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By

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B.A., Anderson University, 2012

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ABSTRACT

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Julie A. Gosney

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The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) that is an integral component of proliferative signaling. When activated by a ligand at the plasma membrane, EGFR undergoes clathrin-mediated endocytosis. This spatial regulation of the receptor is an important regulator of receptor expression as it mediates its degradation. Endocytosis also has implications on EGFR downstream signaling, though the details are not fully understood. The goal of this thesis is to develop a method to isolate early endosomes in order to study downstream effectors associated with activated EGFR in this compartment. HeLa cells were used to test various subcellular fractionation methods, optimizing each step to develop a protocol that enriches early endosomes. The isolated compartments were then analyzed by mass spectrometry to characterize the protein composition of early endosomes, with the goal of further understanding how the spatial regulation of EGFR affects its downstream signaling.

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INTRODUCTION

The study of growth factors and their receptors is a rapidly growing field of research that began in the 1960s when Stanley Cohen and Rita Levi-Montalcini discovered the first growth factors: Nerve Growth Factor (NGF) and Epidermal Growth Factor (EGF) [1, 2]. NGF and EGF are small proteins that stimulate the growth of nerve and epithelial cells, respectively. Before the discovery of growth factors, scientists knew that cells could signal for growth and proliferation, particularly during development—but they didn't understand how this phenomenon occurred [1]. The identification of NGF and EGF was pivotal, as these proteins could now be studied directly in order to elucidate their functions in cellular and organ physiology [3]. These discoveries were a major scientific breakthrough that earned Cohen and Levi-Montalcini a shared Nobel Prize in 1986 [4].

Upon Levi-Montalcini's discovery of NGF, she and Cohen worked diligently to understand its function. Because NGF was discovered in the submaxillary glands of mice, they continued using these extracts to study its effects on neuronal growth [5]. However, injecting mouse salivary gland extracts into newborn pups ended up yielding other, unexpected phenotypes. Cohen noted that these new changes were not due to the induction of nerve growth, but due to changes in epithelial tissues [2]. During Cohen's original experiments

characterizing his novel epithelial tissue-specific growth factor, he referred to the protein as the “tooth-lid factor” [2]. While the title was only temporary until he coined the name EGF, the “tooth-lid factor” was so named because it directly described the effects he saw in mice injected with EGF: it increased the rates of tooth growth and eyelid opening in newborn pups [2].

The discovery and characterization of growth factors led to another essential discovery—growth factor receptors. Once Cohen had discovered EGF, he immediately began working to isolate and identify its receptor. In 1982 Cohen successfully isolated and characterized EGF’s cognate receptor from mouse livers [3]. In this work, the receptor was identified as a 170kDa glycoprotein with intrinsic tyrosine kinase activity. Over the next three decades, the EGF-receptor would be studied extensively, leading to our most current understandings of the signaling, trafficking, regulation, and physiologic implications of this protein.

Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR) is a membrane spanning receptor tyrosine kinase (RTK) that is an integral component of proliferative signaling. Part of the ErbB family of receptors, EGFR is also referred to as ErbB1 or Her1. The other ErbB family members include ErbB2 or Her2, ErbB3, and ErbB4. Structurally, EGFR and the ErbB receptors are made up of three domains: 1) the extracellular ligand binding domain, 2) the transmembrane alpha helices, and 3) the intracellular domain which contains the kinase domain and multiple tyrosine residues on the C-terminus (**Figure 1**). The extracellular domain contains two ligand-binding regions that alternate with two cysteine-rich

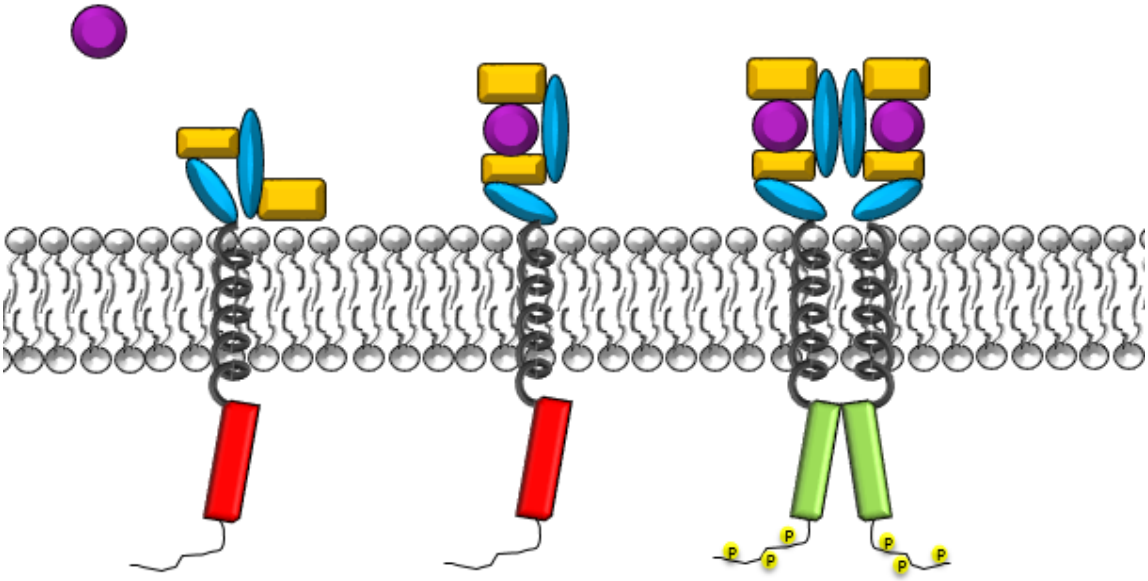

Figure 1. Epidermal Growth Factor Receptor structure. When unbound by a ligand, the receptor is often found in a “closed” conformation in which the cysteine-rich regions of the extracellular region interact. When a ligand is introduced it binds to the two ligand binding domains and a conformational change occurs, exposing a cysteine-rich region which can then interact with an exposed cysteine-rich region of another ligand-bound ErbB family receptor. Red=inactive kinase domain; green=active kinase domain; orange=ligand binding domains; blue=cysteine-rich domains.

regions. Binding of one of EGFR's seven endogenous ligands—epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HBE), epiregulin (EPR), epigen, transforming growth factor alpha (TGFα), amphiregulin (AR), and betacellulin (BTC)—to the extracellular region induces a conformational change in which the ligand binding regions directly interact with the ligand [6]. This structural change exposes the two cysteine-rich domains, allowing the receptor to associate with the exposed cysteine-rich domains of another ligand-bound EGFR or ErbB family RTK monomer to form a dimer [6].

The binding of two ligand-bound receptors causes the formation of a dimer, which is required for receptor activation [7]. The dimer pair interaction structurally induces the activation of the kinase domains. The kinase domain of one receptor then phosphorylates the C-terminal tyrosine residues of its dimer partner (transphosphorylation) [7]. The cytosolic phosphorylated tyrosine residues of the EGFR serve as docking sites for effector molecules that contain phosphotyrosine binding (PTB) or src homology 2 (SH2) domains [8]. Proteins that dock to the phosphorylated tyrosine residues of an activated EGFR will recruit and/or activate other proteins, thus inducing a signaling cascade. For example, at the plasma membrane, an activated EGFR dimer will recruit the scaffolding proteins Shc and Grb2 to bind to phosphotyrosines and the EGFR kinase domains phosphorylate these proteins [8]. Activation of Grb2 leads to the recruitment of SOS and induction of the Ras-ERK pathway which is known to activate cell proliferation [8]. Activation of Shc leads to induction of the JNK pathway which is also known to be involved in the induction of cell proliferation

via the activation of nuclear transcription factors [8].

EGFR Physiology

EGFR signaling plays critical roles in cell proliferation, migration, differentiation, wound healing, development, and tissue homeostasis. Growth factors are mitogens, and the EGFR is an important mitogenic signal transducer. In fact, the EGFR is an essential component of cellular physiology and is critical for proper tissue development. In 1995, Miettinen et al. produced a line of EGFR knockout mice to determine the physiologic importance of the receptor in development [9]. They found that the knockout (-/-) pups only survived for eight days after birth. The mice also had significant developmental impairments in multiple epithelial tissues and organs including the lungs, skin, and gastrointestinal tract [9]. In 1999 Miettinen also documented that EGFR (-/-) pups have compromised craniofacial development (**Figure 2**) [10]. EGFR is clearly a crucial component for normal tissue development and homeostasis throughout the body.

While the absence or reduction of EGFR signaling unquestionably causes severe developmental impairments as discussed, excessive EGFR signaling also has detrimental effects. It has been well documented that the receptor is often overexpressed and/or over-activated in many different cancer types, including non-small cell lung cancer, breast, pancreatic, cervical, head and neck, and colorectal cancer among others [11-16]. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for about 85% of all lung cancers [17]. Many driver mutations have been discovered in NSCLC patients,

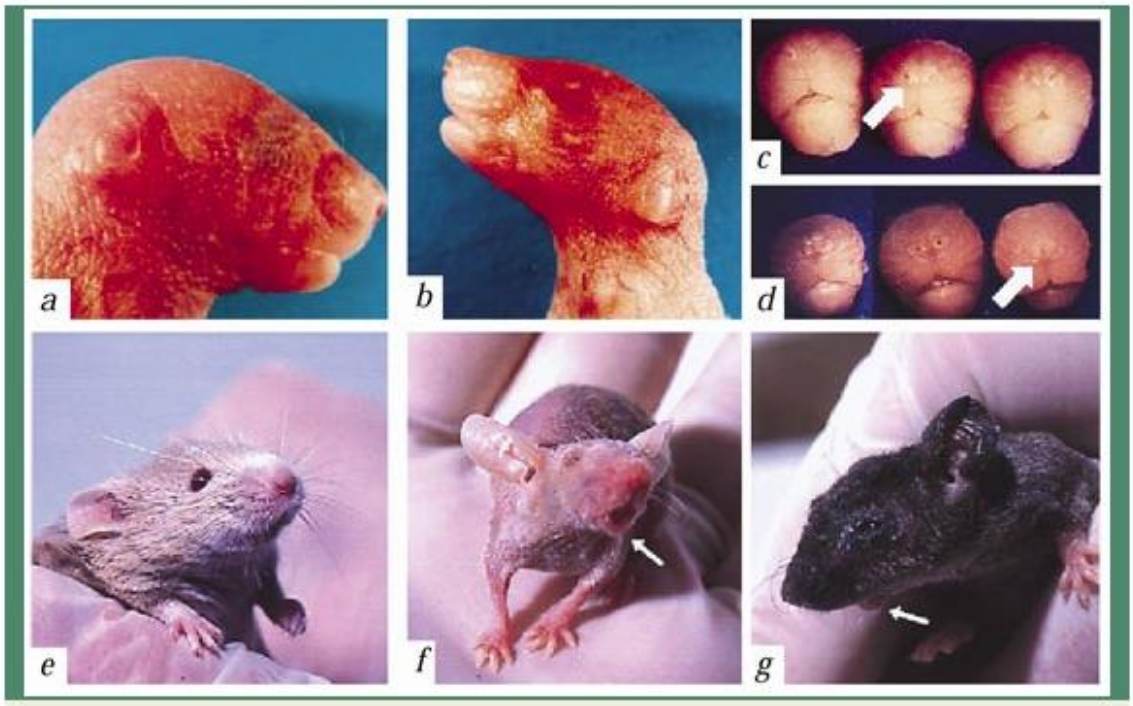

Figure 2. The effects of EGFR knockout on craniofacial development in mice. **a**) A wild-type newborn mouse has a round snout, whereas **b**) an EGFR (-/-) mouse has a narrower snout. **c**) The nostrils of wild-type mice are open (arrow), **d**) but are often closed or narrow (arrow) in EGFR (-/-) mice. **e**) A wild-type mouse at four months of age has long whiskers and a well-shaped snout, whereas **f,g**) EGFR (-/-) mice have smaller lower jaws (arrows), deformed eyes, and short, curly whiskers. (from *Nature Genetics* (1999), 22, 69-73)

and EGFR is the driver mutation in about 50% of patients who are “never smokers” [18]. With a 5-year survival rate of only 17% [19], lung cancer has one of the highest mortality rates of all cancer types [17]. Clearly there is a great need for better lung cancer treatments, and the EGFR is a model candidate to target in these cancers.

