGFR signaling. As a result, EGFR signaling does not occur at the bulk plasma membrane but from within clathrin structures on the plasma membrane, under Tamoxifen-inducible dynamin perturbation. This body of work is extremely important in my research as it sets precedence for clathrin

structures as being necessary microdomains for EGFR signaling from the plasma membrane. PM-EGFR and endocytic sorting have also been implicated in EGF transcriptional responses. In clathrin-endocytic-defective cells there were increases in the abundance of EGF-induced transcripts, comparable to that exhibited by EGFR overexpression, thereby implicating PM-EGFR as the primary agonist of EGF-induced transcriptional response (Brankatschk et al., 2012). These results are in corroboration with previous studies carried out that define distinct roles for endocytosis in EGFR signaling and differences in e-EGFR versus PM-EGFR signaling (Sigismund et al., 2008; Vieira & Schmid, 1996).

However, in the last 10 years of uncovering signaling differences in EGFR, dependent on its spatial organization we have only touched on 2 of 3 possible microdomains, PM-EGFR vs. e-EGFR. However, our lab is suggesting that there is a third microdomain that is being overlooked, and that is EGFR that resides in clathrin-coated pits (CCP-EGFR) (Figure 5).

Rationale

I hypothesize that CCP-EGFR is a signaling microdomain that is necessary for EGF-stimulated AKT activation contingent on a few pieces of information. The most striking of those is the fact that it has been shown that upon EGF-stimulation, EGFR resides within CCPs at a time coincident with AKT activation. That is to say that upon receptor-ligand binding, EGFR is recruited to a CCP, where it remains, directing activation of AKT. This suggests CCPs facilitate EGF-stimulated AKT activation by acting as a microdomain, possibly serving as a scaffold. In order to test whether or not CCP-EGFR is essential for EGF-stimulated AKT activation, preliminary experiments had revealed that clathrin heavy chain silencing successfully perturbs a cells ability to form CCPs thereby isolating PM-EGFR signaling following stimulation with EGF. This was followed with dynamin-II silencing, which allows a cell to form CCPs, however is unable to internalize these structures, thereby isolating EGFR signaling within in CCPs following stimulation with EGF (CCP-EGFR). Importantly, it was shown that dynamin-silenced cells (CCP-EGFR) but not clathrin-silenced cells (PM-EGFR) successfully activated AKT, phosphorylating AKT on residues T308, and S473. This is consistent with the study done by the Di Fiore lab, which demonstrated that CME is essential for sustained EGFR signaling. Therefore the increased levels of active AKT can be due to longer residence times of active EGFR within CCPs, further confirming a role for CCPs as a microdomain necessary for EGF-stimulated AKT activation (Sigismund, 2008). An important caveat is that this study focuses on activity of AKT over hours whereas I will be

focusing on AKT signaling within the first 5min of EGF stimulated activation of EGFR. Schematic representation of differences in EGFR signaling from microdomains FIGURE E.

General Hypothesis

The preliminary work done by Dr Antonescu illustrating the need for CHC and the formation of CCPs for EGF-stimulated AKT activation as well as work done by the Di Fiore lab implicating endocytic proteins in EGFR signaling from the plasma membrane (Puri et al., 2005) is the groundwork to pursue my hypothesis that CCP-EGFR is a necessary microdomain for signaling of pro-cell survival signals, specifically AKT.

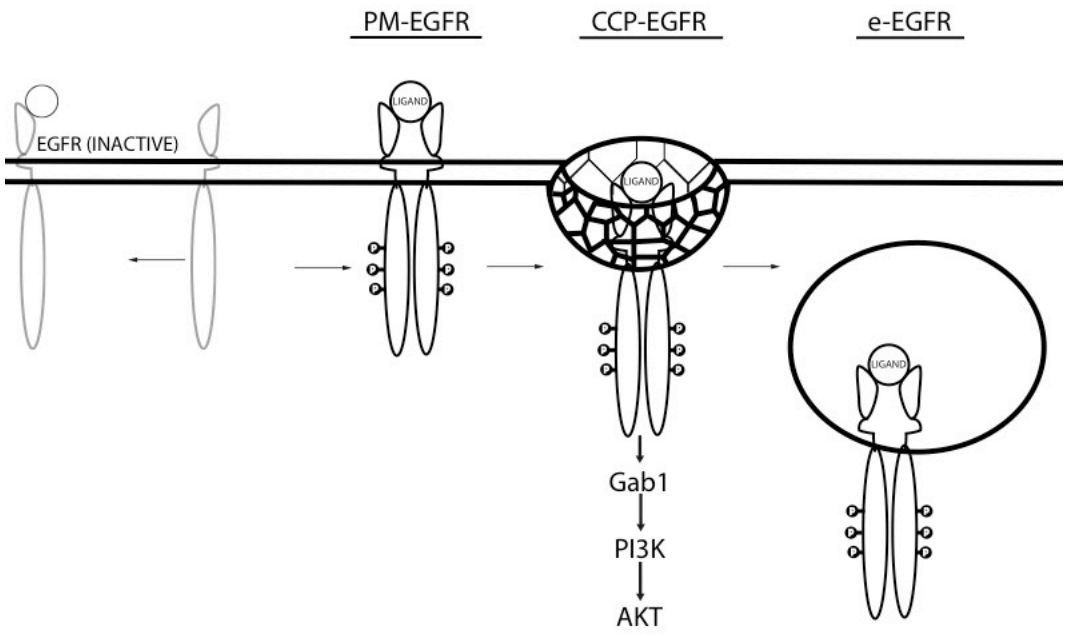

Figure 5. Schematic representation of EGFR signaling from separate microdomains. Upon ligand binding EGFR under goes internalization in order to regulate signaling, thus creating three possible microdomains for signaling. 1) plasma membrane EGFR signaling (PM-EGFR), 2) clathrin-coated pit EGFR

signaling (CCP-EGFR), and 3) endosomal EGFR signaling (e-EGFR). It is my hypothesis that EGF-stimulated activation of AKT is dependent on the CCP microdomains found on the plasma membrane. In order to isolate these three microdomains we use a combination of clathrin and dynamin inhibitors and gene silencing. In order to isolate PM-EGFR signaling we implement clathrin perturbation using clathrin gene silencing and Pitstop2 thereby inducing PM-EGFR signaling exclusively. In order to induce CCP-EGFR signaling exclusive we inhibit the internalization of CCPs with the dynamin inhibitors dynasore and Dyngo4a as well as dynamin gene silencing. Thus granting us the ability to isolate CCP-EGFR signaling from PM-EGFR and e-EGFR signaling

Materials and Methods

Antibodies and probes

EGFR, pEGFR (Tyr^{1068}), Akt, p-Akt(S^{473}, T^{308}), Gab1, p-Gab1(Tyr^{627}, Tyr^{307}), p-Erk1/2 (T^{202}, Tyr^{204}) were from Cell Signaling Technology (Denver, Colorado). The antibodies were from the following companies: Her2, Ambrx (La Jolla, California); Clathrin heavy chain, and Actin I-19, Santa Cruz (Dallas, Texas); Erk1/2, ; Gab1,; Akt, ; EGF antibody used for EGF uptake assay was from Upstate Biotechnology,Inc. Lake Placid, New York . Transferrin antibody used for Immunofluorescence was from Genetex, Irvine, CA. Strep-Cy3, anti-rabbit/mouse-Cy5, secondary antibodies as well as EGF-xx-biotin and Alexa 555 labeled EGF were purchased from Molecular Probes, (Invitrogen, Carlsbad, CA). Secondary antibodies for immunoblotting were from Cell Signaling Technology (Denver, Colorado), and Santa Cruz (Dallas, Texas);

Inhibitors and other reagents

Pitstop2 (final concentration, 10μM), Dynasore (final concentration, 80μM), and Dyngo4a (final concentration, 30μM) were from Abcam (Toronto, Canada). DMSO and EGF (5ng/ml for stimulation experiments, unless otherwise noted) were purchased from Life Technologies, Inc. Carlsbad, Ca.

Plasmids

AKT-RFP plasmid constructed and generously donated by Dr. Grinstein of University of Toronto. pBabepuro-ErbB2 was purchased from AddGene in Cambridge, MA. Gab1-wt, and Gab1-Delta (mutation on p85 docking site) plasmids were a generous gift from Dr Morag Park, McGill University in Montreal,

Canada. p85-GFP plasmid was a gift from Dr John Marshall from Ryerson University, in Toronto,

Cell culture and cell lines

Wild-type human retinal pigment epithelial cells (RPE-wt), and RPE cells expressing clathrin light chain-GFP (RPE-GFP), and clathrin light chain-RFP (RPE-RFP) were cultured in Dulbeccos's modified Eagle's medium (DMEM/F12) (with glucose and glutamine; CellGro, Herndon, VA) with 10% fetal bovine serum (FBS; Life Technologies, Inc. Carlsbad, Ca) and supplemented with 100 I.U./ml of penicillin and 100 µg/ml of streptomycin (ATCC, Inc. Manassas, VA). RPE-ErbB2 cells were also supplemented with 1µM Puromycin. All cells grown at $37^{O}C$ at 5% CO_2. For all EGF stimulations, cells were washed in 1x PBS (CellGro), and maintained in serum-free media (CellGro) for 1 to 2hrs before experimentation. Puromycin was used as a selection marker to create stable cell lines expressing ErbB2.

DNA plasmid preparation

Plasmids were heat shock transformed into DH5α competent *E. coli* and selected for using antibiotics (puromycin to select for pBABEpuro ErbB2; zeocin to select for p85-GFP, kanamycin to select for Gab1-wt; and ampicillin to select for Gab1-delta). Plasmids were prepared using Sigma Aldrich midi kits (Sigma Aldrich, St Louis, MO) or Qiagen mini kits (Qiagen, Valencia, CA) and transfected via Fugene (Promega, Madison, WI). Plasmids were sent out for sequencing confirmation (TCAG, Sick Kids, Toronto, On)

Transfections

In order to prepare for DNA plasmid transfection, cells were washed once in 1x PBS, and replaced with 0.8ml of DMEM containing 10% FBS. Fugene and cDNA was mixed in sterile H_20 at a 3:1 ratio (3µl of Fugene per µg of cDNA for each well in a 6-well plate) to a total volume of 44ul. cDNA mixture was then pipetted 20 times and allowed to sit at room temperature for 20min before pipetting 40ul the cDNA mixture dropwise into each well of a 6-well plate.

siRNA transfection sequences and protocol used in knockdown experiments: clahrin heavy chain I (CHC-I) small interfering RNA (siRNA) duplex (human CLTC, sense GGAAGGAAAUGCAGAAGAAUU; Dharmacon, Lafayette, CO) and CHC-II (human CLTC sense GGGAAUAGUUUCAAUGUUU; Dharmacon, Lafayette, CO) was transfected with Lipofectamine RNAiMax (Life Technologies, Carlsbad, CA) at 220pmol/L for RPE-wt (40-50% confluency to start in a 12-well plate). Cells are then washed 3x in PBS and replaced with 0.9ml of Opti-Mem (CellGro) 100ul of siRNA mixture is then added dropwise to each well and incubated for 3 to 4hrs . Cells are then washed in 1x PBS and then replaced with DMEM containing 10% FBS. RPE-wt cells are treated a second time after 24hrs and lysed after 72hrs, following serum starvation (1-2 hrs) and EGF stimulation (5ng/ml for 5, 10 and 15min). CHC ablation was confirmed by immunoblotting for CHC and compared to the loading control (actin I-19). Dynamin 2 (Dyn2) siRNA duplex (human dyn2, sense GGGCAGGCCUUCUAUAAGUUU; Dharmacon, Lafayette, CO) was transfected as described above. Dyn2 ablation was confirmed by immunoblotting for Dyn2

and comparing to the loading control (actin I-19). siCON7 is a nonspecific, nonscrambled siRNA duplex used as a control in knockdown experiments (human, sense CGUACUGCUUGCGAUACGGUU; Dharmacon, Lafayette, CO). Tranfection of siCON7 was carried out as described previously.

Cell lysis and immunoblotting

Whole cell lysates were prepared using a 2x Laemmli Sample Buffer stock (LSB, 0.5M Tris pH 6.8, Glycerol, 10% SDS) supplemented with a protease and phosphatase inhibitor cocktail (1mM sodium orthovanadate, 10nM okadaic acid, and 20nM Protease inhibitor cocktail) when blotting for phospho-proteins. Following EGF stimulation, cells were washed once with ice-cold PBS and aspirated. LSB was then added to each well (150uL, 12-well plate, 300ul, 6-well plate), scrapped and transferred to an eppendorf. Lysates were then heated at 65^0C for 15min and passed through a 27.5 gauge syringe. Reducing agents (10% β-mercaptoethanol, and 5% Bromophenol blue) were added following measurements of BCA protein assay (Pierce, Fisher Scientific, Waltham, MA). SDS-PAGE (60V, 30min; followed by 120V, 90min), followed by transfer (90V, 2hr) onto a PVDF membrane with a pore size of 0.2µm and binding capacity of 150-160 µg/cm^2 (BioRad, Mississauga, On). Membranes were then washed one in immunoblot wash (Tris-Base, NaCl, Tween-20, and NP40), and incubated at room temperature in blocking solution (3% BSA) for 1hr. Primary antibodies were prepared by adding 1% BSA, 0.02% Sodium Azide and 0.1% of antibody stock in immunoblot wash. BIORAD ChemiDoc XRS+ system was used for detection.