There are currently several FDA-approved anti-EGFR cancer therapies on the market. These drugs are generally split up into two classes—monoclonal antibodies and kinase inhibitors. The monoclonal antibodies (e.g. Cetuximab) target the extracellular portion of the receptor and block the interaction of the receptor with extracellular activating ligands. Cetuximab is approved for the treatment of cancers that express high levels of EGFR, including colorectal and head and neck cancers [20]. Tyrosine kinase inhibitors (TKIs) (e.g. Erlotinib) are small molecules that enter the cell and bind (reversibly or irreversibly) to the kinase domain of the receptor, blocking effector activation and downstream signaling cascades. Erlotinib is approved for use in NSCLC patients whose cancers express EGFR kinase activating mutations, including exon 19 deletion and exon 21 (L858R) substitution mutations [18]. While these drugs tend to be very effective initially for patients whose cancers express over-activated receptors, eventually all of these patients will develop resistance to the drugs [21]. The exact mechanism by which this resistance occurs is unclear, although several studies suggest that the inhibited receptors can form heterodimers with other ErbB family members and even the insulin-like growth factor type-1 receptor (IGF-1R), another RTK with mitogenic effects [22].

Although significant advances in cell biology research have led to the development of targeted therapeutics such as the anti-EGFR cancer drugs, these therapies provide an overall survival increase of only a few months for these subsets of cancer patients [23]. This lack of improvement, coupled with the resistance that develops from these treatments, reveals that there is an enormous gap in our understanding of mitogenic signaling. If more robust cancer treatments are to be developed, it is essential that the mechanisms driving the proliferation and metastasis of these cells are elucidated. Because targeting the EGFR directly has yielded only a minimal benefit to patient outcomes, it would be prudent to find more specific targets within the receptor's signaling pathways. Unfortunately, there are still many facets to EGFR signaling that have yet to be elucidated. One such question that could play an important role in understanding EGFR downstream signaling is: how does the spatial regulation of the receptor affect its signaling? This is the primary question we seek to answer in this work.

EGFR Regulation

The major mechanism through which the EGFR is regulated is the endocytic pathway (**Figure 3**). Once the activated dimer is formed it migrates to a clathrin-rich region of the plasma membrane where it invaginates and pinches off into a clathrin-coated vesicle. The clathrin is then shed, and this intermediate vesicle fuses with an early endosome [24]. The early endosome, sometimes referred to as the signaling or sorting endosome, is the epicenter of endocytic trafficking. This organelle is responsible for determining the fate of its contents, depending on several factors including what ligand is bound and with which ErbB

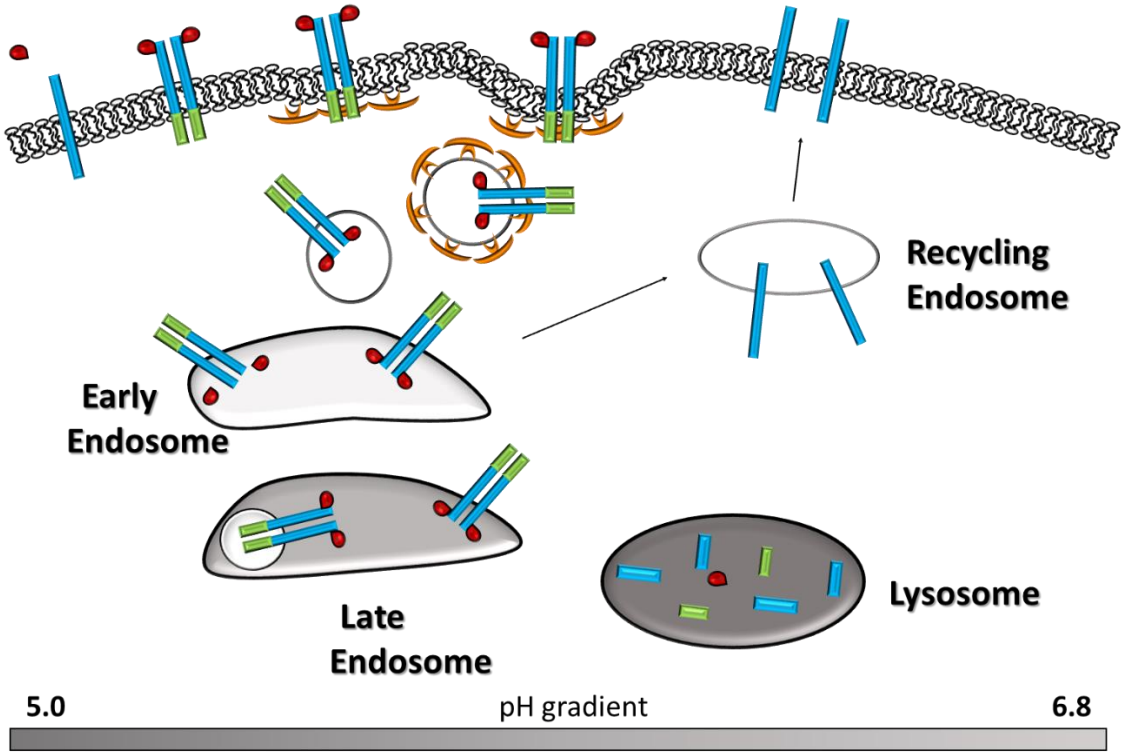

Figure 3. Endocytic trafficking of the EGFR. The EGFR undergoes ligand-dependent, clathrin-mediated endocytosis. Early endosomes either mature into late endosomes where their contents are transported to lysosomes for degradation, or the receptor can be trafficked back to the plasma membrane via a recycling endosome. The increasing acidity of these compartments induces dissociation of the ligand:receptor complex. The EGFR can continue to elicit signaling cascades from the early endosome. Red=ligand, green=active kinase domain, orange=clathrin.

family member the EGFR is dimerized. The early endosome can send proteins back to the plasma membrane (recycling) [25], or sequester cargo to be sent to and degraded in a lysosome [26]. It has been reported that endocytosis can also transport EGFR to the endoplasmic reticulum and the nucleus [27, 28]. Over time early endosomes increase in acidity and “mature” into late endosomes [29]. The late endosomes will fuse with a lysosome, where the receptor is degraded and thus down-regulated. It is important to note that while in the early endosome the kinase domain of the receptor remains exposed to the cytosol, allowing the receptor to continue interacting with other proteins and downstream effectors [30].

Until the 1990s, EGFR spatial regulation by the endocytic pathway was viewed primarily as a mechanism for downregulating receptor expression after activation. Chen et al. discovered in 1989 that an 18 amino acid sequence of the EGFR C-terminus is required for both kinase activation and internalization/ downregulation of the receptor [31]. Shortly thereafter in 1990, Wells et al. discovered that EGFRs that cannot undergo endocytosis enhance cell transformation [7]. They concluded from this study that without endocytosis, the receptor cannot be degraded and thus increases ligand-dependent cell transformation. As such, endocytosis was viewed as a negative regulator of EGFR expression. However, in 1994 Bergeron’s group discovered that certain EGFR scaffolding proteins involved in Ras signaling (i.e. Shc, Grb2, and mSOS) retain their association with active EGFR when it is internalized [25]. Further, in 1996, Vieira et al. created an endocytosis-defective cell line to study the changes

in EGFR downstream effector activation after EGF treatment. They found that blocking EGFR endocytosis enhanced PLCγ and Shc phosphorylation, but decreased ERK1/2, EGFR, & PI3K phosphorylation [26]. These works, among others, pushed the field of EGFR trafficking towards a new line of thinking: endocytosis can positively *and* negatively affect receptor:effector communication. However, there is currently no consensus on how these changes occur, or how they contribute to EGFR signaling and overall cellular physiology.

It has been well established that the endocytic pathway is important in the spatial as well as temporal regulation of the EGFR. The receptor is regulated temporally by the amount of time it takes to traverse the entire endocytic pathway, and how long the receptor is sequestered at each point of the pathway. About 10% of a cell's inactive EGFRs are constitutively recycled into early endosomes and back to the plasma membrane [32]. It has also been shown that different ligands induce varied endocytic responses. For example, it is known that TGFα triggers rapid recycling of the receptors, while EGF triggers the receptor to be maintained in early endosomes, leading to its eventual degradation and downregulation [33].

One explanation for these distinct differences in ligand:receptor trafficking amongst ligands is their affinity for the receptor. EGF is known to have a relatively high affinity for binding EGFR of 0.42nM [34], and thus does not dissociate from the receptor in the acidic environment of early endosomes (pH 6). However, TGFα has a slightly lower affinity for the receptor at 11.9nM [34], causing the ligand:receptor complex to dissociate in early endosomes, permitting

receptor recycling to the plasma membrane [33]. Though both ligands are considered to have “high” affinities for EGFR [35], they do not have the same effects on receptor trafficking. Conversely, the work of Moriai et al. suggests that EGF and TGFα have similar affinities for EGFR, and that certain mutations in the ligand binding domain of some EGFRs may contribute to the different binding affinities and downstream signaling effects of ligands [36].

The Endocytic Pathway

The endocytic pathway is a complex and dynamic system made up of various organelles [37]. Endocytosis is a fundamental cellular process in which extracellular nutrients and portions of the plasma membrane are internalized into the cell [37]. A section of plasma membrane will invaginate and pinch off to form an intracellular vesicle [37]. These preliminary vesicles are typically formed with the assistance of several adaptor and scaffolding proteins that are found near or on the plasma membrane [38]. For example, clathrin-mediated endocytosis (CME) requires the cytosolic protein clathrin, which forms a triskelion coat around the portion of plasma membrane that is to be internalized [38]. This process also requires another protein called dynamin that plays a critical role in the scission of the new vesicle from the plasma membrane [38]. CME is also referred to as receptor-mediated endocytosis, as it occurs when a plasma-membrane receptor is bound and activated by an extracellular ligand, triggering its internalization (e.g. EGF binding to EGFR) [38]. During CME, after the new vesicle is created the clathrin coat is shed. This intermediate vesicle is then trafficked to and fused with an endosome, of which there are several types [38]. The destination of each

vesicle is specific to its cargo and has a direct impact on the fate of that cargo. This process is highly regulated by actin filaments and microtubules, adaptor proteins, and GTPases such as the RAB proteins [38]. The RAB family of proteins are Ras-like GTPases that play an essential role in the endocytic pathway by recruiting effectors that induce the formation and motility of endosomes [39]. There are more than 60 different RAB proteins, and each one is generally specific to a distinct cellular compartment [40]. In 1990, Chavrier and Zerial determined that RAB5 is specific to the plasma membrane and early endosomes, and RAB7 localizes to late endosomes [41]. RAB11 is another member of the RAB family that is specifically localized to recycling endosomes [42]. These three RAB proteins are the major players involved in generating the vesicles involved in the early phases of endocytosis (i.e. early, late, and recycling endosomes).

Clathrin-mediated endocytosis is the primary pathway by which activated EGFR enters early endosomes when stimulated with low, endogenous concentrations of ligand (i.e. ~1ng/mL or 0.16nM EGF) [43]. However, there are other types of endocytosis that utilize adapter proteins similar to clathrin. For example, caveolae are small pits in the plasma membrane made up of lipid rafts and the protein caveolin [44]. Caveolae are also involved in the endocytosis of plasma membrane and extracellular ligands and nutrients. This process is similar to CME, as it also requires dynamin for the scission and formation of vesicles [38]. It also differs from CME in that caveolae cargo can either be delivered to early endosomes *or* to caveosomes [44]. Caveosomes are pH-

neutral intracellular vesicles that strictly contain cargo transported from caveolae, and they do not contain early endosome proteins, although their function is similar to early endosomes [44]. There are other routes of endocytosis that do not involve clathrin or caveolae, which are collectively termed clathrin- and caveolae-independent endocytosis [38]. The major routes of EGFR-endocytosis are CME at low ligand concentrations, and caveolae-mediated endocytosis (CavME) at high ligand concentrations (i.e. >10ng/mL or >1.6nM EGF) [43].

Whether via CME or CavME, most endocytic cargo will be transported into early endosomes. Early endosomes are so named because they are found in the cytosol near the plasma membrane, and they are the first major constituent within the endocytic pathway. These organelles are considered to be slightly acidic, with a pH of ~6.0 and a density of 1.035-1.042g/mL [29, 45]. Early endosomes are the first pit-stop in the pathway, and the sequestration of receptors here is critical to their ultimate fate.

There are two distinct populations of early endosomes: dynamic and static. In 2006 Lakadamyali and Rust discovered and characterized these types of endosomes by their mobility and maturation kinetics [46]. To do this, RAB5 and RAB7 were fluorescently tagged and the association of these proteins with various ligands that undergo CME were tracked using live cell imaging. They found that the static population of early endosomes are the most abundant, and they mature very slowly. The dynamic early endosomes are strongly associated with microtubules and mature rapidly into late endosomes. Remarkably, ligand:receptor complexes that are normally degraded via the endocytic pathway,

such as EGF:EGFR and low density lipoprotein and its cognate receptor (LDL:LDLR), were preferentially trafficked into the dynamic population of early endosomes. On the other hand, complexes that are typically recycled, such as transferrin and its receptor (Tfn:TfnR), were trafficked non-specifically to both populations of early endosomes [46]. This study provides further evidence that the endocytic pathway is highly regulated and the fate of every cargo that enters is tightly monitored.

As evidenced by the fates of the EGFR and TfnR, early endosomal contents can be segregated into various legs of the endocytic pathway. Certain proteins that are marked for recycling back to the plasma membrane can be sent directly to the cell membrane by an intermediate vesicle, or trafficked to a larger specialized vesicle called the recycling endosome [37]. Contents that are not recycled will remain sequestered in early endosomes. The organelles that comprise the endocytic pathway possess proton pumps on their membranes that maintain their luminal pH [29, 47]. However, over time these pumps will increase the acidity of early endosomes. This is a crucial step in the “maturation” process of an early endosome into a late endosome [48].

Late endosomes are also termed “multivesicular bodies” or MVBs, and have an acidic pH of ~5.3 and a density of 1.048-1.070g/mL [29, 45]. The name MVBs comes from the presence of intraluminal vesicles that are created within the organelle [49]. These are small, membrane-bound vesicles that are internalized from the outer membrane of the late endosome itself, which is also referred to as the “limiting membrane” [49]. Receptors and other cargo found in

the late endosome that are to be degraded are marked as such by entering into these intraluminal vesicles [49]. There are specific protein complexes called ESCRTs (endosomal sorting complex required for transport) that are required for the transport of cargo from the limiting membrane into intraluminal vesicles of late endosomes [50]. ESCRT complexes specifically interact with ubiquitinated cargo within the late endosome, as ubiquitination marks proteins for degradation [50]. Once a cargo is sequestered into an intraluminal vesicle, it is destined to be transported to a lysosome where it will be degraded. The late endosome will temporarily fuse with a lysosome and transfer its contents (intraluminal vesicles) to the lysosome [51].

Lysosomes are separate organelles that have a pH of ~5.0 and a density of between 1.070-1.110g/mL [29, 45]. The sole purpose of a lysosome is to degrade proteins, as they are filled with acid hydrolases to break down cargo [51]. This compartment is the final stop in the endocytosis of cargo that is marked for degradation (i.e. ubiquitinated). This degradation process is essential for the down-regulation of a multitude of cellular components, including signaling receptors like EGFR [51].

The focus of this project is the mitogenic signaling of EGFR from early endosomes. EGFR bound and activated by EGF is trafficked through the endocytic pathway to ultimately be degraded by a lysosome. Further, the kinase domain remains active and associated with the cytosol until the receptor is trafficked into the intraluminal vesicles of late endosomes. Our ultimate goal is to understand what specific signals are regulated by the early endosomal

association of an active EGFR. In order to study this, our immediate goal and the focus of this thesis is to develop a protocol for isolating early endosomes.

Endosome Isolation Methods

A multitude of labs have studied the biochemical properties of various endosomes, and have done so by isolating and separating endosomes from cells. The process of breaking open cells to separate out and study specific intracellular compartments is termed subcellular fractionation. Subcellular fractionation can be applied and modified in many ways to study the contents and functions of the various endocytic organelles. In this chapter, these methods will be outlined to determine the strengths and weaknesses of each method.

The process of subcellular fractionation is generally made up of three parts: lysing cells, separating cytosolic organelles, and isolating the target organelle [52]. There are several ways to perform these steps, each of which must also be optimized for the type of cells being used. Subcellular fractionation can be utilized to study virtually any organelle or compartment inside cells. However, the focus of this review will be on the application of these methods for isolating endocytic organelles.

The first step of subcellular fractionation involves breaking open cells to access internal compartments. The two major methods used to achieve this are hypotonic and mechanical lysis. Hypotonic lysis of cells involves incubating cells with a buffer containing lower than physiologic concentrations of either salt or sucrose until enough water moves into the cells via osmosis that the cells swell and eventually burst. This is a very effective method for lysing cells, however, if

the organelles are continuously exposed to a hypotonic buffer, it is possible that the organelles themselves risk being lysed as well. Some organelles, like lysosomes, are sensitive to hypotonic lysis, while others like early endosomes are not [53].

The second option for lysing cells is to use mechanical disruption. This can be achieved by passaging cells through a syringe and a small needle (typically 20-25 gauge), a ball-bearing homogenizer, or exposing cells to sonication. All of these methods work to lyse cells by applying physical force to the membrane of the cell. This method is less invasive than a hypotonic buffer and is generally considered to have little effect on the integrity of the intracellular compartments. However, it has been documented that these mechanical techniques can cause the formation of new, non-physiologically relevant vesicles as a result of hybrid fusion of distinct organelles [54]. The pros and cons of both of these lysis methods should be considered when selecting a lysis method for subcellular fractionation.

The second step of subcellular fractionation is separating intracellular components. Typically after lysis, the cell lysates will be gently centrifuged to pellet and remove large debris and nuclei. The nuclei can be discarded or used for further analysis of nuclear proteins or DNA. The resulting supernatant contains all cytosolic organelles, proteins, cytoskeleton, and the broken plasma membrane. This is referred to as the post-nuclear supernatant (PNS). The contents of the PNS must then be separated out to make the target organelle more accessible for the final isolation step of subcellular fractionation.

The most common methods of organelle separation utilize centrifugation. There are two widely used types of centrifugation—rate zonal or differential, and isopycnic. Rate zonal/differential centrifugation separates samples by size, and isopycnic/density centrifugation separates samples by density. Creating a PNS from cell lysates utilizes differential centrifugation. This type of separation can also be used to separate any other subcellular compartments based on size. Generally, increasingly higher speeds are required to pellet increasingly smaller organelles. Large nuclei require low speeds to pellet (~600 x g), while much smaller mitochondria and endosomes require much higher speeds to pellet (~10,000-20,000 x g), and still smaller ribosomes and endoplasmic reticulum fragments require extremely high speeds to pellet (~100,000 x g) [52]. Differential centrifugation is typically applied in sequence, beginning with low speeds to pellet large organelles and collecting the supernatant to spin at higher speeds to pellet smaller organelles. This process allows rapid and distinct separation of target compartments. However, because several organelles can sediment together due to size similarities, further separation methods may be necessary for isolation of a pure population of the target organelle. Differential centrifugation has been used for early/late endosome isolation [53, 55], but recently isopycnic centrifugation has been more commonly used.

Isopycnic centrifugation requires the use of media to create a density gradient. One type of density gradient is a continuous gradient. A continuous gradient is typically created with the use of a commercially available heterogeneous media. During centrifugation, the media creates a spontaneous,

self-forming gradient throughout the sample tube—the least dense materials will migrate to the top of the tube, and the densest materials will migrate to the bottom. Percoll is an example of a commonly used density gradient media for isopycnic centrifugation. Percoll is a mixture of colloidal silica coated with polyvinylpyrrolidone. When cell lysates are mixed with and centrifuged in a continuous gradient, organelles migrate to their isopycnic point within the gradient. The gradients can then be collected in multiple “fractions” to separate the contents with varying densities. An advantage of using a continuous gradient is the ability to resolve compartments with minute differences in density. However, a distinct disadvantage is that samples are diluted within the media, decreasing their concentration. This becomes more of an issue when the target organelle exhibits a range of densities and migrates within several fractions of the gradient, further decreasing their concentration. For example, early and late endosomes exhibit two separate ranges of densities (i.e. 1.035-1.042g/mL and 1.048-1.070g/mL, respectively) [45]. Although this increases the range of fractions within the gradient that will contain these vesicles, their densities are distinct enough to still separate both, with minimal overlap. Percoll gradients have been utilized for decades to separate and isolate endosomes [56-58].

The second type of density gradient is a discontinuous gradient. Discontinuous gradients are pre-formed and made of layers of media with increasing densities. Sucrose is the most common media used to create a discontinuous gradient—also referred to as a “step” gradient. The final products of a discontinuous gradient are distinct “fractions” that can be collected from the

“interface” between each layer of media. The number of interfaces/fractions in the gradient is dependent upon the number of layers in the gradient, and the quantity and density of the layers can be optimized based on the target subcellular compartment being collected. This is a distinct advantage of using a step gradient over a continuous gradient. The fractions from a discontinuous gradient can also be collected in much smaller volumes, providing more concentrated samples. While samples still migrate to their isopycnic point in a step gradient, there are a finite number of isopycnic points as they correlate to each distinct interface. As such, compartments collected at each fraction can exhibit a wide range of densities. This feature can serve as either an advantage or a disadvantage to this technique, depending on the target compartment. A disadvantage is the increased potential for samples to be contaminated with other subcellular organelles. Sucrose step gradients are also commonly used to isolate endosomes [58, 59].

The third and final step of subcellular fractionation is purification of the target organelle. Technically, this step is not a requirement for subcellular fractionation. In fact, depending on the scientific question being asked, this step is frequently omitted altogether. In many cases, the separation and enrichment of target organelles with density gradients is sufficient for further study with biochemical techniques [60, 61]. However, obtaining a pure organelle sample is essential for analyzing the proteome of a compartment. Multiple platforms can be used for this step, and it is arguably the most important component of organelle isolation. Typically, in order to isolate a particular cellular

compartment, a protein specific to the compartment of interest will be targeted. For example, antibodies against RAB11a, a protein specifically associated with recycling endosomes, were conjugated to magnetic beads, incubated with subcellular fractions, and placed on a magnet to purify recycling endosomes in the work of Silvis et al. [59]. The affinity of the antibody for its antigen, as well as the substrate to which the antibody is conjugated, are two critical components of this method [52]. Magnetic (Dynabeads), sepharose, and agarose beads are commercially available binding substrates with either Protein A or Protein G (or a mixture of both) coupled to the beads. Protein A & G are immunoglobulin-binding proteins that should be selected based on the source of the monoclonal antibody they will bind. The material make-up of the beads (Dynabeads, agarose, or sepharose) can also be selected based on their properties. Agarose and sepharose beads must be centrifuged or loaded on a column to isolate the beads and their bound organelles. Dynabeads, however, can be placed on a magnet and the supernatant removed with a pipette. Magnetic beads generally provide a gentler platform for isolating the target organelle, however they tend to be more expensive. The target organelles can be eluted off of the beads using either pH washes or a protein solubilizing buffer.