Transferrin uptake assay

RPE-wt cells were plated in 6-well plates 24 hrs before uptake assay to achieve 80-90% confluence. Cells were serum starved and incubated with pitstop2, Dynasore, and Dyngo4a for 30min at 37^0C with 5% CO_2. There were three sets of cells: Background, Total, and Uptake. *Uptake*: cells were stimulated with Tfn-Biotin (20ng/ml for 7.5min at 37^0C), and then washed 3x with ice-cold supplemented PBS (PBS^2: 1x PBS supplemented with 1mM $MgCl_2$ and 1mM $CaCl_2$). 1ml of a 1/175 avidin in PBS solution was then added to each well and allowed to sit at room temperature for 20min, following 6 washes with PBS before being fixed with paraformaldehyde (PFA) for 30min. *Background*: cells were placed on ice and washed 2x with ice-cold PBS then stimulated with EGF-Biotin (20ng/ml for 7.5min on ice), and then quenched with avidin for 20min. cells were then washed 6x with PBS before being fixed with PFA for 20min. *Total*: cells were placed on ice and washed 2x with ice-cold PBS^{2+} then stimulated with EGF-Biotin (20ng/ml for 7.5min on ice), and then washed 6x with PBS before being fixed with PFA for 20min. following fixation of all three sets of cells (Background, Total, and Uptake) 100nM was added to each well for 10min, followed by permeabilization of the cells with 0.1% Triton x-100. Subsequently, cells were blocked with a solution of 30% Superblock Lite (Fisher Scientific, Waltham, MA) before incubation with a 1/500 solution of strep-Cy3 in Superblock Lite for 1hr in the dark. Cells were then treated with 1/14,000 DAPI solution and mounted in DAKO (Sarstedt, Newton, USA)

EGF uptake assay.

Preparation of Anti-EGF ELISA plates: ELISA plates were prepared 24hrs before uptake assay. EGF antibody was added to a solution of 0.05M carbonate buffer, pH9.6 and incubated overnight at 40^0C in an advanced moisture controlled chamber. The antibody solution was then washed 3x in PBS and incubated in blocking buffer for 1hr at 37^0C prior to being loaded with samples.

Plating: RPE-wt cells were plated on 6-well plates 24hrs before uptake assay to achieve 90% confluence. Cells were serum starved and incubated with pitstop2, Dynasore, and Dyngo4a for 30min at 37^0C with 5% CO_2.

EGF uptake assay: There were three sets of cells: Background, Total, and Uptake. Background and Total: cells were placed on ice and were 2x with ice-cold supplemented PBS (PBS^{3+}: 1x PBS supplemented with 1mM $MgCl_2$, 1mM $CaCl_2$, and 5mM glucose). Cells were then stimulated with EGF-xx-biotin (5ng/ml). Uptake: uptake plate was then stimulated with EGF-xx-biotin (5ng/ml for 5, 10 and 15min). Uptake and Background: Following stimulation of uptake plates for each time point both uptake and background plates were washed 3x with PBS, and quenched with a solution of 1/1000 avidin diluted in PBS^{3+} and incubated for 30min on ice. Cells were subsequently washed and treated with a 1/40000 dilution of Biotin (Invitrogen, Carlsbad, CA in PBS^{3+} and incubated for 15min on ice. Total, uptake, and Background: cell lysis buffer (Superblock supplemented with 0.1% TX-100 and 0.05% SDS) was added to each well and cells were scrapped. 200ul of each condition was then transferred to anti-EGF-ELISA plate. EGF-biotin standards (0ng/ml to 0.5ng/ml EGF-xx-biotin) were

prepared using remaining EGF-xx-biotin. Sample-loaded anti-EGF plate was incubated at 4^0C overnight.

Detection of EGF-xx-biotin bound ELISA Plates: a solution of 1/5000 dilution of Streptavidin-OPD in blocking buffer is added to the ELISA plate and allowed to incubate at room temperature in the dark for 1hr, following 4 washes in PBS. Cells are then washed 3x with PBS, and 150ul of OPD assay solution (10mg of 0-phenylenediamine, 10ul H_2O_2 in 25ml OPD reaction buffer) is added to each well. Upon sufficient color development the reaction was terminated by adding 50ul (3M HCl). Absorbance of each well was then recorded at 492nm.

Microscopy and image analysis.

Samples were visualized by using an Olympus IX81 TIRF microscope (IX81; Olympus, Inc,; Richmond Hill, On), and widefield epifluorescence microscopes (Axiovert 200M; Carl Zeiss, Inc.; Thornwood, NY. Leica DM5000B, Leica Microsystems, Wetzlar, Germnay)

TIRF microscopy was carried out using a 150x oil immersion objective lens with a numerical aperture of 1.45. Images were captured using a Hamamatsu C9100-13 EM-CCD, Li2CAM iCCD (w/GenIII GaAs intensifier) and rendered using Perkin Elmer Volocity software. Laser lines used were 491nm (GFP) and 561nm (RFP/A55), and two single-band bandpass filters were used 520/35, and 624/40. TIRF-Analysis: Coated pits as well as Gab1 and EGF punctate structure were detected by software developed in Matlab (Mathworks, Matick, MA), as described in (Aguet et al., 2013). Briefly, clathrin-coated pits that are diffraction limited were detected using a Gaussian-based model.

Fluorescence intensity of proteins conjugated to red or green fluorophores were determined by the amplitude of the Gaussian model. Data was then used to render 2D histograms of intensity and differences in intensity in Matlab, using 35 intensity bins for each channel.

Epifluorescence microscopy was carried out using a 100x oil immersion objective lens with a numerical aperture of 1.45 on the Zeiss microscope, and 63X objective and 1.49NA on the Leica. Images were captured using a Hamamatsu Flash 4.0 and rendered using Perkin Elmer Volocity software. Excitation filters chosen were 340/25 for DAPI, 481/15 for GFP, and 531/40 for RFP/Cy3, emission filters chosen were 480,40 for DAPI, 520/35 for GFP, and 610/60 for RFP/Cy3. Widefield epifluorescence image analysis was carried out using ImageJ software (National Institutes of Health, Bethesda, MA). Briefly, EGF-stimulated gain in intensity was calculated as the difference in mean fluorescence between EGF-stimulated cells and basal (unstimulated cells) of the same condition.

Immuno-Fluorescence.

RPE-GFP cells were plated in 6-well plates 24 hrs before assay to achieve 90% confluence. Cells were serum starved and stimulated with EGF. Cells were then incubated at room temperature (RT) in the dark with 4% PFA for 20min. 100mM glycine was then used to wash the fixative and 0.1% Triton x-100 solution was used for cellular permeabilization. Following 3 washes in PBS cells were washed with 3% BSA for 20min in the dark at RT. Following 3 washes in PBS cells were treated with 1/400 antibody solution (P110α, P110β, P85, p-Gab1;Tyr^{627} and

ErbB2, Cell Signaling, Danvers, MA) for 1hr in the dark at RT. Following 10 washes with PBS over 20min with shaking they were treated wit a solution of secondary antibody (1/400 Cy3; goat-anti-mouse, goat-anti-rabbit) for 30mn in the dark at RT. Cells were then washed for 20min with shaking and incubated with 1µg/ml DAPI solution for 5min, followed by 5 washes in PBS and mounting in DAKO.

Results

In order to determine if EGFR signaling requires localization of the receptor to specific membrane locales such as clathrin microdomains or endosomes, methods capable of selectively perturbing EGFR transit through these membrane locales are required. Using this approach, EGFR can be isolated to or restricted within specific microdomains, allowing examination of the impact of this spatial compartmentalization of EGFR. By perturbing clathrin using Pitstop2, and by using clathrin siRNA gene silencing, I isolated EGFR signaling within the bulk plasma membrane. Within this compartment, EGF stimulation did not elicit stimulation of AKT phosphorylation. In contrast, perturbing dynamin using dynasore/Dyngo4a or dynamin siRNA gene silencing, I successfully isolated CCP-EGFR signaling. Localization of EGFR within this microenvironment allowed EGF-stimulated of AKT phosphorylation. Thus, my results indicate that CCPs are necessary scaffolds for EGF-stimulated activation of AKT, and as such, form signaling microdomains. I also demonstrated that some signaling components are recruited to CCPs following stimulation with EGF.

siRNA gene silencing of clathrin but not dynamin impairs EGF-stimulated Akt phosphorylation.

In order to determine if clathrin heavy chain and dynamin silencing selectively perturb EGFR transit to specific membrane locales, I monitored EGF internalization (**Figure 6A**). Briefly, the cell-surface receptors were stimulated with EGF-biotin to allow CME of the receptor following treatment with clathrin and dynamin inhibitors. Subsequently, cell surface EGF-biotin was quenched with avidin in order to isolate cytosolic EGF. We saw that gene silencing of either clathrin heavy chain or dynamin inhibited EGF transit into endosomes compared to control, thus trapping EGFR on the plasma membrane following stimulation with EGF ($n=3$, $p < 0.05$). Of note, while both clathrin and dynamin 2 siRNA prevented transit of EGF to endosomes, silencing of clathrin by this method resulted in PM-EGFR, while silencing of dynamin resulted in both PM-EGFR and CCP-EGFR. In order to determine whether perturbation of EGFR transit to specific microdomains impacted EGFR signaling from the bulk plasma membrane, I examined the phosphorylation of the downstream target AKT on S473. I found that inhibition of EGFR transit to clathrin-coated pits by clathrin siRNA significantly reduced AKT phosphorylation, however AKT phosphorylation was not affected by dynamin siRNA gene silencing (**Figure 6B & C**). These results suggest a requirement for clathrin structures in EGF-stimulated AKT phosphorylation, and also indicate that EGFR endocytosis is not required for EGF-stimulated activation of Akt.

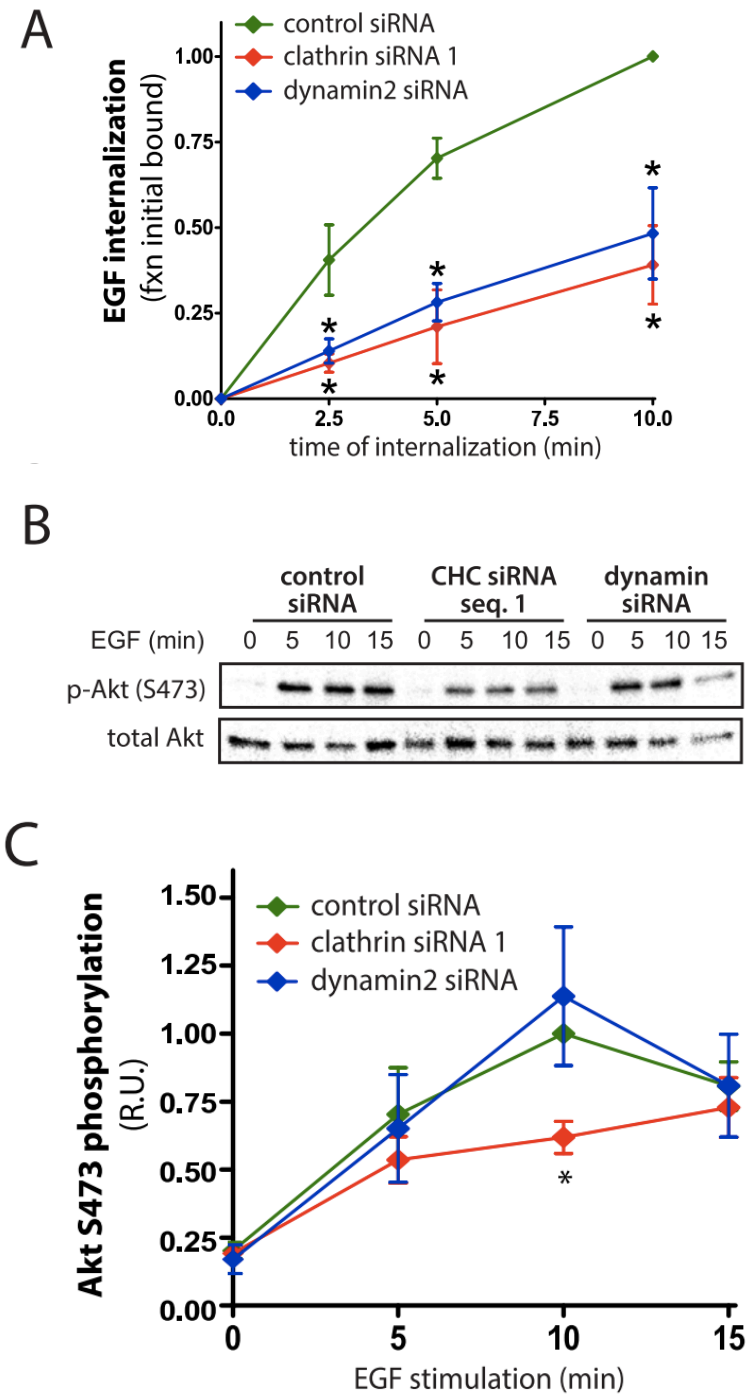

Figure 6. SiRNA gene silencing of clathrin heavy chain but not of dynamin2 inhibits EGF- stimulated Akt phosphorylation. Experiment performed by Dr. Costin Antonescu and Camilo Garay. RPE cells were with transfected with siRNA sequences targeting clathrin heavy chain, dynamin2 or non-targeting sequence (control). (A) Cells were stimulated with 5ng/ml EGF; EGF internalization was monitored over 10min. Both clathrin and dynamin silencing showed a significant decrease in internalized EGF (1-way ANOVA: n=3, $p<0.05$) relative to the control.