Various modifications of all three steps of subcellular fractionation detailed in this chapter were tested in order to develop a protocol optimized for the isolation of early endosomes from HeLa cells to study EGFR signaling from these compartments.

MATERIALS AND METHODS

Cell Culture

HeLa cells were acquired from American Type Culture Collection (ATCC). Cells were cultured at 37°C in 5% CO_2 and maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 5% Fetal Bovine Serum (FBS, Invitrogen), 100U/mL streptomycin, 100U/mL penicillin, and 2mM glutamine [62].

Cell Lysis Preparation

Cells were lysed using either mechanical lysis or osmotic lysis as indicated in the text and figure legends. Cells were grown to confluency in 15cm dishes, serum starved for 2 hours at 37°C, then incubated with the indicated concentration of EGF ligand (Invitrogen, #PHG0311) and/or carbocyanine dye "DiI" (Molecular Probes, #D-282) (dissolved in EtOH) for the indicated amount of time immediately prior to harvest.

1. Mechanical Lysis

Cell lysates were prepared by washing twice with room temperature (RT) PBS and equilibrating to 4°C on ice, followed by scraping into 4mLs of ice-cold lysis buffer (PBS supplemented with a cocktail of protease and phosphatase inhibitors—2mM PMSF [phenylmethylsulfonyl fluoride], 1μM Na_3VO_4 [sodium orthovanadate], 10μM pepstatin, and 1μM aprotinin). Cell suspensions were pipetted into 0.5mL aliquots (~2.0 x 10^6 cells) and each aliquot was passaged 15

times through an Isobiotec metal ball-bearing cell homogenizer (Heidelberg, Germany) with a clearance of 14μm at 4°C. Lysates were pooled together and centrifuged at 200 x g for 10' at 4°C in a JA25.50 rotor (Beckman-Coulter). The post-nuclear supernatant (PNS) was collected from each sample [45].

2. Osmotic Lysis

Cell lysates were prepared by washing twice with RT PBS and equilibrating to 4°C on ice, followed by equilibrating in ice-cold lysis buffer (TES-10mM triethanolamine, 1mM EDTA, 0.25M sucrose pH 7.2). Cells were incubated on ice with TES buffer (supplemented with 2mM PMSF, 1mM Na_3VO_4, 10μM pepstatin, and 1μM aprotinin) until cells began to swell, but before bursting (approx. 5 minutes), and scraped with a rubber policeman. The collected cells were pipetted up and down 40 times with a P1000 pipet and centrifuged at 200 x g for 10' at 4°C in a JA25.50 rotor to create a post-nuclear supernatant (PNS) which was subsequently collected [45].

Percoll Gradient Fractionation

Twenty-four hours prior to experimentation, stock Percoll (GE Healthcare) was equilibrated with 2.5M sucrose at a ratio of 9:1. The 90% Percoll/0.25M sucrose solution was stored at 4°C until use. Samples were prepared as indicated (by PNS preparation via either osmotic lysis or mechanical lysis, or by sucrose gradient fractionation), and each sample was mixed with the 90% Percoll solution (final concentration 17% Percoll) and Percoll buffer (250mM sucrose, 1mM EDTA, 10mM HEPES in PBS, pH 7.2) or TES to a total volume of 11.5mLs. Buffers were spiked with 2mM PMSF, 1mM Na_3VO_4, 10μM pepstatin, and 1μM

aprotinin before use. PNS/Percoll/Buffer mixtures were pipetted into 16mm x 67mm OptiSeal™ polypropylene tubes (Beckman Coulter) and loaded into a pre-chilled VTi65.1 vertical rotor. Density beads (with known densities in 17% Percoll/250mM sucrose) (GE Healthcare) were loaded into a separate tube containing 17% isotonic Percoll in buffer and mixed. Samples were spun in a Beckman Coulter Optima L-100 XP Ultracentrifuge at 50,000 x g for 25' with max acceleration and brake. Samples were then fractionated from the bottom of the centrifuge tube in 10-drop aliquots (~330μL) into pre-chilled Eppendorf tubes (~30 fractions per gradient) using a peristaltic pump and a glass pipet at 4°C [45]. For cells that were labeled with DiI, the collected fractions were loaded into a 96-well dish and read on a fluorescence plate reader (BioTek) (excitation 530/25, emission 590/35). For experiments where fractions were subjected to affinity purification, the fractions in which DiI fluorescence peaked were pooled together (~6 fractions per condition, ~2mL total) and mixed by inverting and gently pipetting up and down. For experiments where fractions were not pooled but analyzed directly via immunoblot, each fraction was diluted in 6X SDS sample buffer containing 10% β-mercaptoethanol (βME), boiled at 100°C for 3', and centrifuged at 21,000 x g to pellet Percoll. The tube containing density beads was imaged and Rf values were calculated based on bead migration in the gradient.

Affinity Purification of Early Endosomes

Protein G Dynabeads (Invitrogen) were resuspended and washed three times in PBS before use. Approximately 0.22μg of EEA1 monoclonal antibody

(Cell Signaling, #3288) was pre-conjugated to ~2.0 x 10^7 washed magnetic beads in an equivalent volume of buffer used for cell lysis by rotating at 4°C for at least 2 hours. The beads were washed again in ice-cold buffer three times to remove unbound antibody. Either pooled DiI peak fractions from Percoll gradient samples or HeLa cell PNS (as indicated) were incubated with the EEA1 antibody-conjugated magnetic beads (~1mL sample per tube of EEA1-conjugated magnetic beads) and rotated at 4°C for 1 hour. Magnetic beads were isolated and the first supernatant (pass through, PT) was collected. The beads were then washed two times in ice-cold buffer and eluted in 6X SDS buffer containing 10% βME and boiled at 100°C for 3'. Remaining samples collected were diluted in 6X SDS buffer with 10% βME and boiled. Samples containing Percoll were centrifuged at 21,000 x g to pellet Percoll.

Sucrose Gradient Fractionation

Cells were grown to confluency in 15cm dishes, serum starved for 2 hours, and incubated with the indicated concentration of EGF ligand and/or DiI for 15' immediately prior to harvest. Cells were washed twice in RT PBS before equilibrating to 4°C on ice. Dishes were equilibrated in ice-cold lysis buffer (1mM Tris, 2mM EDTA, pH 7.4) followed by incubation on ice with ice-cold lysis buffer (supplemented with 2mM PMSF, 1mM Na_3VO_4, 10μM pepstatin, and 1μM aprotinin) until cells begin to swell, but before they burst (~5'). Cells were scraped with a rubber policeman and pelleted at 800 x g for 3' in an Allegra 25R centrifuge (Beckman Coulter). The cell pellet was resuspended in 1mL ice-cold lysis buffer supplemented with protease inhibitors and passaged 20 times

through a 22-gauge (22G) needle and syringe. Cell lysates (750µL) were loaded on top of a pre-loaded sucrose gradient (SW41-14 x 89mm thinwall polypropylene tubes [Beckman Coulter]) supplemented with protease and phosphatase inhibitors as follows, beginning with the bottom layer: 3.7mL 60% sucrose, 4.0mL 38% sucrose, 3.2mL 5% sucrose, 750uL cell lysates. Tubes and buckets were weighed to ensure balance, loaded on an SW41 rotor (Beckman), and centrifuged in a Beckman Coulter Optima L-100 XP Ultracentrifuge at 210,000 x g for one hour with max acceleration and max brake at 4°C. The milky interface between each layer of sucrose was collected, with intracellular vesicles at the 5%-38% interface and membranes at the 38%-60% interface. Samples were diluted in 6X SDS buffer with 10% βME and boiled for 3' at 100°C.

Immunoblotting

Samples were diluted in 6X SDS buffer with 10% βME and boiled at 100°C for 3' prior to gel loading. Samples were loaded as a percentage of total sample volume and resolved by SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane and blocked with 5% milk before probing overnight at 4°C with primary antibody, diluted 1:1000. The following antibodies were used for immunoblot detection: EGFR (Santa Cruz, #sc-03), TfnR (BD Biosciences, #612124), LAMP2 (University of Iowa Hybridoma Bank, #H4B4), EEA1 (BD Biosciences, #610456), Na/K-ATPase (Sigma, #A276-only used where indicated), Na/K-ATPase (Cell Signaling, #3010), and Calnexin (BD Transduction, #610524). Membranes were then washed 3 x 10' in TBS-Tween20 (TBST) and incubated with the appropriate horseradish peroxidase

conjugated secondary antibody (anti-mouse or anti-rabbit, Thermo Fisher-Pierce) diluted 1:2500, for one hour at RT. Membranes were washed again 3 x 10' in TBST and imaged using Enhanced Chemiluminescence on a Fotodyne imaging system. Western blots were quantified using Image J software.

Indirect Immunofluorescence

HeLa cells were grown to confluency on NaOH treated, sterile 12mm round glass coverslips. Cells were serum starved for 2 hours and incubated with the indicated concentration of EGF ligand and/or DiI for 15 minutes. Coverslips were washed gently in PBS^{++} (PBS, 0.5mM $CaCl_2$, 0.5mM $MgCl_2$) and cells were fixed in 4% paraformaldehyde/PBS^{++} for 5' at RT and 15' on ice. After fixation, coverslips were removed from ice and washed 3 x 5' in PBS^{++}. Cells were permeabilized in PBS^{++++} (0.1% saponin/5% FBS/PBS^{++}) for 20' at RT and washed 3 x 5' in PBS^{++}. The indicated primary antibody (1°Ab) [EGFR (Ab-1, EMD Millipore, #GR01), EEA1 (BD Biosciences, #610456)] was prepared by a 1:1000 dilution in PBS^{++++} and centrifuged for 5' at 21,000 x g. Coverslips were placed, cell-side down, on top of 30µL aliquots of 1°Ab on a piece of parafilm for 1 hour at RT. Coverslips were returned to dishes and washed 3 x 5' in PBS^{++}. The appropriate secondary antibody (2°Ab), either Alexa488- or Alexa568-labeled (Life Technologies) was diluted 1:200 in PBS^{++++} and centrifuged at 21,000 x g for 5'. Cells were incubated with 2°Ab for 1 hour at RT as described for the 1ºAb. Free Ab was removed with 6 x 10' washes in PBS^{++} and coverslips were rinsed in ddH_2O prior to mounting onto glass slides with Prolong + DAPI (Life Technologies) [63]. Slides were cured in the dark overnight before imaging

with a 60X oil immersion objective lens on a Nikon Eclipse Ti-E Inverted fluorescence microscope, using Nikon NIS Elements software.

Coomassie Staining

Early endosomes immunoprecipitated from HeLa cell PNS were resolved on a 7.5% SDS-PAGE. The gel was rinsed once in ddH_2O and covered with Coomassie (50% MeOH, 0.05% Coomassie Brilliant Blue R [Sigma], 10% acetic acid, 40% ddH_2O) and microwaved for 5 seconds. The gel was incubated with Coomassie at RT with gentle rocking for 15'. The Coomassie was removed and the gel was rinsed twice in ddH_2O. The gel was then covered in Destain solution (7% glacial acetic acid, 5% MeOH, 88% ddH_2O) and incubated overnight at RT with gentle rocking. The gel was rinsed in ddH_2O, imaged using a Fotodyne imaging system, and stored in 7% acetic acid/ddH_2O at 4°C.