(B) Immunoblot demonstrating whole cell lysates that have been stimulated with 5ng/ml EGF over 15min following transfection relative to non-stimulated conditions (basal). (C) Graphical representation of immunoblot demonstrating significant reduction if AKT phosphorylation (S473) following clathrin but not dynamin gene silencing (2-way ANOVA: n=5, $p<0.005$), also shown is the mean ±SD.

In order to determine the efficiency of clathrin and dynamin perturbation, I examined the reduction in translation of clathrin and dynamin in RPE-wt cells following gene silencing of two siRNA sequences targeting clathrin heavy chain (CHC-1, CHC-2), and one targeting dynamin2. We confirmed that siRNA treatment resulted in robust reductions in expression of CHC and dynamin II, thus perturbing the formation of clathrin-coated pits and the transit of EGF to endosomes (**Figure 7A & C**). Further to determine the efficacy of perturbation of clathrin-mediated endocytosis under clathrin and dynamin gene silencing conditions, the internalization of the CME exclusive receptor transferrin was monitored over 10min. We saw that Tfn internalization underwent robust inhibition following both clathrin and dynamin gene silencing (1-way ANOVA: n=3, $p<0.05$) (**Figure 7B**). These results confirm that perturbation of clathrin and dynamin specifically and robustly inhibit clathrin-mediated endocytosis, thereby prevented transit of Tfn to endosomes. Further, we examined the effect of clathrin perturbation on EGF-stimulated AKT phosphorylation by two siRNA sequences targeting clathrin heavy chain. CHC-1 and CHC-2 had a robust inhibition of AKT phosphorylation (S473) (2-way ANOVA: n=3, $p<0.05$) (**Figure 7D & E**). These results suggest clathrin and dynamin perturbation significantly inhibited clathrin-mediated endocytosis. Further, two independent siRNA sequences targeting clathrin heavy chain perturbed EGF-stimulated AKT

phosphorylation (S473), further implicating clathrin structures as necessary microdomains for EGFR signaling from the plasma membrane.

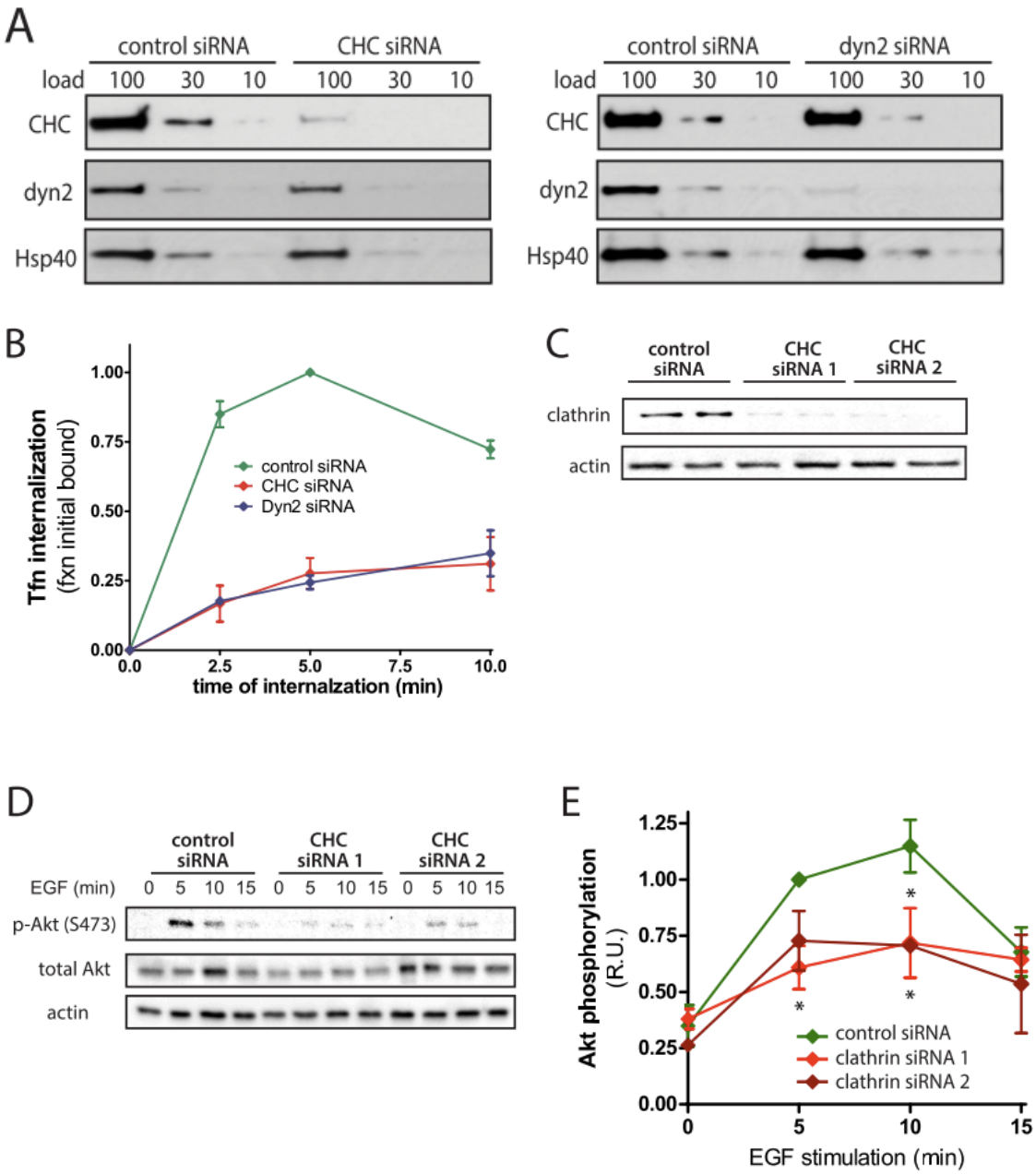

Figure 7. Effective target protein knockdown by siRNA gene silencing of clathrin and dynamin each inhibit Tfn internalization, yet only clathrin silencing impacts Akt phosphorylation.
Experiment performed by Dr Costin Antonescu and Camilo Garay. RPE cells were transfected with siRNA sequences targeting clathrin heavy chain (CHC-1, CHC-2), dynamin2 or non-targeting sequence (control). (A) Whole cell lysates were prepared immediately and a serial dilution was immunoblot resolved for

clathrin heavy chain, dynamin2, or Hsp40 (n=4). (B) Tfn internalization was measured in cells transfected with siRNA targeting clathrin heavy chain and dynamin2 (2-way ANOVA: n=3, $p<0.05$), also shown is the mean ±SD. (C) Representative image of whole cell lysates that were immunoblot resolved for clathrin heavy chain or actin (n=3). (D & E) RPE cells were with transfected with siRNA sequences targeting clathrin heavy chain (CHC-1, and CHC-2), followed by stimulation with 5ng/ml EGF over 15min, or non=stimulated (basal). (D) Representative image of immunoblot of phospho-AKT (S473), total AKT, or actin (n=3). (E) Graphical depiction of clathrin gene silencing on AKT phosphorylation (S473) for two independent siRNA sequences (2-way ANOVA: n=3, $p<0.05$), also shown are the mean ± SE.

Acute inhibition of clathrin but not dynamin by small molecules impairs EGF-stimulated Akt phosphorylation.

In order to determine the working concentration of the small molecule Pitstop2, which is an acute inhibitor of clathrin, I performed a dose curve analysis of the effect of this drug on EGF-stimulated Akt phosphorylation. I treated RPE-wt cells with varying concentration of Pitstop2, followed by stimulation with 5ng/ml EGF for 5min. Whole cell lysates were then immediately prepared, and immunoblot techniques were implemented to detect phospho-AKT levels (S473, T308) or actin (as a loading control) (**Figure 8**). AKT phosphorylation was inhibited in a dose-dependent manner by treatment with the clathrin inhibitor pitstop2. Total loss of Akt phosphorylation was seen at 5-10 μM of pitstop2, suggesting that 10 μM of pitstop2 significantly perturbs Akt phosphorylation. Interestingly, Pitstop2 has been shown to inhibit the formation of clathrin-coated pits in a dose dependent manner, with complete inhibition of clathrin-dependent endocytosis occurring at 5-10 μM (von Kleist et al., 2011), suggesting that the effects of Pitstop2 on the inhibition of AKT phosphorylation may be a direct cause

of the inhibition of clathrin by this drug, and not secondary effects caused by non-specificity of Pitstop2.

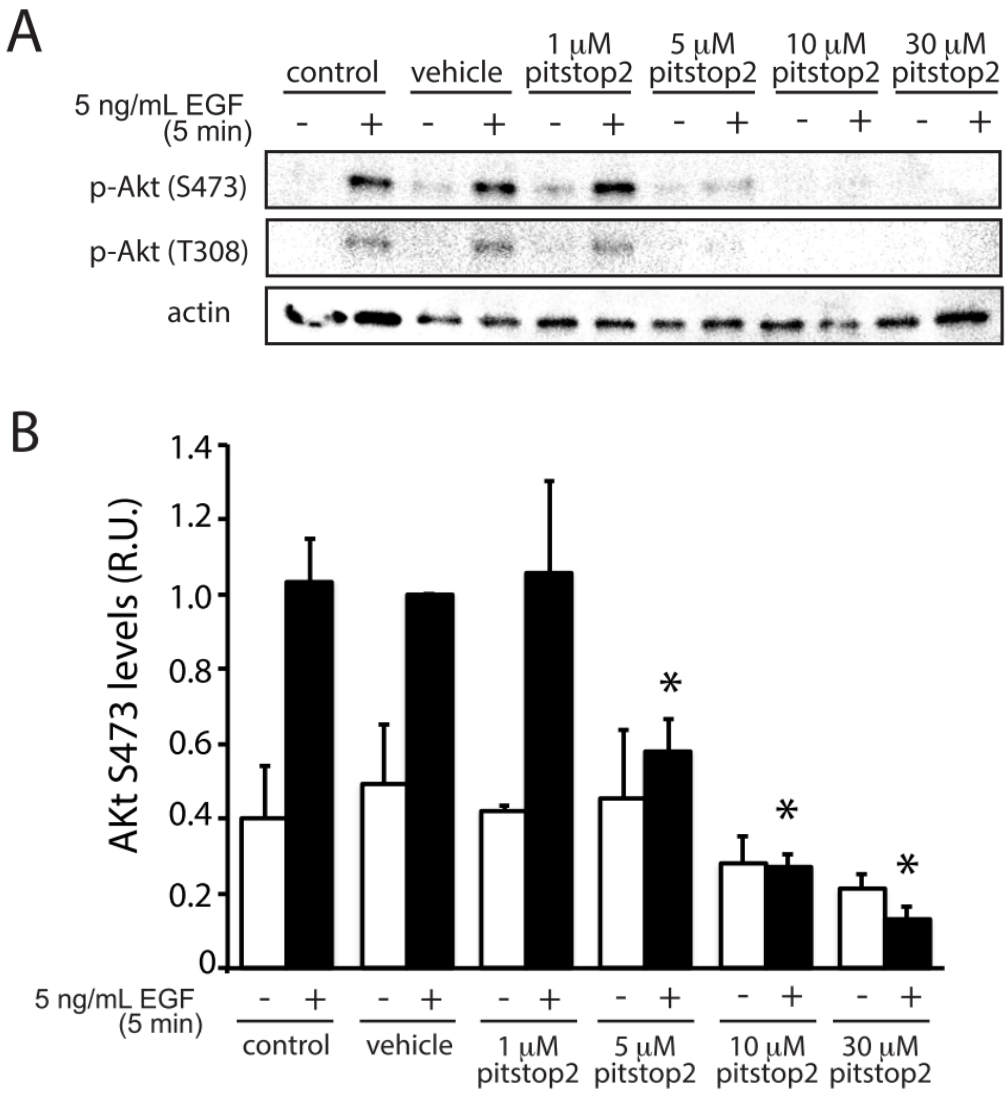

Figure 8. Pitstop2 inhibits EGF-stimulated Akt phosphorylation with a similar potency as clathrin-mediated endocytosis. RPE cells were treated with Pitstop2 at varying concentrations (1μM, 5μM, 10μM or 30μM) or with vehicle control DMSO (0.1% v/v) for 30 min. Cells were stimulated with 5ng/ml of EGF for 5min following drug treatment or left unstimulated (basal). Whole cells lysates were prepared following stimulation. (A) Immunoblot to resolve levels of AKT phosphorylation (S473, T308), or actin. (B) Graphical representation of AKT phosphorylation (S473) in RPE-Ewt cells at 5min of EGF stimulation in the presence of Pitstop2 relative to the control EGF-stimulated condition (1-way ANOVA: n=5, $p<0.05$), also shown the mean ± SD.

By implementing a pharmacological approach we can ensure the effect of clathrin and dynamin perturbation on AKT phosphorylation demonstrated previously is not due to compensatory effects of long-term loss of clathrin or dynamin. I then proceeded to use a pharmacological strategy to perturb clathrin and dynamin acutely respectively, using Pitstop2 (clathrin inhibitor), or dynasore and dyngo4a (dynamin inhibitors). I monitored EGF internalization to confirm that each of these selective inhibitors effectively perturbed EGFR transit to endosomes. I observed that Pitstop2, dynasore and Dyngo4a significantly perturbed EGFR transit to specific endosomes (**Figure 9A**), demonstrating that each of these inhibitors had a similar magnitude of perturbation of endocytosis. Importantly, only clathrin perturbation by Pitstop2 inhibited AKT phosphorylation (**Figure 9B & C**), and treatment with dynasore or dyngo4A was without effect on EGF-stimulated Akt phosphorylation. Therefore, my pharmacological experiments complement our gene silencing experiments, further indicating that clathrin acts as a necessary scaffold for EGFR signaling from the plasma membrane, directly involved in EGF-stimulated AKT phosphorylation.