Mass Spectrometry

1. In-Gel Protein Digestion

This protocol is modified from Jensen, et al., 1999 [64]. A Coomassie stained SDS-PAGE gel was cut into $1mm^3$ plugs and incubated in 100mM triethylammonium bicarbonate (TEA-BC, Sigma) at RT for 15'. Acetonitrile (ACN) was added to the TEA-BC solution and the gel plugs were incubated at RT for 15' with gentle vortexing. The solvent was removed and the washing process was repeated until the Coomassie blue stain was no longer visible. Solvent was removed and the gel plugs were dried in a SpeedVac for 5'. The dried plugs were incubated in DTT (20mM DTT [BioRad],100mM TEA-BC) at 56°C for 45', followed by iodoacetamide (55mM iodoacetamide [Sigma], 100mM

TEA-BC) at RT for 30' protected from light. Iodoacetamide was removed and gels were washed in 50mM TEA-BC at RT for 15', followed by the addition of ACN for 15' at RT with gentle vortexing. The gel plugs were again dried for 5' in a SpeedVac, and incubated in digestion buffer (20ng/µL modified Trypsin [Promega] in 50mM TEA-BC) for approximately 10' until the gel plugs swelled. After swelling, 50mM TEA-BC was added to the plugs, followed by 37°C overnight incubation in a shaker. Digestion supernatants from the upper and lower half of the gel were combined for each sample.

2. Extraction of Peptides

This protocol is modified from Shevchenko, et al. 2006 [65]. LC-MS grade water was added to the digested gel plugs to give a final concentration of 25mM TEA-BC. Two volumes of 1:2 5% v/v formic acid:acetonitrile was added and incubated at RT for 15' in a shaker (100rpm in a C25 Incubator Shaker [New Brunswick Scientific]). Liquid surrounding the gel pieces was transferred to a clean microtube and dissolved in Chromatography Buffer A (2% v/v acetonitrile/0.1% v/v formic acid). The dissolved sample was filtered through a 0.45µm regenerated cellulose syringe filter (Thermo #F2504-7) to remove any remaining gel material. Resolubilized gel band digests were desalted and concentrated using C18 PROTO™, 300 Å Ultra MicroSpin Column (The Nest Group, Inc., Southborough, MA, USA). Samples were cooled to -80°C, dried using a SpeedVac, and redissolved in Chromatography Buffer A. Sample absorbance was read at 205nm using a NanoDrop 2000 spectrophotometer to determine peptide concentration. Sample volumes were adjusted in Buffer A to

normalize peptide concentrations to 0.1µg/µL.

3. Liquid Chromatography/Mass Spectrometry (LCMS)

Gel band digests (0.5µg) were separated on 12cm of Aeris Peptide XB-C18, 3.6µm, 100Å material (Phenomenex, Torrance, CA, USA) packed into a 360µm OD x 100µm ID fused silica tip that was pulled using a Model P-2000 Micropipette Puller (Sutter Instrument Co., Novato, CA, USA). Peptides were eluted from the column using an EASY n-LC UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) in an 80' linear gradient using Buffer A and Buffer B (80% v/v acetonitrile/0.1% v/v formic acid) as mobile phases (from 0% Buffer B to 50% Buffer B). The samples were then separated by a 5' linear gradient from 50% Buffer B to 95% Buffer B, followed by a 5' wash in 95% Buffer B. The sample was introduced into the LTQ-Orbitrap Elite (ThermoElectron) mass spectrometer by nanoelectrospray using a Nanospray Flex source (ThermoElectron). The ion transfer capillary temperature was set to 225°C and the spray voltage was set to 1.6kV. An Nth Order Double Play was created in Xcalibur v2.2. Scan event one of the method obtained an FTMS MS1 scan (normal mass range; 240,000 resolution, full scan type, positive polarity, profile data type) for the range 300-2000m/z. Scan event two obtained ITMS MS2 scans (normal mass range, rapid scan rate, centroid data type) on up to twenty peaks that had a minimum signal threshold of 5,000 counts from scan event one. The lock mass option was enabled (0% lock mass abundance) using the 371.101236m/z polysiloxane peak as an internal calibrant.

Proteome Discoverer v1.4.1.14 (Thermo Fisher Scientific) was used to

analyze the mass spectrometer data. MS2 scan data were extracted from the Xcalibur RAW file, CID MS2 scans were searched in Mascot v2.5.1 (Matrix Science, Inc., Boston, MA, USA) and SequestHT, and results were collected in a single file. The protein database UniprotKB *Homo sapiens* version 3/9/2016 reference proteome canonical and isoform sequences, with cRAP database (thegpm.org) version 1/1/2012 appended to it, were used in the Mascot and SequestHT searches. The resulting files from Proteome Discoverer were loaded into Scaffold Q+S v4.4.5 (Proteome Software, Inc., Portland, OR, USA). The peptide false discovery rate was calculated with Scaffold Local FDR algorithm, and protein probabilities were calculated using the Protein Prophet algorithm. Results were annotated with human gene ontology information from the Gene Ontology Annotations Database (ftp.ebi.ac.uk).

RESULTS

The overarching goal of these experiments was to develop a strategy to isolate EGFR-containing early endosomes. I hypothesized that biochemical enrichment of endosomes, followed by immunoisolation of an early endosome specific protein would lead to a preparation enriched with early endosome marker proteins but devoid of plasma membrane, late endosome, and endoplasmic reticulum candidate proteins.

The experiments described below reflect my findings during the optimization process. All experiments were performed using HeLa cells, a human adenocarcinoma cell line. HeLa cells express physiologic levels of EGFR (~50,000 EGFRs/cell) [66], are amendable to cDNA transfection, adenoviral and lentiviral transduction, grow rapidly, and are relatively easy to culture. The final protocol that was developed based upon these data is outlined in the Discussion section.

EGFR associates with EEA1-positive vesicles upon EGF stimulation.

I first wanted to determine the extent of EGF-mediated EGFR redistribution to early endosomes in HeLa cells. To monitor EGFR localization, HeLa cells were treated with EGF for increasing amounts of time, fixed, and subjected to indirect immunofluorescence probing for the EGFR (using a mouse monoclonal antibody, Ab-1 that recognizes the amino terminal, extracellular domain of the receptor) and Early Endosome Autoantigen 1 (EEA1) (using a

rabbit polyclonal antibody) (**Figure 4**). Localization of the primary antibodies were visualized with Alexa 488-conjugated goat anti-rabbit (EEA1) and Alexa 586-conjugated goat anti-mouse (EGFR) secondary antibodies. Cells treated without EGF were used as a negative control.

In the absence of EGF, the EGFR is localized primarily to the plasma membrane of cells. The addition of EGF induces a time-dependent redistribution of EGFR that peaks with an accumulation of EGFR co-staining with early endosomes at 20 minutes. These kinetics of endocytic trafficking are consistent with previous reports [67, 68]. Importantly, neither the intensity nor the distribution of EEA1 changes over time. After 30 minutes of EGF treatment, there is a decrease in EGFR and EEA1 co-staining, which is consistent with reports that the EGFR is trafficked out of the early endosome 20-30 minutes after EGF stimulation [67, 68].

DiI fluorescence can be used as a marker for early endosomes.

Next, I wanted to determine if 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindo-carbocyanine Perchlorate (DiI) could be used as a marker for early endosomes. DiI is a commercially available indocarbocyanine dye that binds to the plasma membrane and laterally diffuses into all lipid membranes. The redistribution of the dye can be used as a measure of membrane trafficking. When membrane domains containing the EGF:EGFR internalize, the DiI stained membrane would co-localize with the EGFR.

HeLa cells were equilibrated to 4°C to halt membrane trafficking and incubated with 10μM DiI. After the indicated amount of time, cells were warmed

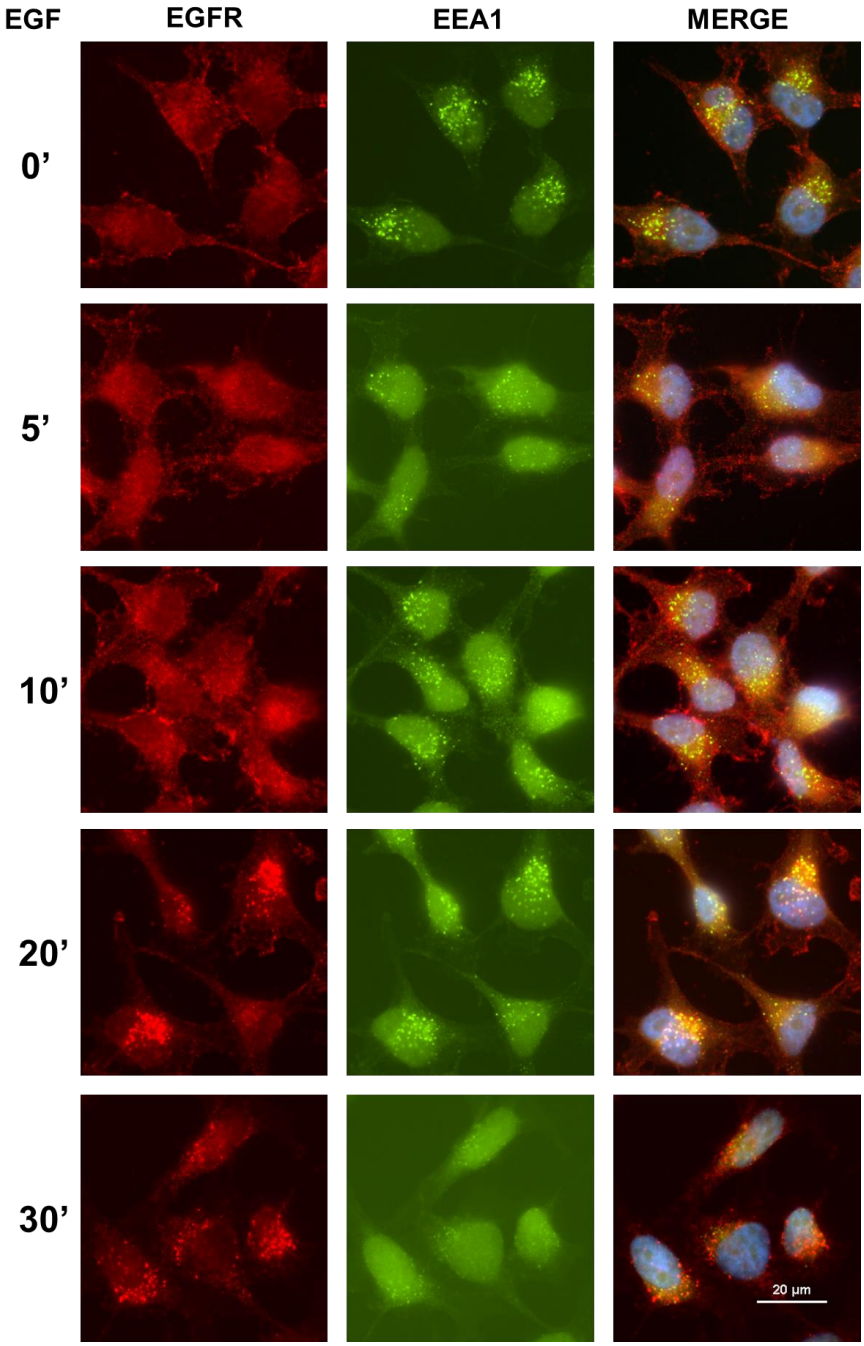

Figure 4. EGFR co-localization with EEA1-positive vesicles upon EGF stimulation. HeLa cells were serum starved for 2 hours at 37°C and incubated with 10ng/mL EGF for 0', 5', 10', 20', and 30'. Cells were fixed with 4% paraformaldehyde and stained for EGFR (Alexa-568) and EEA1 (Alexa-488) using indirect immunofluorescence. Scale bar=20µm.

to 37°C and treated with 10ng/mL EGF for 10'. The distribution of DiI stained membranes relative to EEA1 was determined (**Figure 5**). Although there was an increase in total fluorescence that was proportional to the incubation time with DiI, there was no co-localization of DiI with early endosomes. It is possible that the 4°C incubation may have reduced the ability of DiI to fully incorporate into membranes. Further, the 10' incubation at 37°C may not have been sufficient to re-equilibrate cells to a high enough temperature to re-initiate physiologic membrane trafficking.