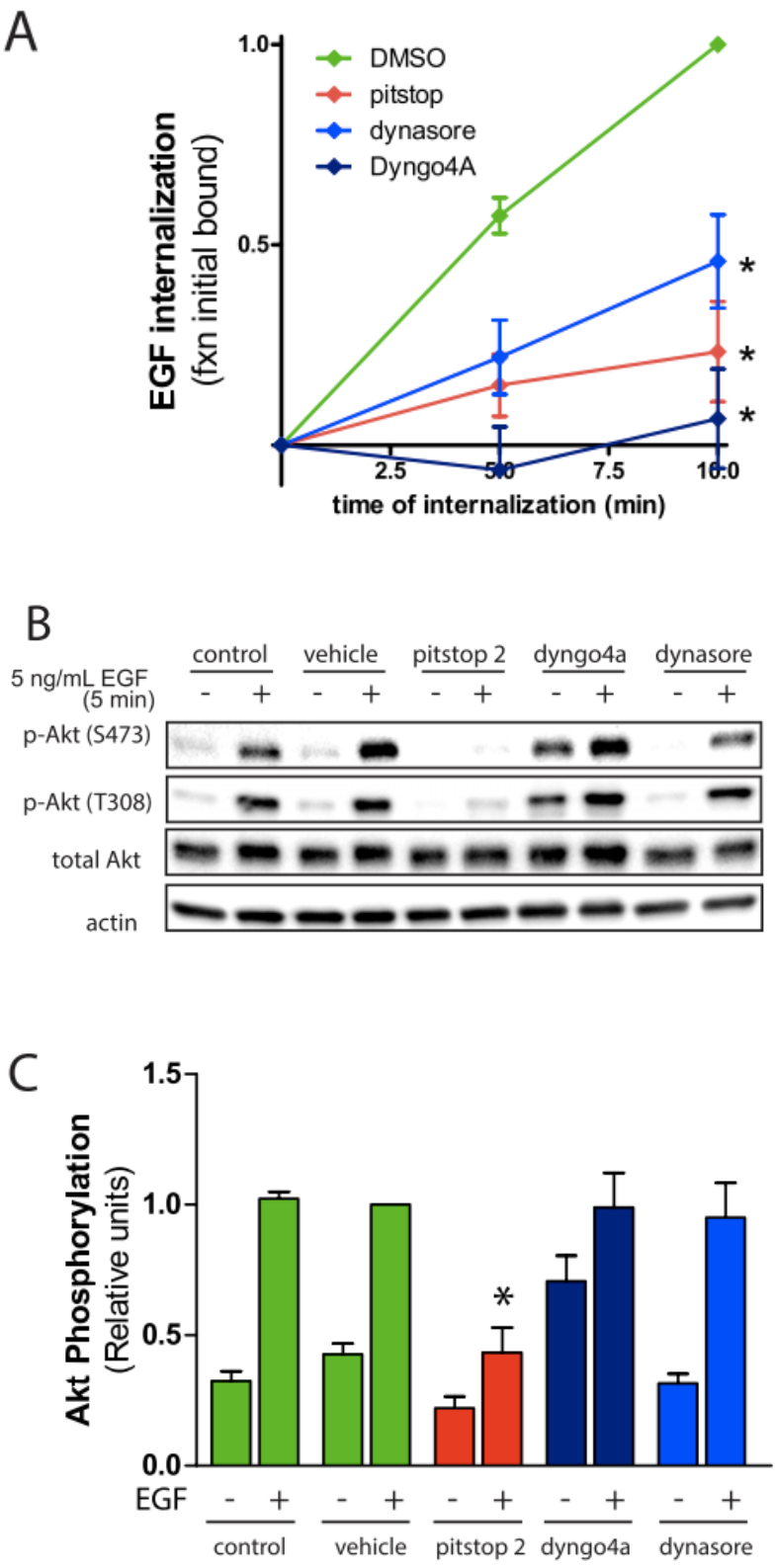

Figure 9. Treatment of RPE cells with the clathrin inhibitor pitstop2 but not the dynamin inhibitors dynasore or dyngo4A inhibits EGF-stimulated Akt phosphorylation. RPE cells were treated with the clathrin inhibitor Pitstop2

(10μM), the dynamin inhibitors Dyngo4a (80μM) and dynasore (30μM) or with a vehicle control DMSO (0.1% v/v) for 30min. (A) Cells were stimulated with 5ng/ml EGF; EGF internalization was monitored over 10min. Both clathrin and dynamin perturbation showed a significant decrease in internalized EGF (1-way ANOVA: $n=4$, $p<0.05$) relative to the vehicle control. (B) Immunoblot demonstrating whole cell lysates that have been stimulated with 5ng/ml EGF for 5min following transfection relative to non-stimulated conditions (basal), p-AKT (S473, T308, and total AKT). (C) Graphical representation of immunoblot demonstrating significant reduction in AKT phosphorylation (S473) following clathrin but not dynamin perturbation (1-way ANOVA: $n=5$, $p<0.005$), also shown is the mean ±SD.

In order to determine whether pitstop2, dynasore, and dyngo4a globally inhibit clathrin-mediated endocytosis (CME) in RPE-wt cells, the effect of clathrin and dynamin perturbation on the internalization of the CME exclusive Transferrin receptor (Tfn-R) was examined. Tfn-R internalization was assayed through a technique that monitors cell surface levels of Tfn in comparison to cytosolic levels of Tfn-R outlined in the materials and methods. Briefly, the cell-surface receptors were stimulated with Tfn-biotin to allow CME of the receptor following treatment with clathrin and dynamin inhibitors. Subsequently, cell surface biotin-Tfn was quenched with avidin in order to isolate cytosolic Tfn. Internalized Tfn-R was then labeled with Strep-Cy3 following fixation and permeabilization. Measuring and comparing difference in fluorescence intensities between conditions determined the amount of internalized Tfn-R. I found that perturbation of clathrin and dynamin with small molecule inhibitors resulted in inhibition of Tfn-R internalization mediated by CME (**Figure 10**). These results suggest a global inhibition of CME as a result of pitstop2, dynasore, and dynago4a treatment. Therefore, acute treatment with pharmacological inhibitors of clathrin and dynamin significantly inhibit CME, thereby inhibiting Tfn transit to endosomes

under clathrin and dynamin perturbed conditions. Thus, trapping Tfn on the bulk plasma membrane under clathrin perturbed conditions, and trapping Tfn in CCPs under dynamin perturbed conditions.

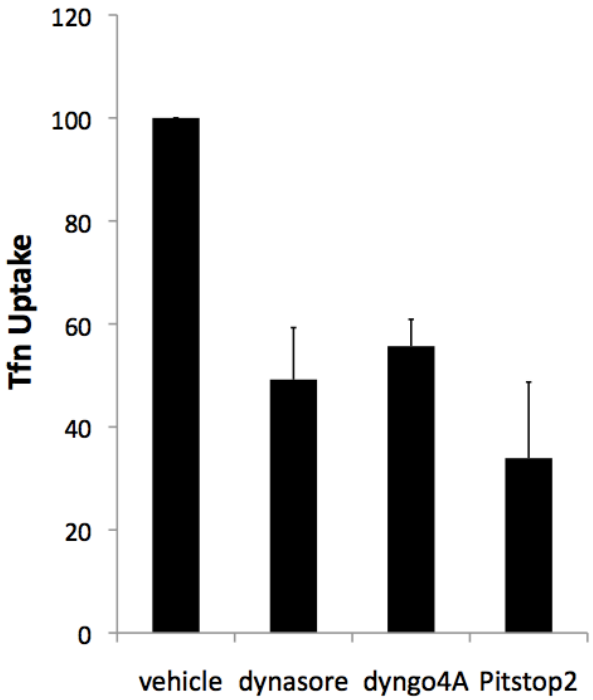

Figure 10. Treatment of RPE cells with the clathrin inhibitor pitstop2 and the dynamin inhibitors dynasore or dyngo4A inhibits Tfn internalization. RPE cells were treated with Pitstop2 (10 µM), dyngo4A (30 µM), dynasore (80 µM) or with vehicle control DMSO (0.1% v//v) for 30 min. Cells were stimulated with 5 ng/mL Tfn-biotin for 5 min following treatment, or non-stimulated (basal). Cells were then fixed with 4% PFA, permeabilized and labeled with Strep-Cy3 for microscopy. Graphical depiction of internalized Tfn under clathrin and dynamin perturbed condition (1-way ANOVA: n=3, $p<0.05$), also shown the mean ± SD.

Impairment of EGFR signaling by clathrin perturbation is rescued by co-expression of ErbB2.

In order to determine if the requirement for clathrin microdomains are a global phenomenon common to all cell types that express EGFR, I examined the effect of clathrin perturbation in HeLa cells. I perturbed clathrin in HeLa cells with Pitstop2 for 30min, stimulated with 5ng/ml EGF for 5min and monitored AKT

phosphorylation levels by immunoblotting (**Figure 6A**). We saw that perturbation of clathrin in HeLa cells had no effect of EGF-stimulated phosphorylation of AKT, suggesting that the role of clathrin as a necessary scaffold for plasma membrane signaling is cell type specific. While HeLa cells have similar levels of EGFR as in RPE cells, they also express high levels of ErbB2 (**Figure 6B**). These results indicate that one of the major differences between HeLa cells and RPE cells that may explain the difference in sensitivity to clathrin perturbation is the fact that HeLa cells exhibit signaling from EGFR:ErbB2 hetero-dimers while RPE cells signal exclusively from EGFR homodimers.

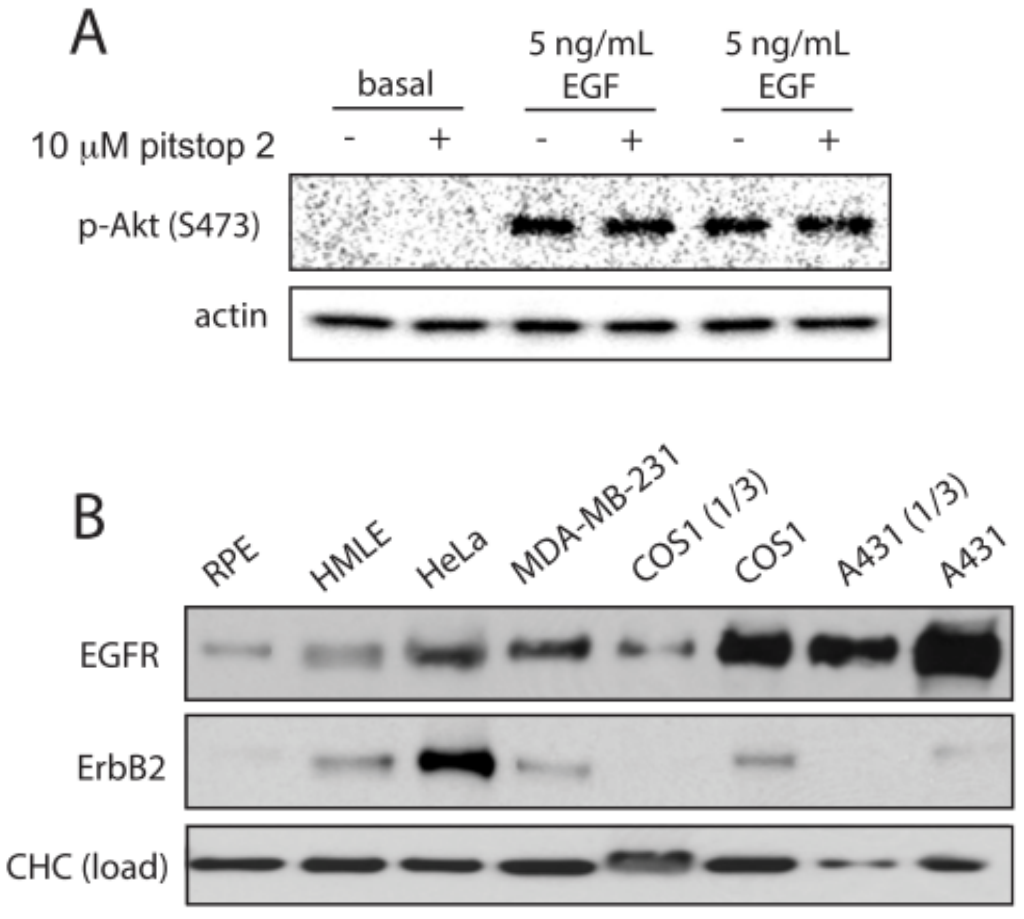

Figure 11. Expression of ErbB2 correlates with pitstop2-insensitive EGF-stimulated Akt activation. Experiments performed by Dr Costin Antonescu and

Camilo Garay. (A) HeLa cells were treated with Pitstop2 (10μM) or control vehicle DMSO (0.1% v/v) for 30min, followed by stimulation with 5ng/ml of EGF for 5min, or non-stimulated (basal) (n=3). Whole lysates were prepared immediately. Representative immunoblot resolved phospho-AKT (S473) or actin. (B) whole cell lysates prepared from multiple cell lines, immunoblot to resolve EGFR, ErbB2 or clathrin heavy chain (n=3).