Next, I asked if treating cells with EGF ligand and DiI concomitantly at 37°C would enhance co-localization of DiI with early endosomes. HeLa cells were incubated with 10μM DiI and 10ng/mL EGF at 37°C for 15'. Cells were fixed and monitored for EGFR localization using indirect immunofluorescence. EGFR was monitored rather than EEA1 as I wanted to monitor newly formed endocytic internal compartments. Cells treated without DiI and/or EGF were used as a negative control. EGF treatment induced a redistribution of EGFR into punctate intracellular compartments. DiI fluorescence was also observed in punctate intracellular compartments that co-localized with EGFR staining (**Figure 6**).

Having confirmed that DiI labels compartments containing internalized EGFR after 15' treatment with DiI and EGF at 37°C, I wanted to determine whether endosomal DiI labeling was restricted to early endosomes. Serum-starved HeLa cells were treated with 10uM DiI and 10ng/mL EGF for 15'. Cells were then harvested using mechanical lysis and the PNS was subjected to a

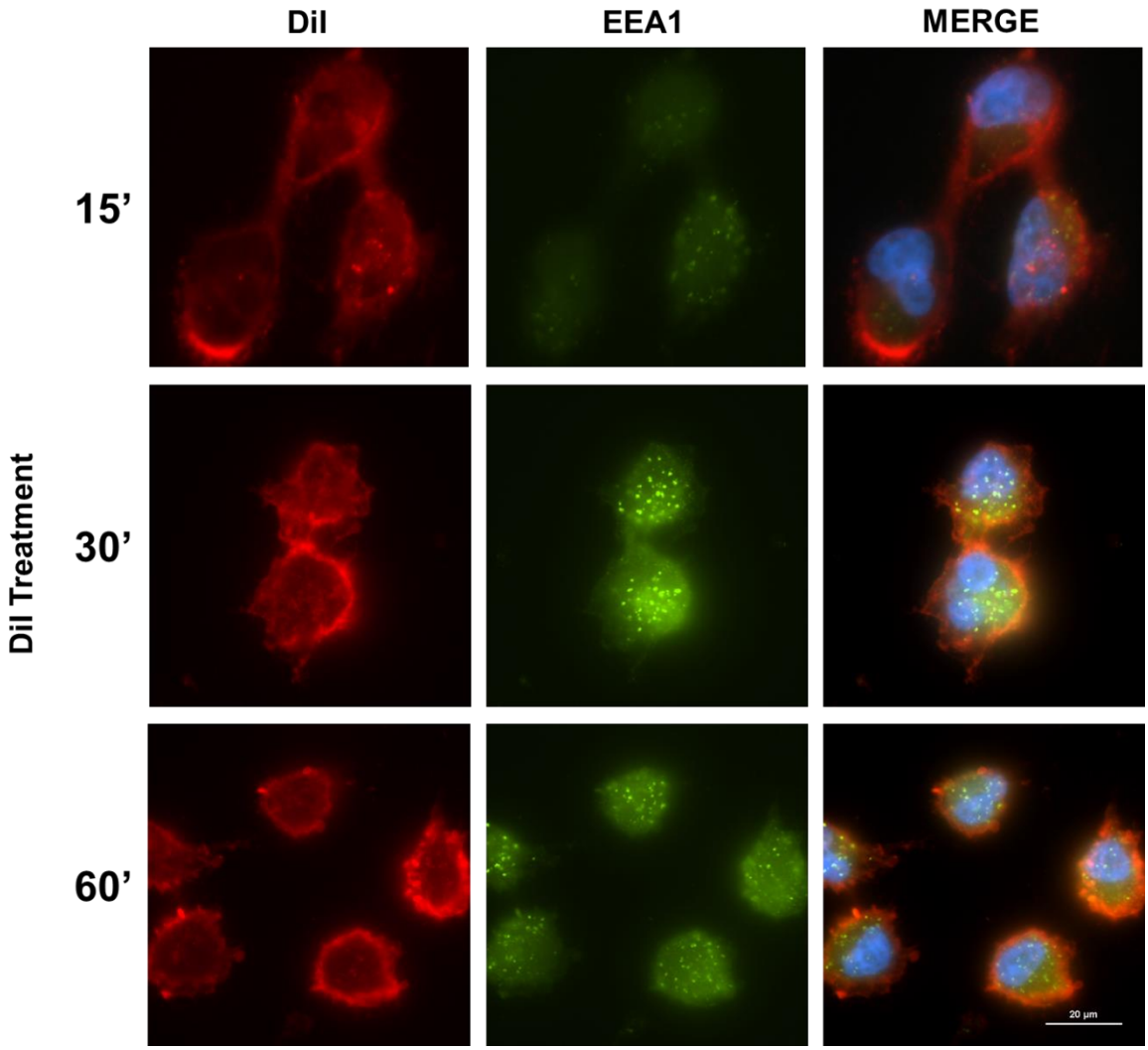

Figure 5. DiI does not co-localize with early endosomes when incubated at 4°C. HeLa cells were serum starved for two hours at 37°C and pre-treated with 10µM DiI on ice (4°C) for the indicated amount of time, followed by treatment with 10ng/mL EGF at 37°C for 10'. Cells were fixed with 4% paraformaldehyde and stained for EEA1 (Alexa-488) using indirect immunofluorescence. Scale bar=20µm.

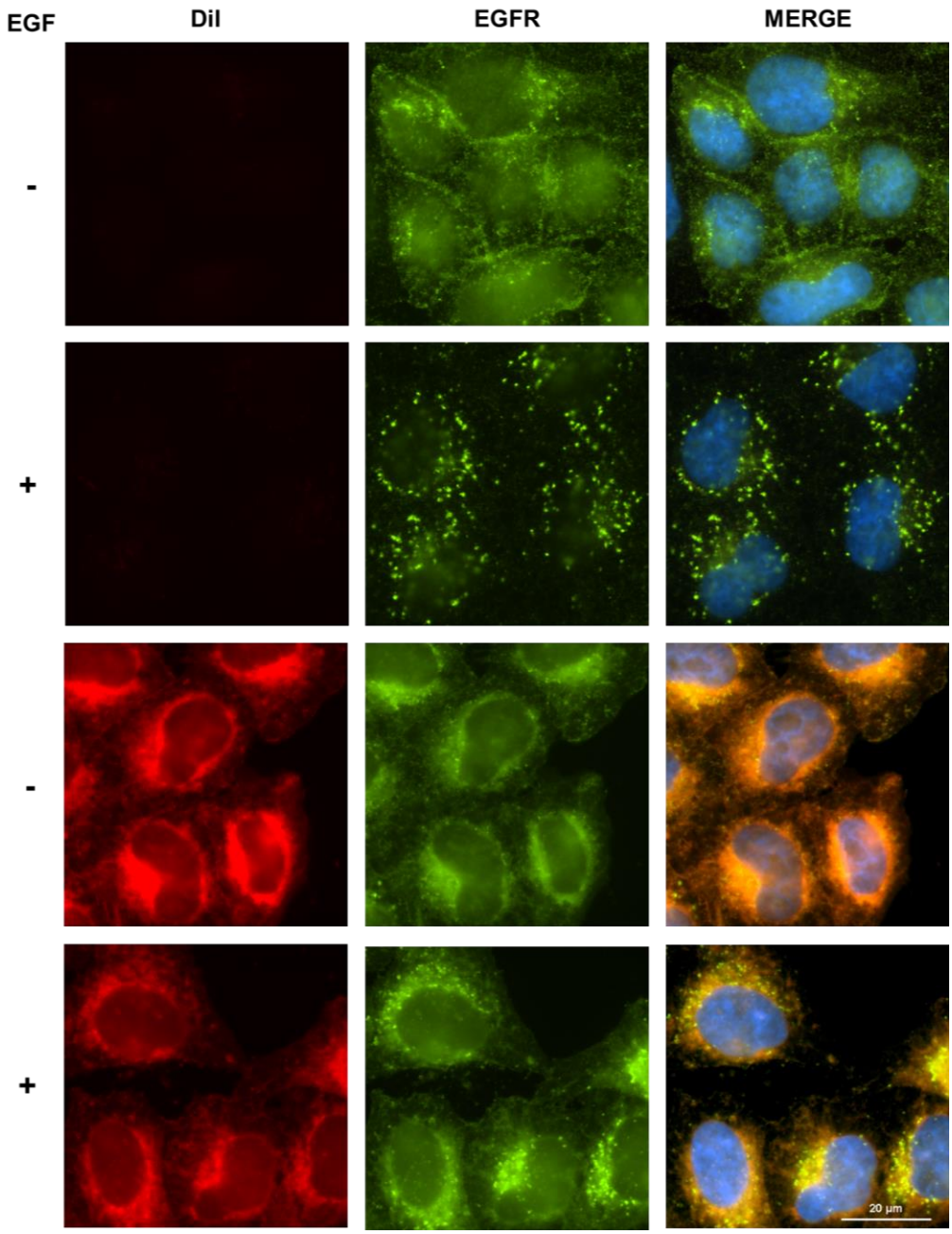

Figure 6. Co-localization of DiI and EGFR fluorescence at 37°C. HeLa cells were serum starved for 2 hours at 37°C and incubated ±10µM DiI, ±10ng/mL EGF for 15'. Cells were fixed with 4% paraformaldehyde and stained for EGFR (Alexa-488) using indirect immunofluorescence (DiI fluorescence was measured using a 568nm filter). Scale bar=20µm.

17% Percoll gradient in 10mM triethanolamine/1mM EDTA/250mM sucrose (TES) buffer. The gradient was fractionated into ~330µL (10 drop) fractions and assayed for DiI fluorescence on a plate reader. A distinct peak of DiI fluorescence was measured at approximately the same Rf value in gradients of cells treated EGF (**Figure 7**).

EGFR protein concentration peaks with TfnR protein concentration and DiI fluorescence in Percoll gradient fractions.

In order to determine the endosome composition of the DiI peak fractions, immunoblotting was used to measure the distribution of early and late endosomal markers. Using the same protocol described above, every other fraction of Percoll gradients were subjected to immunoblot for EGFR, TfnR (early/recycling endosome marker), and LAMP2 (late endosome/lysosome marker) (**Figure 8A**). In both the –EGF and +EGF samples, EGFR and TfnR peaked in the same fractions in which DiI fluorescence also peaked (**Figure 8B**), and LAMP2 was not present in these fractions. Interestingly, the peak of EGFR was independent of EGF treatment. Since the immunofluorescence data indicated that the endosomal accumulation of the EGFR was EGF dependent, this opened the possibility of plasma membrane contamination in these fractions.

EEA1 can be targeted to purify early endosomes.