However, there may be many other differences between RPE and HeLa cells that could account for the different sensitivity to clathrin perturbation with regards to EGFR signaling. In order to determine if EGFR:ErbB2 hetero-dimerization is responsible for HeLa cells insensitivity to Pitstop2, I stably expressed ErbB2 (using the cDNA construct pBABEpuro-ErbB2) in RPE cells using the antibiotic puromycin as a selection marker. I then perturbed clathrin using Pitstop2 and monitored AKT phosphorylation (S473) following EGF stimulation with 5ng/ml for 5min. Immunoblot band intensity was quantified and compared to levels of AKT phosphorylation in RPE-wt cells. While pitstop2 inhibited EGF-stimulated Akt phosphorylation in REP-wt cells, I observed that RPE cells stably expressing ErbB2 had minimal perturbation of EGF-simulated Akt phosphorylation by pitstop2 (**Figure 12**). Hence, expression of ErbB2 in RPE cells lead to a robust rescue of AKT phosphorylation following perturbation of clathrin. This result suggests that hetero-dimerization of EGFR may be sufficient to bypass the requirement for clathrin structures in EGF-stimulated phosphorylation of AKT. Further, the requirement for clathrin, as a necessary scaffold for AKT phosphorylation may be a global cell phenomenon, not RPE-wt specific, but limited to cells that do not express ErbB2.

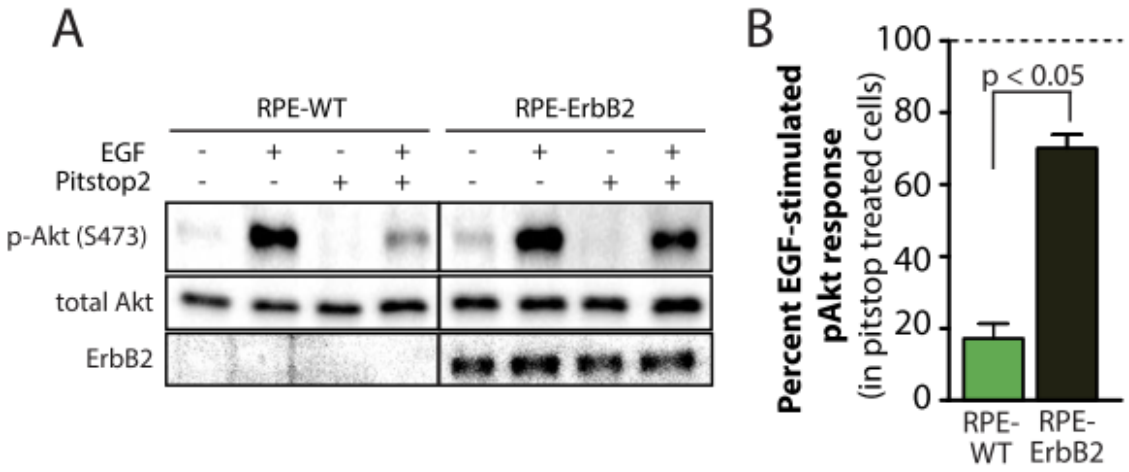

Figure 12. Stable expression of ErbB2 in RPE cells rescues EGF-stimulated Akt phosphorylation from inhibition by pitstop2. RPE cells were transfected to stably express ErbB2 (RPE-ErbB2). RPE-ErbB2 or RPE-wt cells were treated with Pitstop2 (10μM) or with vehicle control DMSO (0.1% v/v) for 30min. Cells were stimulated with 5ng/ml of EGF for 5min following drug treatment or left unstimulated (basal). Whole cells lysates were prepared following stimulation. (A) Immunoblot to resolve levels of AKT phosphorylation (S473), total-pan AKT, or ErbB2. (B) Graphical representation of AKT phosphorylation (S473) in RPE-ErbB2 cells and RPE-wt cells at 5min of EGF stimulation in the presence of Pitstop2 (1-way ANOVA: n=4, $p<0.05$)

Clathrin is required for EGF-stimulated phosphorylation of Gab1 but not auto-phosphorylation of EGFR.

In order to determine how clathrin perturbation impacts EGFR signaling leading to Akt phosphorylation, I examined the effect of Pitstop2 on EGFR autophosphorylation. I treated RPE-wt cells with Pitstop2 for 30min and stimulated for 5min with 5ng/ml of EGF. Further, I implemented immunoblot techniques to detect the phosphorylation of EGFR on 6 different tyrosine residues (**Figure 8**). Clathrin and dynamin perturbation was shown to have no effect on EGFR phosphorylation, suggesting that pharmacological inhibition of clathrin and dynamin does not disrupt the intrinsic kinase abilities of EGFR.

Importantly, clathrin perturbation had no effect on the phosphorylation of Y1068 on EGFR, which is the key signaling stage required for phosphorylation of Akt. These observations support that the loss of AKT phosphorylation seen under clathrin perturbed conditions was in fact as a direct cause of a loss of clathrin structures and not a non-specific action of the drug on the receptor itself.

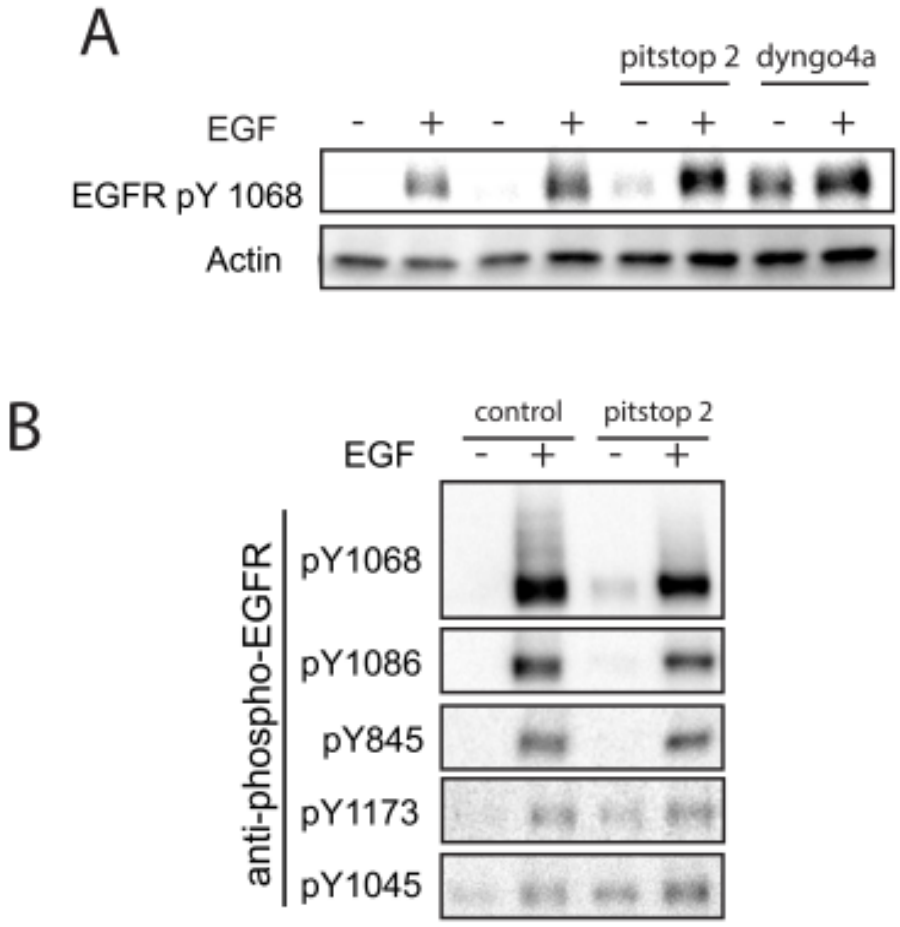

Figure 13. Perturbation of clathrin by pitstop2 treatment or siRNA gene silencing does not alter EGF-stimulated EGFR phosphorylation. Experiments were done in collaboration with Stefanie Lucarelli. RPE cells were treated with the clathrin inhibitor Pitstop2 (10µM), the dynamin inhibitor Dyngo4a (80µM), or with a vehicle control DMSO (0.1% v/v) for 30min. Further, cells were stimulated for 5min with 5ng/ml of EGF, or non-stimulated (basal), whole cell lysates were prepared immediately. (A) Representative immunoblot resolving phospho-EGFR (Y1068) following clathrin and dynamin perturbation (n=3). (B)

Representative immunoblot resolving phospho-EGFR (Y1068/1086/845/1173/1045) following clathrin (n=3).

In order to determine where the requirement for clathrin lays in the PI3K-AKT signaling pathway, I implemented immunoblotting techniques and widefield epifluorescence microscopy to monitor EGF-stimulated Gab1 phosphorylation and PI3K localization. RPE cells were treated with Pitstop2 or vehicle control for 30min followed by stimulation with 5ng/ml EGF for 5min. We saw that clathrin perturbation significantly perturbed EGF-stimulated Gab1 phosphorylation as well as PI3K transit to punctate structures (**Figure 14A & B**). These findings support the findings of the experiments presented in Figure 6 and suggests that the requirement for clathrin structures in the EGF-stimulated activation of AKT lays downstream of receptor activation but upstream of Gab1 activation. These results were confirmed using gene silencing of clathrin and dynamin, where Gab1 phosphorylation (Y627) was significantly perturbed when clathrin but not dynamin was silenced (**Figure 14D**). As the p85 regulatory subunit of PI3K is known to bind Y472 of Gab1 (Gu & Neel, 2003), I sought to monitor the phosphorylation of Gab1 Y472 upon EGF stimulation. However, there are currently no available antibodies that specifically bind this specific residue. Nonetheless, fluorescence microscopy allows observation of the cellular localization of GFP-p85 as a surrogate to monitor Gab1 phosphorylation of Y472 (**Figure 14C**). While GFP-p85 exhibited a diffuse, cytosolic distribution in the absence of EGF stimulation, treatment with the hormone caused a dramatic redistribution of GFP-p85 to punctate structures. Importantly, under clathrin

perturbed conditions (pitstop2), EGF failed to elicit the formation of GFP-p85 puncta, suggesting that clathrin perturbation impairs Gab1 phosphorylation on the Y427 residue.

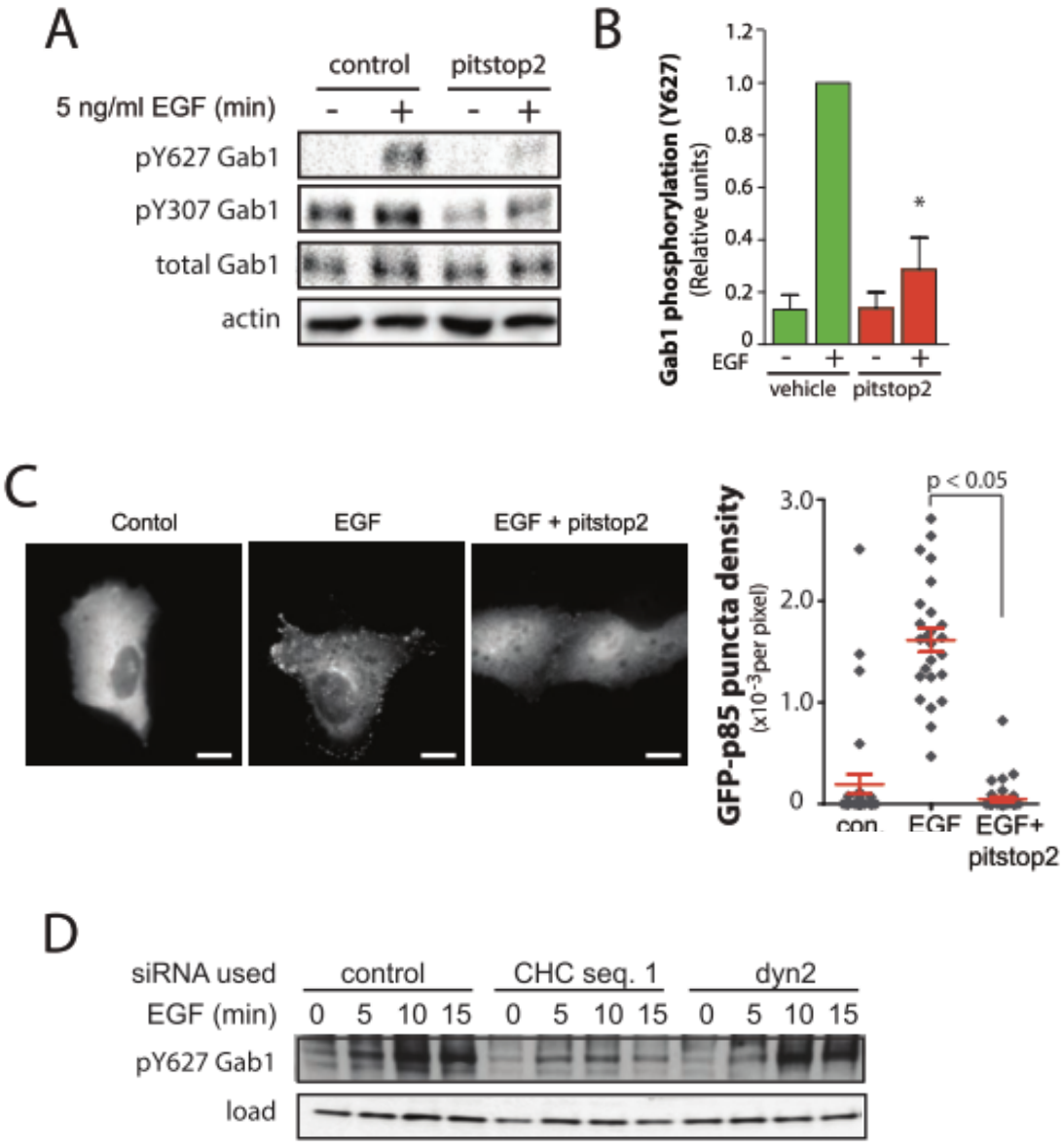

Figure 14. Perturbation of clathrin by pitstop2 treatment of siRNA gene silencing inhibits EGF-stimulated Gab1 phosphorylation. Experiments done by Costin Antonescu and reproduced with permission (A & B) RPE cells were treated with the clathrin inhibitor Pitstop2 (10μM), or with a vehicle control DMSO (0.1% v/v) for 30min. Further, cells were stimulated for 5min with 5ng/ml of EGF,

or non-stimulated (basal), whole cell lysates were prepared immediately. Shown in (A) Representative immunoblot resolving phospho-Gb1 (Y627, Y307) and total Gab1 following clathrin perturbation (n=3), (B) are graphical representations of phospho-Gab1 (Y627) immunoblots (1-way ANOVA: n=4, $p<0.05$), also shown are ± SE. (C) RPE cells were treated with the clathrin inhibitor Pitstop2 (10µM), or with a vehicle control DMSO (0.1% v/v) for 30min. Further, cells were stimulated for 5min with 5ng/ml of EGF, or non-stimulated (basal). Cells were then fixed with 4% PFA and mounted in DAKO for fluorescence microscopy. (Left panel) Representative images of p85-GFP fluorescence throughout RPE-wt cells (scale = 10µm). (Right Panel) Density of p85-GFP punctate structure detected by automated image analysis in individual cells (1-way ANOVA: n=4, $p<0.05$), also shown is the means ± SE. (D) RPE cells were with transfected with siRNA sequences targeting clathrin heavy chain, dynamin2 or non-targeting sequence (control). Immunoblot demonstrating whole cell lysates that have been stimulated with 5ng/ml EGF over 15min following transfection in relation to non-stimulated conditions (basal). Representative image of Phospho-Gab1 (Y627) or actin in clathrin perturbed cells (n=3)

In order to determine whether perturbation of dynamin function by dynasore and dyngo4a inhibits the intrinsic kinase ability of EGFR, Gab1 phosphorylation or AKT phosphorylation, the effect of dynamin disruption on each signaling intermediate was examined. I implemented immunoblotting techniques to assay phosphorylation of signaling intermediates. Cells were treated with dynasore, and dyngo4a for 30min, and subsequently stimulated with EGF allowing activation of signaling intermediates. Lysates were then probed with a p-EGFR (Y1068) p-Gab1 (Y627), and p-AKT (S473) antibodies. Dynamin perturbation had no effect on the phosphorylation of all the signaling intermediates (**Figure 15**). These results suggest pharmacological inhibition of dynamin does not disrupt the intrinsic kinase abilities of EGFR, more importantly, phosphorylation of Y1068 the key stage in AKT phosphorylation, as well as the downstream phosphorylation of Gab1 and AKT. Therefore, under conditions in

which dynamin is perturbed, thus trapping EGFR in CCPs and isolating CCP-EGFR signaling, EGF-stimulated signaling from EGFR to AKT is unperturbed.

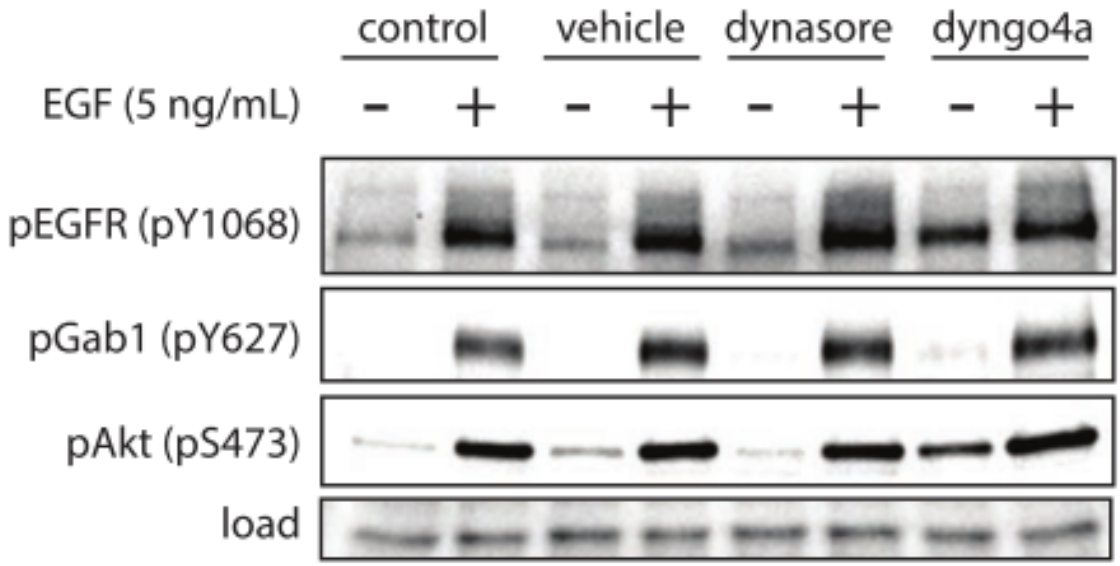

Figure 15. Inhibition of dynamin with dynasore or dyngo4a does not affect EGF-stimulated EGFR, Gab1 or Akt phosphorylation. RPE cells were treated with dyngo4A (30 µM), dynasore (80 µM) or with vehicle control DMSO (0.1% v//v) for 30 min. Cells were stimulated with 5 ng/mL EGF for 5 min following treatment, or non-stimulated (basal). Whole cell lysates were prepared immediately, immunoblotting resolved for phospho-EGFR (pY1068), phospho-Gab1 (pY627), phospho-Akt (S473) or actin. Representative image of immunoblot (n=3)

siRNA gene silencing of clathrin and dynamin do not impair EGF-stimulated ERK phosphorylation.

Having characterized clathrin as a necessary scaffold for EGF-stimulated phosphorylation of AKT, I then proceeded to use this strategy to determine whether the effect of AKT phosphorylation as a result of clathrin and dynamin gene silencing was a global effect on EGFR signaling or specific to EGF-stimulated AKT phosphorylation. I examined levels of ERK phosphorylation under clathrin and dynamin silenced conditions. I saw that neither clathrin nor

dynamin gene silencing inhibited ERK phosphorylation (**Figure 16**) as there was no change in EGF-stimulated ERK phosphorylation levels between control, clathrin and dynamin silenced conditions. Taken together with the results of clathrin and dynamin silencing on AKT phosphorylation (**Figure 6B & C**), this suggests that a role of clathrin as a necessary scaffold for plasma membrane signaling is specific to EGF-stimulated AKT phosphorylation, and not necessary for global cell signaling of EGFR.

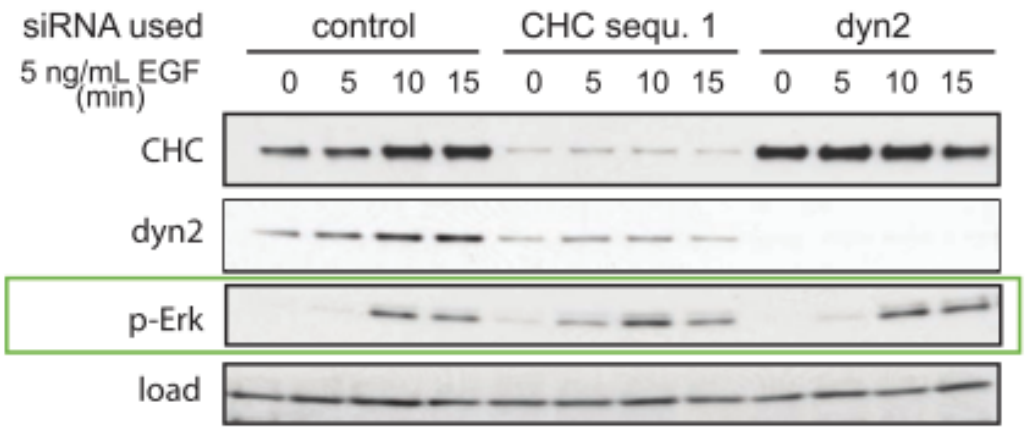

Figure 16. EGF-stimulated phosphorylation of EGFR or Erk is unaffected by siRNA gene silencing of clathrin or dynamin.
Experiment performed by Dr Costin Antonescu and reproduced with permission. RPE cells were transfected with siRNA sequences targeting clathrin heavy chain (CHC-1), dynamin2 or non-targeting sequence (control), followed by stimulation with 5ng/ml EGF over 15min, or non-stimulated (basal). Whole cell lysates were prepared immediately. Representative image of immunoblot resolving clathrin heavy chain, dynamin2, phospho-ERK, or Hsp40 (n=3).

Clathrin perturbation by Pitstop2 does not inhibit the recruitment of EGFR to clathrin structures

The results thus far indicate that clathrin perturbation does not affect the autophosphorylation of EGFR. However in order to determine whether EGFR recruitment to clathrin structures is clathrin dependent, we perturbed clathrin with Pitstop2 and examined degree of colocalization between EGFR and clathrin. EGFR recruitment to clathrin structures was assayed through TIRF microscopy. RPE cells stably expressing a clathrin-light-chain-GFP construct were serum starved and stimulated with an EGF conjugated to A555. Cells were then fixed and mounted for TIRF microcopy. The degree of colocalization between EGF-A555 and clathrin was determined using automated analysis that involved detection of clathrin structures using Gaussian modeling of the point-spread function of diffraction-limited clathrin structures, followed by systematic quantification of EGF-A555 fluorescence within these detected objects. I saw that clathrin perturbation by Pitstop 2 has no effect on the recruitment of EGF to the detected clathrin objects (**Figure 17**). However, clathrin disruption resulted in the recruitment of EGF to larger and brighter clathrin structures, suggesting pitstop2 results in the stabilization of old clathrin structures trapped in an unknown phase of CCP maturation, therefore cannot be considered the canonical clathrin-coated pit necessary for EGF-stimulated phosphorylation of AKT. Together with the experiments presented in **Figure 9A & B,** these results suggest that in pitstop-2 treated cells, clathrin structures may not have the signaling properties necessary for EGF-stimulated phosphorylation of AKT. As is detailed in the Discussion,

pitstop2 impairs the dynamics and composition of clathrin structures at the cell surface, but not the recruitment of EGFR therein. Interestingly, these findings are suggestive that EGFR can be recruited to pre-existing clathrin structures upon stimulation of ligand, as opposed to clathrin structures forming around active EGFR at the plasma membrane, this is supported by a study that demonstrated that EGFR does localize to clathrin structures upon activation, but activation does not result in the formation of new clathrin structures (Rappoport & Simon, 2009).

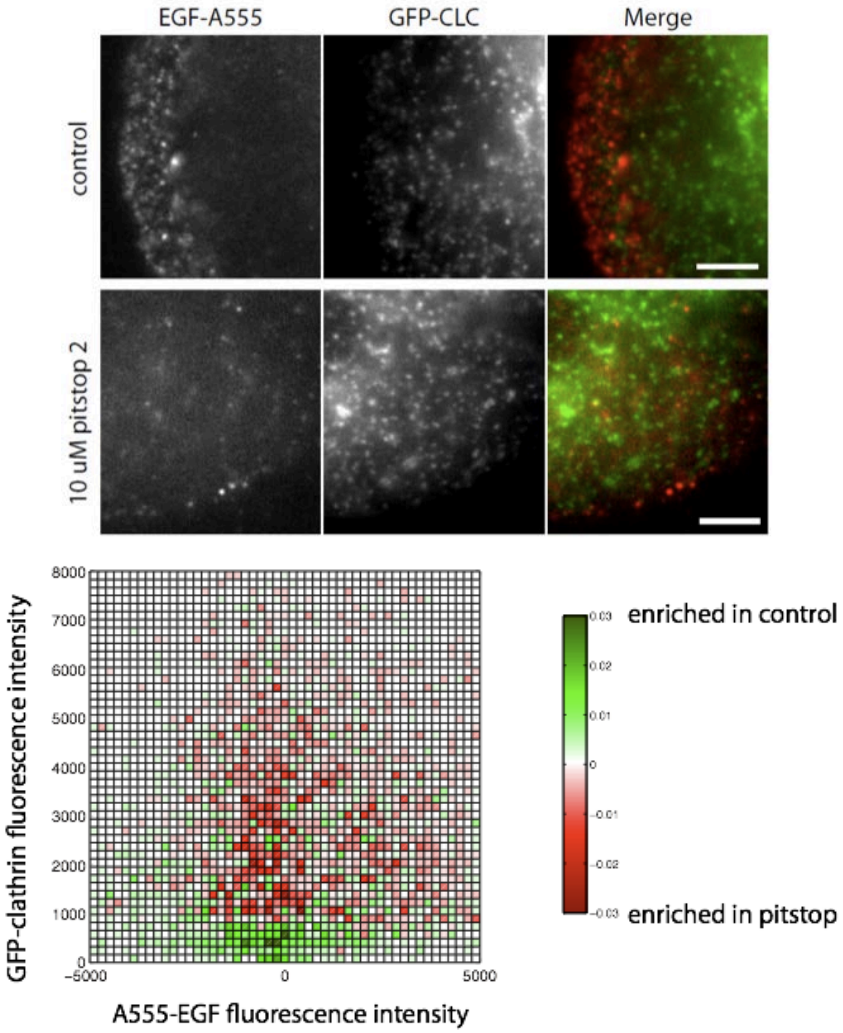

Figure 17. EGFR is recruited to clathrin-coated pits upon EGF stimulation. RPE stably expressing clathrin-light-chain-RFP construct (RPE-RFP) were stimulated with 5ng/ml EGF-A555 for 3min or non-stimulated (basal), fixed

immediately with 4% PFA and chamber mounted for TIRF microscopy. (Top) Representative images depicting recruitment of EGF-A555 to CCPs under stimulated conditions (scale bar = 5 μm). (Below) 2D histogram depiction of the difference in EGF-A555 and RPE-RFP intensity frequencies between stimulated and basal conditions (1-way ANOVA: n=3, $p<0.05$, >45 cells from 3 independent experiments). Green depicts structures that are more frequently detected in control conditions. Red depicts structures that are more frequently detected in Pitstop2 conditions.