In order to further enrich the early endosomes present in the Rf ~0.35-0.58 Percoll fractions (~1.045g/mL density), I performed immunoisolation of these vesicles with various proteins predicted to be in the early endosomes: EEA1, EGFR, and TfnR. Magnetic Dynabeads were utilized because they provide a

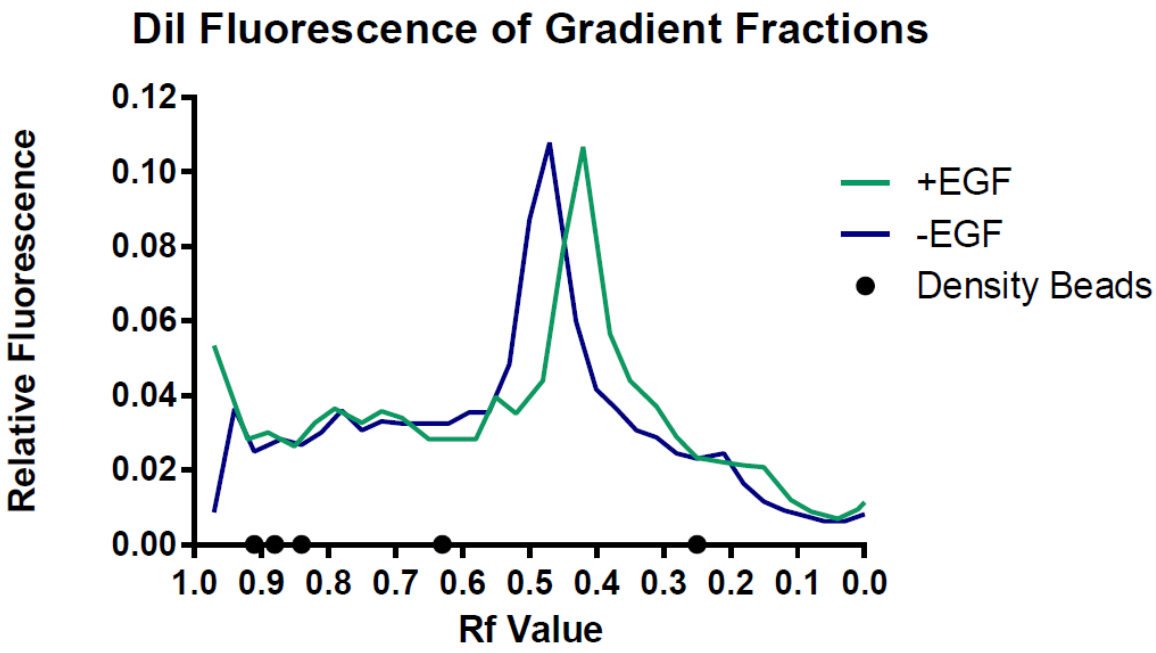

Figure 7. Relative DiI Fluorescence as a function of Percoll gradient fraction Rf Values. HeLa cells were serum starved for 2 hours at 37°C and incubated with 10μM DiI ±10ng/mL EGF for 15'. Cells were harvested using mechanical lysis and the PNS was loaded on a Percoll gradient and fractionated as outlined in the Materials and Methods. DiI fluorescence of each fraction was measured on a plate reader (Ex- 530/25, Em-590/35). Rf values were calculated as a function of total drops collected per sample. Marker beads with known densities were also separated with a Percoll gradient and plotted against Rf values. Fractions decrease in density from left to right (1.0-0.0). Closed circles on the x-axis represent density bead migration (Rf ~0.91=1.109g/mL, ~0.88=1.070g/mL, ~0.84=1.057g/mL, ~0.63=1.049g/mL, ~0.25=1.042g/mL).

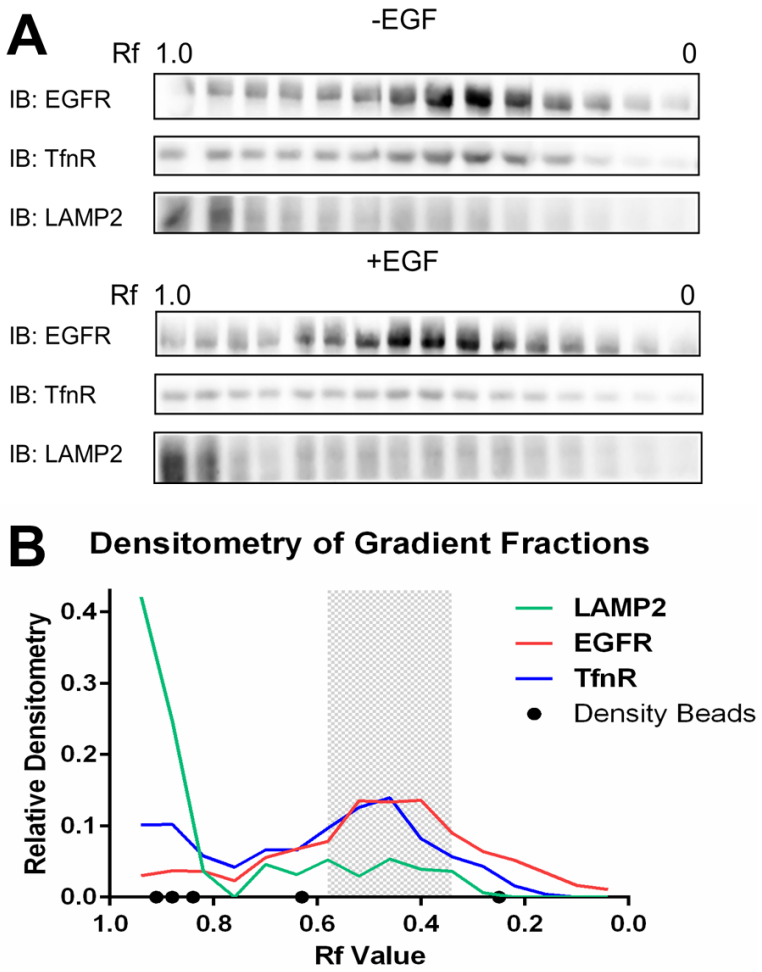

Figure 8. EGFR containing compartments migrate with DiI-positive and TfnR-positive compartments in Percoll gradients.

A. Equivalent volumes from every other fraction of Percoll gradients from HeLa cells treated with 10µM DiI, ±10ng/mL EGF were loaded and resolved on a 7.5% SDS-PAGE. Membranes were immunoblotted for EGFR, TfnR, and LAMP2. Immunoblots were imaged using enhanced chemiluminescence. **B.** Relative intensity of the +EGF immunoblots in **A**. calculated using Image J Software. Shaded region=Rf values where DiI fluorescence peaked in these samples. Circles on the x-axis represent density bead migration (Rf ~0.91=1.109g/mL, ~0.88=1.070g/mL, ~0.84=1.057g/mL, ~0.63=1.049g/mL, ~0.25=1.042g/mL).

rapid, gentle, and high-throughput technique for affinity purification that would maximize the likelihood of keeping endosomes intact. As an initial test of the efficiency of the antibodies, each was separately conjugated to Protein G Dynabeads and incubated with the PNS (from cells harvested by mechanical lysis) of HeLa cells treated ±10ng/mL EGF. Three antibodies were selected based on the manufacturer's rating for its IP capabilities and its protein target's association with early endosomes. The antibodies tested were targeted to: the C-terminus of EGFR (sc-03, Santa Cruz), TfnR (BD Biosciences), and EEA1 (Cell Signaling). The pass through (PT) is defined as the remaining sample that did not bind to the beads, and the elution (E) is defined as the sample that bound to the beads after incubation (**Figure 9**).

The sc-03 and TfnR antibodies did not effectively pull down EGFR, TfnR, or LAMP2. The EEA1 antibody pulled down EGFR in the +EGF treated cells but not in cells that were not treated with EGF. This is consistent with the notion that EGF:EGFR complexes are trafficked to the early endosome. Further, the EEA1 antibody yielded an approximately 50% pull down of TfnR, and virtually no pull down of LAMP2.

Based on these results, the EEA1 antibody was selected to immunoprecipitate early endosomes from Percoll gradient fractions. I hypothesized that affinity purification after density gradient fractionation would increase the yield of early endosomes, as they are enriched within the fractions, and decrease the amount of plasma membrane and late endosomal markers.

Serum starved HeLa cells were treated with 10μM DiI ±10ng/mL EGF for

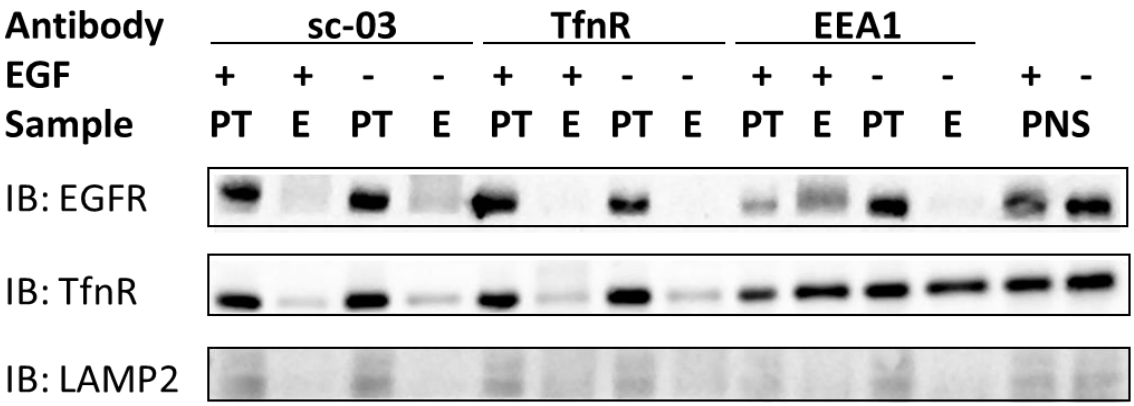

Figure 9. EEA1 mAb conjugated to Protein G Dynabeads precipitates early endosome-specific proteins. HeLa cells were serum starved for 2 hours and incubated ±10ng/mL EGF for 15'. Cells were harvested using mechanical lysis and the PNS was incubated at 4°C with rotation for one hour with the specified antibody pre-conjugated to Protein G Dynabeads. The beads were washed in buffer and eluted as outlined in the Materials and Methods. Samples were loaded based on percent of the total sample volume (12.5% PT, 1.25% PNS, 100% E) and proteins were resolved on a 7.5% SDS-PAGE and immunoblotted with the indicated primary antibody. PT=pass through; E=elution.

15'. Cells were then harvested using mechanical lysis and subjected to a 17% Percoll gradient. Gradients were fractionated, and DiI-positive peak fractions were pooled and immunoisolated with EEA1-conjugated Protein G Dynabeads. Samples were then immunoblotted using the indicated primary antibodies (**Figure 10**).

Approximately 50% of TfnR was pulled down, EGFR was only pulled down in the EGF treated sample, and LAMP2 did not appear to be precipitated. EEA1 was almost 100% pulled down in the elution, suggesting that using the EEA1 antibody for affinity purification is highly effective.

Plasma membrane and Endoplasmic Reticulum contamination of Percoll gradient fractions.

Although EGFR was precipitated in the EGF treated sample in Figures 9 and 10, there was still a significant fraction of EGFR remaining in the pass through. To determine if this excess EGFR in the pass through was an artifact of contamination from other organelles, particularly plasma membrane and endoplasmic reticulum (ER), immunoblot membranes from previous experiments were re-probed for both a plasma membrane specific protein (Na/K-ATPase) and an ER specific protein (Calnexin) (**Figure 11**).

Previously probed membranes from a Percoll gradient experiment (**Figure 8**) and an early endosome affinity purification experiment (original data not shown) were stripped and re-probed for Na/K-ATPase and Calnexin. Briefly, membranes were stripped of antibodies by rocking in stripping buffer (0.06M Tris, 0.07M SDS, 0.7% BME, pH 6.8) at 50°C for 30', followed by extensive washing in

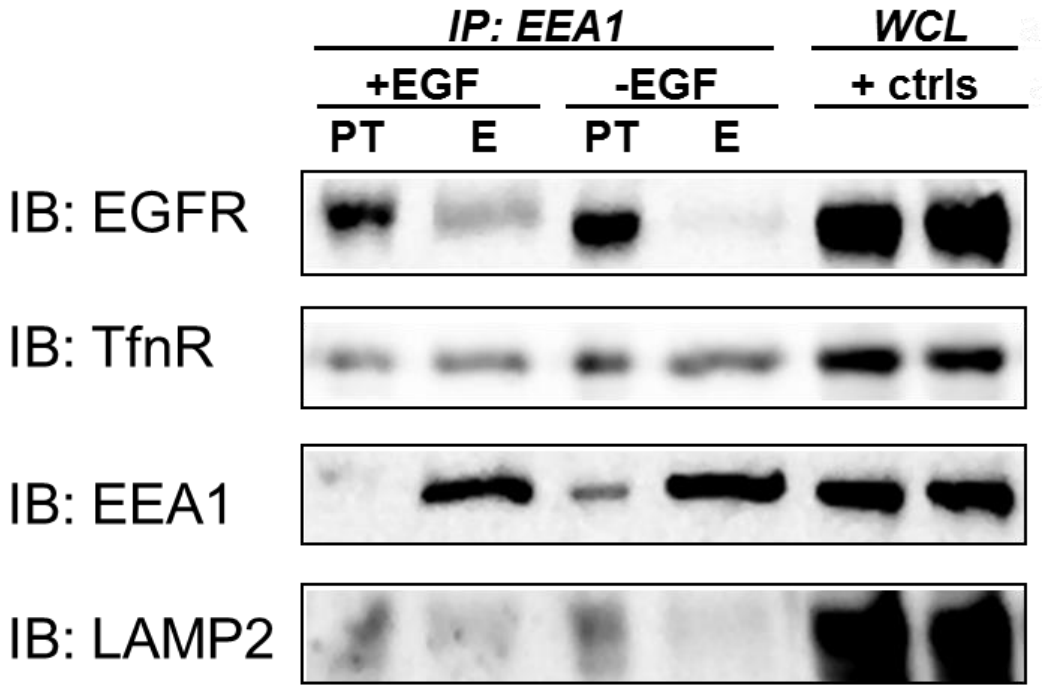

Figure 10. Affinity purification of early endosomes from Percoll gradient fractions. Percoll gradient fractions of HeLa cells treated with 10μM DiI ±10ng/mL EGF were pooled based on DiI fluorescence peaks. Affinity purification was performed as outlined in the Materials and Methods. Samples were loaded based on percent of total sample volume (5% PT, 50% E, & 2.5% WCL) and proteins were resolved on a 7.5% (EEA1 & TfnR) and 12% (EGFR & LAMP2) SDS-PAGE. Membranes were immunoblotted for early and late endosomal proteins using the indicated primary antibodies. WCL=whole cell lysates, PT=pass through, E=elution, IP=immunoprecipitation.