EGF triggers an enrichment of Gab1 to clathrin structures.

The results presented thus far indicate that clathrin is required for EGF-stimulated phosphorylation of Gab1, which eventually leads to phosphorylation of Akt. These results may suggest that clathrin functions as a scaffold to locally enrich signaling intermediates within clathrin-coated pits. In order to determine the spatial orientation of Gab1 in relation to clathrin structures, Gab1 recruitment to clathrin structures was assayed through TIRF microscopy. RPE cells stably expressing a clathrin-light-chain-RFP construct were transiently transfected with a Gab1-wt-GFP plasmid followed by stimulation with 5ng/ml EGF for 3min. Cells were then fixed and mounted for TIRF microcopy. The degree of colocalization between Gab1-wt and clathrin was determined using automated analysis that involved detection of clathrin structures using Gaussian modeling of the point-spread function of diffraction-limited CCPs, followed by systematic quantification of Gab1 fluorescence within these detected objects. I saw that stimulation of cells with EGF lead to an increase in the recruitment of Gab1 to clathrin-coated pits (**Figure 18)**. Gab1 recruitment to clathrin structures following EGF stimulation is evident in the 2D-histogram shown in (**Figure18C)**. This histogram demonstrates the differences in Gab1 recruitment to clathrin structures between basal and

stimulated conditions. Following stimulation with EGF there is an evident right shift in the 2D-histogram, indicating a robust enrichment of Gab1 in clathrin structures. Gab1 transit to clathrin-coated pits and taking in account previous results showing the requirement for clathrin in Gab1 phosphorylation, further suggests that clathrin may be involved in mediating Gab1 phosphorylation following EGF stimulation.

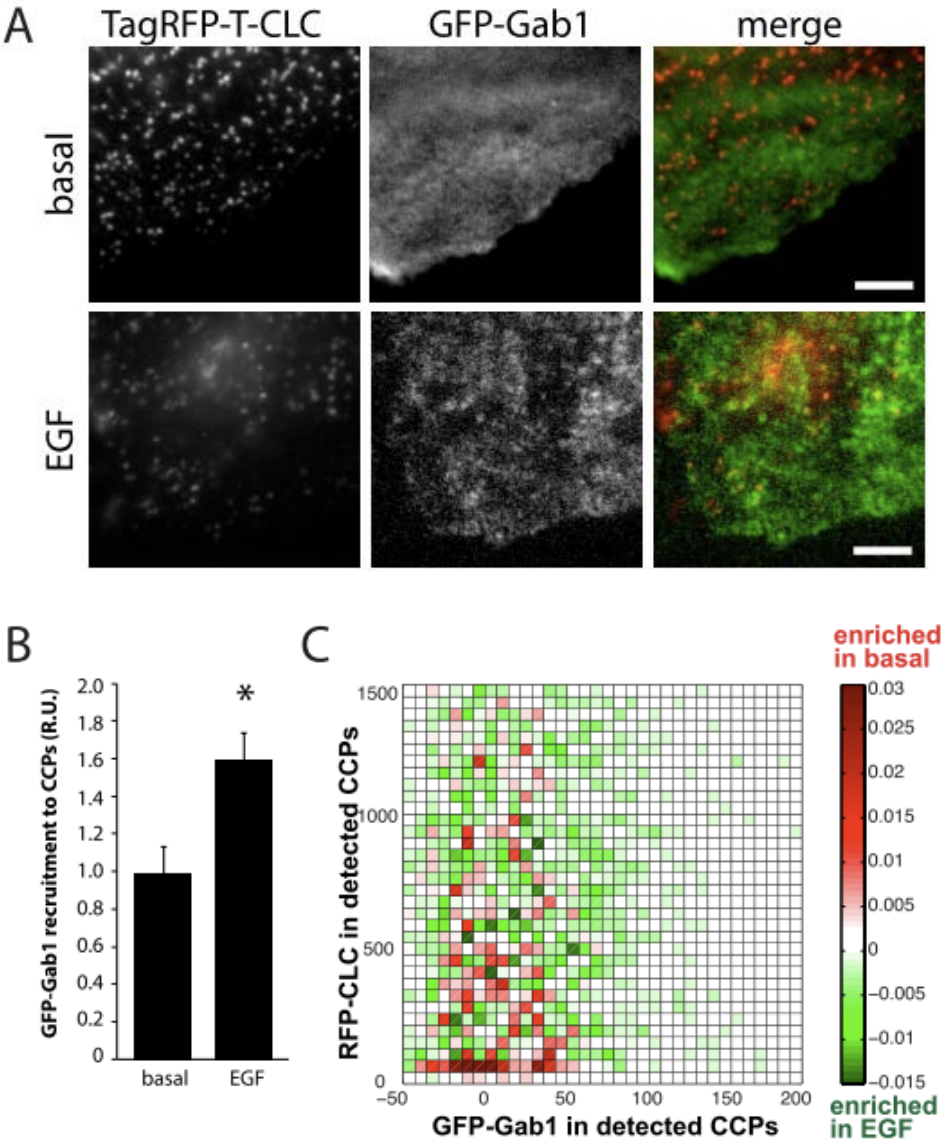

Figure 18. Gab1 is recruited to clathrin-coated pits upon EGF stimulation. RPE stably expressing clathrin-light-chain-RFP construct (RPE-RFP) were transiently transfected with GAB-wt-GFP construct. Cells were then stimulated with 5ng/ml EGF for 3min or non-stimulated (basal), fixed immediately with 4% PFA and chamber mounted for TIRF microscopy. (A) Representative images depicting recruitment of Gab1 to CCPs under stimulated conditions (scale bar = 5 µm). (B) Graphical depiction of the significant increase in Gab1 recruitment to CCP following EGF stimulation for 3min determined by automated detection of clathrin structures (n=4, $p<0.05$, >45 cells from 4 independent experiments), also shown is the mean ± SE. (C) 2D histogram depiction of the difference in Gab1-GFP and RPE-RFP intensity frequencies between stimulated and basal conditions. Green depicts structures that are more frequently detected in stimulated conditions. Red depicts structures that are more frequently detected in basal conditions.

Discussion

Taken together, the results presented thus far show that perturbation of CCP assembly and recruitment of endocytic accessory proteins to the plasma membrane (within CCPs) results in a loss of AKT phosphorylation following growth factor stimulation. In addition, EGFR retained its kinase capabilities despite perturbation of CCPs, as there was robust autophosphorylation of EGFR in CCP-perturbed cells. This CCP-independent autophosphorylation of EGFR occurred specifically on the docking site of Grb2, the Y1068 residue on EGFR. Further, it appears that by inhibiting the maturation of CCPs the break in the PI3K-AKT signaling axis occurs at Gab1 phosphorylation, where a significant loss in Y627 phosphorylation was seen. As there was no loss in AKT phosphorylation when EGFR endocytosis was defective by perturbation of dynamin-II, these results suggest a distinct role for clathrin in regulating EGFR signaling from the plasma membrane, instead of a requirement for EGFR internalization for this process. Interestingly, the inhibition of EGF-stimulated AKT phosphorylation upon perturbation of CCPs was rescued by co-expression of ErbB2 in RPE cells. This suggests that the requirement for clathrin is not a universal feature of all RTKs or ErbBs, but instead is at least partly specific to a subset of receptor tyrosine kinases including EGFR. Determination of the molecular mechanism by which clathrin controls EGF-stimulated AKT phosphorylation remains to be determined. Based on the experiments presented in this thesis, I propose that clathrin may function as a scaffold necessary to spatially coordinate the proximity of crucial proteins to mediate the progression of

EGFR signaling. As such, clathrin may facilitate the local enrichment within CCPs of non-receptor tyrosine kinases required for the EGFR phospho-relay. Finally, substantial evidence argues that clathrin is required for the phosphorylation of Gab1 in CCPs necessary to activate PI3K and subsequently phosphorylate AKT.

Excluding e-EGFR signaling in order to compare and contrast PM- and CCP-EGFR signaling.

In order to distinguish EGFR signaling from three separate microdomains 1) PM-EGFR signaling, 2) CCP-EGFR signaling and 3) e-EGFR signaling, I used specific methods that allowed perturbation of EGFR localization to each of these compartments. Since all three microdomains are traversed sequentially by EGFR upon ligand binding, by inhibiting two specific endocytic proteins, I was able to experimentally segregate the properties of EGFR within each of these three signaling domains. In order to isolate PM-EGFR signaling, I implemented two independent methods to perturb clathrin: siRNA gene silencing and pharmacological inhibition of clathrin. siRNA silencing of clathrin by two different siRNA sequences successfully depleted >80-90% of total cellular clathrin found in RPE-cells. This disruption of clathrin inhibits the transition of EGFR from PM-EGFR to CCP-EGFR signaling, allowing for robust separation of the signaling properties of PM-EGFR from CCP-EGFR. Pitstop2 is a small molecule inhibitor of clathrin, which was found to bind to the N-terminal domain of clathrin inhibiting the recruitment of endocytic proteins necessary for the maturation of CCPs, such as amphiphysin (von Kleist et al., 2011), thereby providing an additional method for the isolation of PM-EGFR signaling from that of CCP-EGFR. Pitstop2 was

shown to dose dependently inhibit the formation of CCPs, therefore we carried out a dose curve of Pitstop2 on the effect of AKT phosphorylation on S473 and T308 residues. As expected, there was a decrease in AKT phosphorylation with increasing concentrations of Pitstop2, which suggests that clathrin may facilitate AKT phosphorylation from the plasma membrane. However, Pitstop2 may also have off-target or indirect effects (Dipannita Dutta, Chad D. Williamson, Nelson B. Cole, 2012; Willox, Sahraoui, & Royle, 2014) that could account for the loss in AKT phosphorylation. However, since the effects of pitstop2 on EGF-stimulated Akt phosphorylation were corroborated with similar findings using siRNA gene silencing to perturb clathrin, it is likely that the inhibition of EGFR signaling by pitstop2 was due to selective inhibition of CCPs. These methods were also shown to significantly inhibit the internalization of EGFR therefore successfully inhibiting e-EGFR, making these two methods a robust measure of signaling by PM-EGFR.

In order to isolate CCP-EGFR signaling from e-EGFR signaling, we implemented siRNA gene silencing and pharmacological inhibition of dynamin-II. Dynamin-II is necessary for scission of CCP from the plasma membrane, therefore by successfully inhibiting the internalization of CCPs we effectively inhibited the formation of endosomes and e-EGFR signaling. siRNA gene silencing of dynamin-II significantly inhibited the internalization of EGFR, thereby successfully isolating CCP-EGFR signaling by inhibiting e-EGFR signaling. Dynasore and Dyngo4a are small molecule inhibitors of dynamin-II, therefore serving as a secondary method to inhibit the internalization of EGFR. I confirmed

that treatment of cells with dyngo4A or dynasore had a similar inhibition of EGFR internalization to endosomes as that observed with pitstop2 (**Figure9A**). Dyngo4A may have some off-target effects as treatment with this inhibitor resulted in an increase in basal levels of EGFR, Gab1 and Akt phosphorylation; however, both dynasore and Dyngo4A had no effect in the net-gain of Akt phosphorylation upon EGF stimulation. Thus, these two dynamin inhibitors are robust inhibitors of EGFR internalization, thereby isolating CCP-EGFR signaling, and inhibiting e-EGFR signaling.

The inhibition of EGF internalization by pitstop2, dynasore and dyngo4A was robust but not complete. I confirmed that this represented an incomplete inhibition of CME by examining the effect of these drugs on the internalization of Transferrin receptor (TfR), which internalizes exclusively through CME. Using this assay, I was able to demonstrate that pitstop2, dynasore, and dynago4a similarly inhibit TfR internalization. These results show that treatment of cells with each of these inhibitors results in robust inhibition of clathrin-dependent endocytosis of EGFR and TfnR, with minimal compensation of EGFR internalization by other endocytosis mechanisms.

Taking together, these results indicate that I have successfully devised a systematic way of isolating the three plausible signaling domains of EGFR and developed a means to attribute signaling differences depending on the specific microdomain in which EGFR resides.

Multiple independent methods identify a specific role for clathrin but not dynamin-II in EGF-stimulated AKT phosphorylation

As mentioned above, pharmacological inhibition of clathrin and dynamin may have non-specific effects that could potentially alter receptor signaling from the cell surface, so we corroborated our pharmacological studies with siRNA gene silencing studies. However, siRNA gene silencing of clathrin or dynamin-II over a few days can have indirect effects as well, possible cell adaptive effects to compensate for the inability to internalize any plasma membrane protein through CME. Nonetheless, that each of these two independent methods of perturbation of clathrin and dynamin showed very similar effects on EGF-stimulated Akt phosphorylation strongly suggests that the effects of each on EGFR signaling were due to selective inhibition of clathrin.

I also examined the intrinsic EGFR kinase activity under various perturbation conditions. I showed that siRNA of clathrin, as well as pharmacological inhibition of clathrin and dynamin did not inhibit EGF-stimulated phosphorylation of EGFR. Further, EGF-stimulated phosphorylation of ERK1/2 was not effected in either clathrin- or dynamin –perturbed cells. This suggests that long-term loss of clathrin and dynamin, as well as acute incubation with pharmacological inhibitors of clathrin and dynamin do not perturb PM-EGFR or CCP-EGFR autophosphorylation capabilities.

When monitoring phosphorylation of the downstream adaptor protein Gab1 on Y627 residue we saw the first difference in signaling between PM-EGFR, and CCP-EGFR. Under PM-EGFR signaling parameters (perturbed

clathrin), Gab1 phosphorylation was significantly reduced compared to control cells. However, under dynamin perturbed conditions (CCP-EGFR signaling), Gab1 phosphorylation was comparable to control cells. This was corroborated with siRNA gene silencing of clathrin and dynamin. These results suggest that clathrin may be facilitating the phosphorylation of Gab1, perhaps serving as a scaffold in order to bring necessary endocytic proteins in close proximity.

This difference in PM-EGFR and CCP-EGFR signaling was also noted when investigating the phosphorylation of AKT on residues T308 and S473. Phosphorylation of AKT residues T308 and S473 were significantly perturbed in PM-EGFR signaling conditions. However, under CCP-EGFR signaling conditions phosphorylation of AKT was comparable to control cells. This is of specific interest because AKT has been shown to be overactive in many cancers. Therefore, this evidence implicates clathrin as a mediator of EGF-stimulated AKT activation provides an alternative target for the development of cancer therapeutics that target aberrant EGFR signaling from clathrin structures.

Localization of signaling components in relation to CCP

Upon ligand binding EGFR is recruited to CCPs where it resides until internalized into cells. Therefore, following the above data that suggest clathrin structures are necessary for EGF-stimulated AKT activation we carried out co-localization assays in order to determine whether or not EGFR recruitment to CCPs is perturbed or if these clathrin structures differ under Pitstop2 conditions. By using TIRF microscopy, we were able to selectively image the most membrane proximal 65nm of the ventral side of a cell, thus successfully

eliminating any fluorescence that may be emitted by endosomes. To accomplish this, I used RPE-GFP cell line that stably express GFP-clathrin light chain; these cells have been well characterized to have normal assembly of clathrin (Aguet et al., 2013). By implementing TIRF microscopy we switch from direct light to an evanescent wave that propagates in the z-direction and gradually degrades, which arises from the glass/water/specimen interface. This allows us to image the most proximal 200nm to the cell surface, this depth is called the penetration depth and can be fixed anywhere from 60-200nm by adjusting the angle of incident at which the laser light meets the coverslip.

Using these cells, I first found that Pitstop2 did not effect the recruitment of EGF:EGFR to CCPs. These may seem counterintuitive since Pitstop2 inhibits the formation of CCPs, however it does not facilitate the disassembly of old CCPs, therefore any CCP present before the onset of incubation with Pitstop2 will remain arrested at the plasma membrane (von Kleist et al., 2011). The recruitment of EGFR to CCP in cells treated with pitstop2 is consistent with previous studies that found that pitstop2 did not prevent EGFR or TfR recruitment to CCPs (but inhibited subsequent internalization) (von Kleist et al., 2011). Importantly, pitstop2 does inhibit the recruitment of numerous cytosolic proteins to CCPs (e.g. amphiphysin), as well as the maturation of old CCPs (von Kleist et al., 2011). Indeed pitstop2 selectively binds to the N-terminal beta-propeller domain of clathrin, which is responsible for interaction of clathrin with over 50 other proteins (von Kleist et al., 2011), but does not bind to the regions of clathrin involved in self-assembly of clathrin into lattice structures or to the region of

clathrin that interacts with the cargo adaptor AP-2. Therefore, while pitstop2 treatment allows formation of CCPs at the plasma membrane, these assemblies are severely defective in numerous functions, including, I presume the scaffolding of cytosolic factors important for endocytosis and receptor signaling. As such, pitstop2-treated cells represent PM-EGFR signaling because the clathrin structures in these conditions are unable to recruit or bind to necessary endocytic proteins. Interestingly, EGFR was shown to localize in bigger brighter clathrin structures compared to control cells, which exemplifies the importance of clathrin as a scaffold in EGF-stimulated signaling, as well suggests a role for the requirement of proper orientation of clathrin. Collectively, these results confirm that perturbation of clathrin by pitstop2 or clathrin heavy chain gene silencing have the expected effects on EGFR traffic.

I next investigated the recruitment of Gab1 to CCPs using TIRF microscopy. Interestingly, I found that in RPE-RFP cells stimulated with 5ng/ml, Gab1 was quantitatively recruited to clathrin structures. This is consistent with my overall hypothesis that clathrin functions to scaffold certain EGFR signaling components. EGFR phosphorylation on Y1068 in clathrin-perturbed cells was comparable to that observed in control cells; therefore we expect that within ~ 1 min of EGF stimulation, the adaptor protein Grb2 would be rapidly recruited to ligand-bound EGFR. Further, Grb2 recruits Gab1 to the membrane from a cytosolic fraction, and in doing so localizes Gab1 within clathrin structures along with activated EGFR. These results suggest a novel finding of robust recruitment

of Gab1 to CCPs following stimulation with EGF necessary for the progression of EGFR signaling from the plasma membrane.

Although examining Y627 is not a direct measure of a loss in the Y446/472/589 sites that bind the p85 regulatory subunit of PI3K, we found that Pitstop2 and clathrin gene silencing inhibit EGF-stimulated phosphorylation of Y627. Interestingly, Y627 residue on Gab1 recruits the phosphatase SHP2 that dephosphorylates Y446/472/589 sites on Gab1. However, SHP2 is also a positive regulator of PI3K activation as it can bind directly to PI3K (Wu et al., 2001). This evidence suggests that clathrin may be required for the phosphorylation of Gab1 on multiple residues, which are implicated in both the activation and inhibition EGF-stimulated AKT phosphorylation, thus increasing the complexity in the role of clathrin in EGF-stimulated activation of AKT.

We then investigated the spatial localization of the p85 subunit of PI3K in RPE-wt cells using epifluorescence microscopy under clathrin-perturbed cells. Under control conditions, it was demonstrated that p85 would localize to punctate structures, however upon further investigation these structures did not colocalize with clathrin and are thus not clathrin-coated pits. However, formation of p85 puncta is a good measure of PI3K activation, but not a good indication of endogenous p85 localization as these foci are aggregates of p85 devoid of membrane, are not sites of PI(3,4,5)P3 production, and are the result of overexpressed GFP-P85 (Luo, Field, Lee, Engelman, & Cantley, 2005). While these are artificial structures that result from overexpression and do not contribute to signaling they are food reporters of Gab1 phosphorylation,

specifically Y472. Interestingly, under clathrin-perturbed conditions, this phenotypic punctate localization of p85 was abolished, and these cells demonstrated a phenotype similar to basal cells, showing diffuse, presumably P85. This is entirely consistent with my previous findings that suggest that Gab1 phosphorylation and recruitment of PI3K to phosphorylated Gab1 is lost upon perturbation of clathrin using Pitstop2. As such, this further provides evidence for a specific role of clathrin in the EGF-stimulated activation of AKT. From this data we suggest that clathrin may be responsible for recruiting a kinase responsible for Gab1 phosphorylation and P85 binding.

Clathrin structures are necessary for Gab1 phosphorylation

Following perturbation of clathrin either by the small cell molecule Pitstop2 or gene silencing Gab1 phosphorylation was perturbed on Y307, and Y627, suggesting a role for clathrin in EGF-stimulated Gab1 phosphorylation. Although RTKs are required for Gab1 phosphorylation, EGFR autophosphorylation is unaffected following clathrin perturbation, thus the receptor itself does not phosphorylate all Gab1 residues. Therefore non-receptor tyrosine kinases such as Src, Fyn, Yes, other Src Family Kinases (SFks), or serine/threonine kinases must be involved in EGF-stimulated Gab1 phosphorylation (Gu & Neel, 2003). Therefore, our findings suggest that clathrin structures may form as scaffolds for some initially cytosolic non-receptor tyrosine kinase necessary for EGF-stimulated Gab1 phosphorylation.

A role for clathrin coated structures as microdomains for plasma membrane signaling is not completely novel as it is has been shown to regulate

G-protein signaling by sequestering Gα-interacting protein (GAIP) under unstimulated conditions, as well as serve as a scaffold regulating G-protein signaling sequestering δ-opioid receptor, Gαi3, and GAIPs under stimulated conditions (Elenko et al., 2003). Therefore, we hypothesize that a similar role for clathrin is necessary in EGF-stimulated phosphorylation of AKT. As clathrin has no catalytic properties itself, the requirement for clathrin as a microdomain must rely on its ability to recruit a number of proteins including kinases. Specifically, we are interested in TOM1L1, which forms a direct interaction with clathrin, is tyrosine phosphorylated by SFKs, and forms a transient interaction with EGFR mediated by Grb2/Shc necessary for endocytosis of the receptor upon EGF-stimulation (Liu, Loo, Loh, Seet, & Hong, 2009). TOM1L1 also stably interacts with some SFKs such as Fyn, which suggests that TOM1L1 may help recruit Fyn to clathrin structures. Further, the non-receptor tyrosine kinase Ack1 forms direct contacts with clathrin and is concentrated in CCPs when stimulated with EGF under conditions in which cells lack dynamin, thus isolating CCP-EGFR signaling (Shen et al., 2011). Therefore, we hypothesize that clathrin serves as a scaffold to prevent access to phosphatases, or a scaffold to locally cluster signaling intermediates such as TOM1L1 and Fyn as well as receptors necessary for progression of signaling from the plasma membrane as seen for Fc-ϒ receptors (Duchemin, Ernst, & Anderson, 1994).

EGFR:ErbB2 hetero-dimerization account for HeLa cell insensitivity to Pitstop2

While I performed in-depth analysis to understand the sequence of events of EGFR signaling in RPE cells that required clathrin, I found that HeLa cells are insensitive to Pitstop2 inhibition of EGF-stimulated AKT activation. This posed the question of why do HeLa cells not require clathrin as a scaffold for PM-EGFR signaling. Following further analysis, we showed that HeLa cells express very high levels of ErbB2 and EGFR compared to RPE-wt cells. Since EGFR has been shown to prefer hetero-dimerization with ErbB2 when the latter is co-expressed, it is likely that HeLa cells preferentially exhibit EGFR:ErbB2 heterodimers. Importantly, either EGFR:ErbB2 or EGFR: ErbB3 heterodimers may have the ability to bypass the requirement for Gab1 in ligand-simulated PI3K-Akt activation, as they have direct docking sites for PI3K, ErbB2 has one PI3K docking site, whereas ErbB3 has 6 docking sites (Campbell, Amin, & Moasser, 2010).

. Given the ability of these heterodimers to directly bind PI3K I hypothesize that EGFR:ErbB2 hetero-dimers may be the reason for HeLa cell insensitivity to Pitstop2 with respect to EGF-stimulated Akt phosphorylation. Further, ErbB2 has been shown to bind c-Src directly, while EGFR cannot (Marcotte, Zhou, Kim, Roskelly, & Muller, 2009), thereby suggesting that another signaling parameter that is unique to EGFR:ErbB2 heterodimers. Therefore, it is possible that EGFR:EGFR homo-dimers require clathrin structures for Gab1 phosphorylation,

whereas EGFR:ErbB2 hetero-dimers may bypass the need for clathrin structures and phosphorylate Gab1 directly.

To test this hypothesis, I created stable RPE cell lines expressing ErbB2. Consistent with this hypothesis, RPE cells stably expressing ErbB2 were largely insensitive to pitstop2 treatment with respect to EGF-stimulated in AKT phosphorylation. These results demonstrate that EGFR:ErbB2 heterodimers do not require additional signaling scaffolds to activate Akt, suggesting that the ability of EGFR:ErbB2 heterodimers to directly bind either Src or PI3K allows bypass of the requirement for clathrin in signaling. Moreover, since EGFR homodimers and EGFR:ErbB2 heterodimers both use a common set of signaling intermediates to activate Akt these results indicate that inhibition of EGFR homo-dimer signaling with pitstop2 is not due to an off-target effect of this drug on a downstream signaling intermediate. Instead, these results indicate that pitstop2-dependent perturbation of clathrin signaling microdomains is selective for signaling by EGFR homodimers but not EGFR:ErbB2 heterodimers.

Conclusion

In summary, I used multiple independent methods to provide evidence in support of a novel role for clathrin mediating EGF-stimulated AKT phosphorylation. This function exist outside the realm of clathrin mediated endocytosis as we demonstrated that endocytosis of EGFR is not necessary to activate AKT. Further examination is required to determine whether clathrin plays a direct role in the signaling cascade or is required for spatial organization of microdomains that contributes to context-specific signaling of EGFR. However, a novel role for clathrin has been identified, as a mediator of Gab1 phosphorylation, and activation of EGF-stimulated AKT.

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