TBST. Membranes were blocked overnight in 5% milk at 4°C and immunoblotted using the indicated primary antibodies.

Calnexin was present in similar levels throughout the gradient, suggesting that ER contamination was present, but non-specific (**Figure 11A**). However, Na/K-ATPase protein levels had distinct peaks within the gradient. These fractions corresponded to the same peak fractions of DiI, TfnR, and EGFR from this experiment (see **Figure 8**) and are consistent with the notion that plasma membranes are enriched with early endosomes. Despite the presence of both ER and plasma membrane contamination in these peak fractions, Calnexin and Na/K-ATPase were not present in the elution after affinity purification of pooled Percoll gradient fractions (**Figure 11B**). However, since the plasma membrane contamination corresponded to the DiI peaks used to pool early endosomes, a discontinuous sucrose gradient was used to remove plasma membrane contamination from the PNS.

Discontinuous sucrose gradients separate plasma membrane and intracellular vesicles.

A discontinuous sucrose gradient, commonly referred to as a step gradient, was used to remove plasma membrane fragments from harvested cells. While Percoll gradients are a type of continuous gradient created during centrifugation, a step gradient is prepared prior to centrifugation and yields distinct fractions between each layer. For this particular sucrose gradient, a step gradient with 60%, 38%, and 5% sucrose was used to separate intracellular vesicles and plasma membrane.

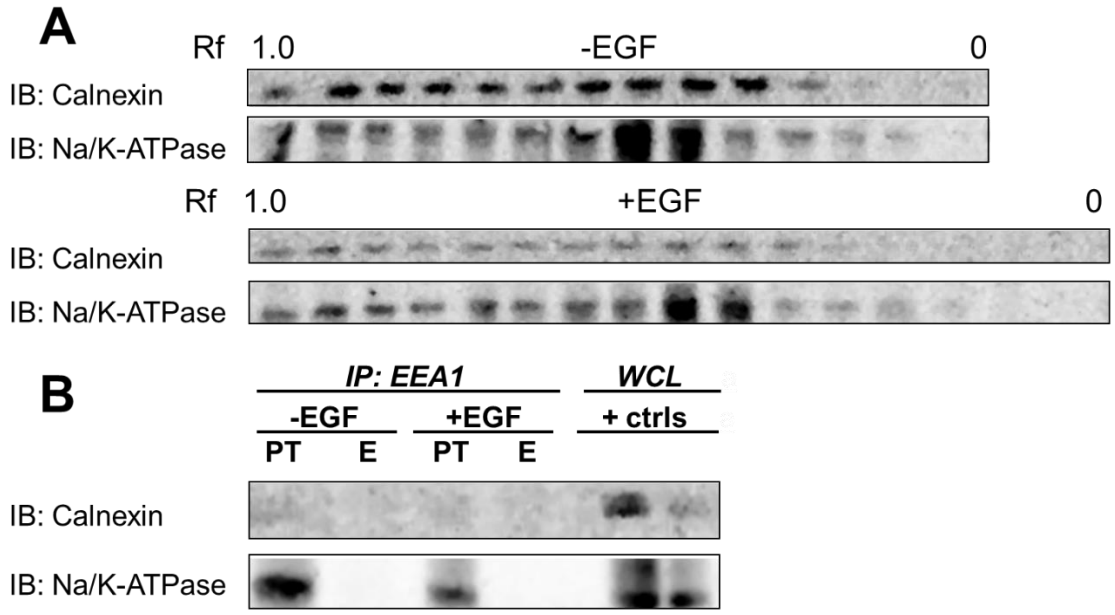

Figure 11. Plasma membrane and ER contamination of Percoll gradient fractions. A. Immunoblot membranes from a previous Percoll gradient were stripped and re-probed for Calnexin and Na/K-ATPase (Sigma). See Figure 8 for experimental conditions. **B.** Immunoblot membranes from a previous affinity purification experiment were stripped and re-probed for Calnexin and Na/K-ATPase (Sigma). Samples from the original immunoblot experiment were collected from HeLa cells lysed with mechanical lysis followed by Percoll gradient fractionation in Percoll buffer. Percoll gradient fractions were pooled based on DiI fluorescence and subjected to affinity purification with an EEA1 antibody conjugated to Protein G Dynabeads. Samples were loaded based on total sample volume (PT=5%, E=50%, WCL=1.25%) and proteins were resolved on a 10% SDS-PAGE.

Serum starved HeLa cells were incubated ±10ng/mL EGF for 15'. Cells were harvested via a form of osmotic lysis and scraping as outlined in the Materials and Methods. Lysates were then loaded on top of the sucrose gradient (i.e. on top of the 5% sucrose layer) and centrifuged. Fractions were collected at both the 5%/38% interface, referred to as "vesicles", and the 38%/60% interface, referred to as "membranes". The fractions were then immunoblotted for markers of early and late endosomes as well as plasma membrane (**Figure 12**).

EEA1 was detected exclusively in the vesicles fractions. EGFR protein concentrations were higher in the vesicles fraction compared to the membranes fraction in both EGF treated and untreated cells. There was also an increase in EGFR in the vesicles fraction after EGF treatment. TfnR and LAMP2 were both more concentrated in the vesicles fractions of both samples. Although Na/K-ATPase was also more concentrated in the vesicles fractions, there was still a distinct (albeit reduced) population of the protein within the membranes fraction.

Discontinuous sucrose gradient followed by continuous Percoll gradient fractionation yields discrete early endosome enrichment.

Due to the distinct separation of endosomal marker proteins into the vesicles fractions and plasma membrane marker proteins into the membranes fractions on the sucrose gradient, I wanted to know if loading the vesicles fractions onto a Percoll gradient would result in diminished plasma membrane contamination in the early endosome fractions. Serum starved HeLa cells were treated with 10ng/mL EGF for 15' immediately prior to harvest. Cells treated without EGF were used as a negative control. Cells were harvested and loaded

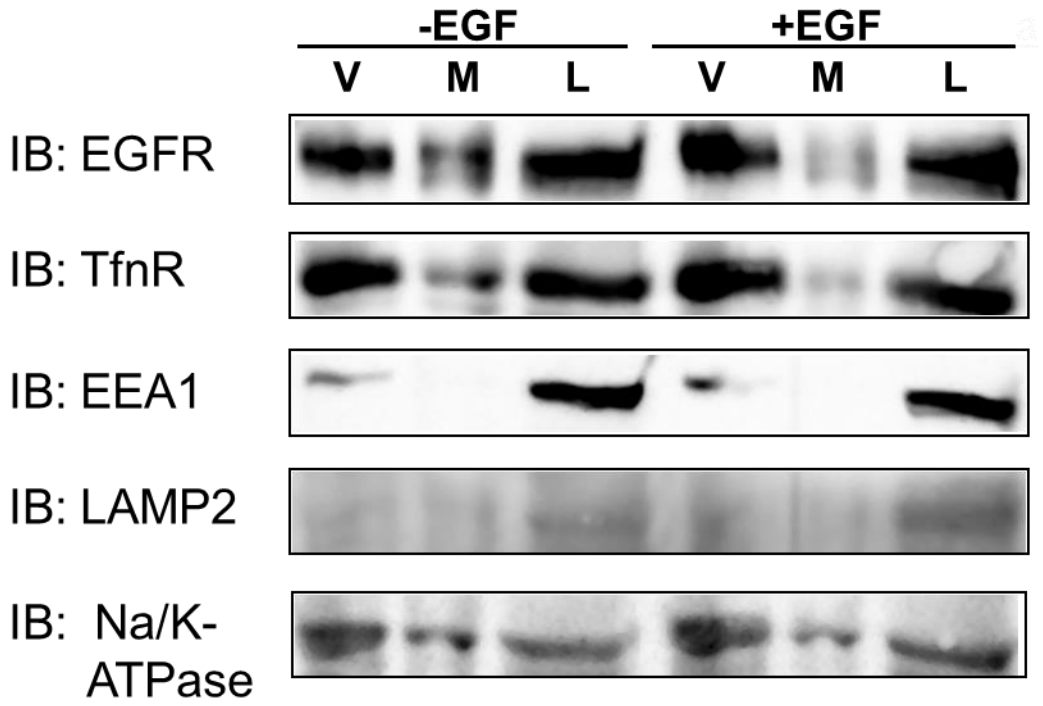

Figure 12. Discontinuous sucrose gradient separates plasma membrane and vesicle marker proteins. HeLa cells were serum starved for two hours, followed by 15' incubation with ±10ng/mL EGF. Cells were harvested and lysates were loaded on a discontinuous sucrose gradient as outlined in the Materials and Methods. Samples were loaded based on total sample volume (13% of V, M, & L) and resolved on a 7.5% SDS-PAGE. Membranes were immunoblotted using the indicated primary antibody. V=vesicles fraction (5%/38% interface), M=membranes fraction (38%/60% interface), L=lysates.

onto a pre-formed sucrose step gradient and centrifuged as outlined in the Materials and Methods. Vesicles fractions were collected and loaded onto a 17% Percoll gradient adjusted to a final concentration of 0.25M sucrose. Sucrose gradient and Percoll gradient fractions were immunoblotted for markers of early endosomes, late endosomes, and plasma membrane, as well as EGFR (**Figure 13)**.

The sucrose gradient separated approximately 55% of plasma membrane proteins into the membranes fraction, as evidenced by both Na/K-ATPase and EGFR (without EGF treatment) protein levels detected by immunoblot. Approximately 100% of EEA1 and LAMP2, positive controls for endosomes, were separated into the vesicles fraction. After loading the vesicles fractions onto Percoll gradients, EEA1, EGFR, and Na/K-ATPase protein levels all peaked in fractions with an Rf value of approximately 0.15-0.30. These Rf values corresponded with estimated vesicle densities of ~1.040g/mL. LAMP2 protein concentration peaked in fractions with an Rf of approximately 0.87, and was also present in low levels in the EGFR, EEA1, and Na/K-ATPase peak fractions.

Mass spectrometry analysis of purified early endosomes.

The ultimate goal of this thesis work is to isolate EGFR-containing early endosomes in order to analyze their protein make-up with mass spectrometry. Understanding the complete protein composition of early endosomes that contain activated EGFR will provide important insights into the influence of EGFR spatial regulation on downstream signaling. Mass spectrometry will provide an unbiased platform to determine what proteins—including signaling, trafficking, scaffolding,

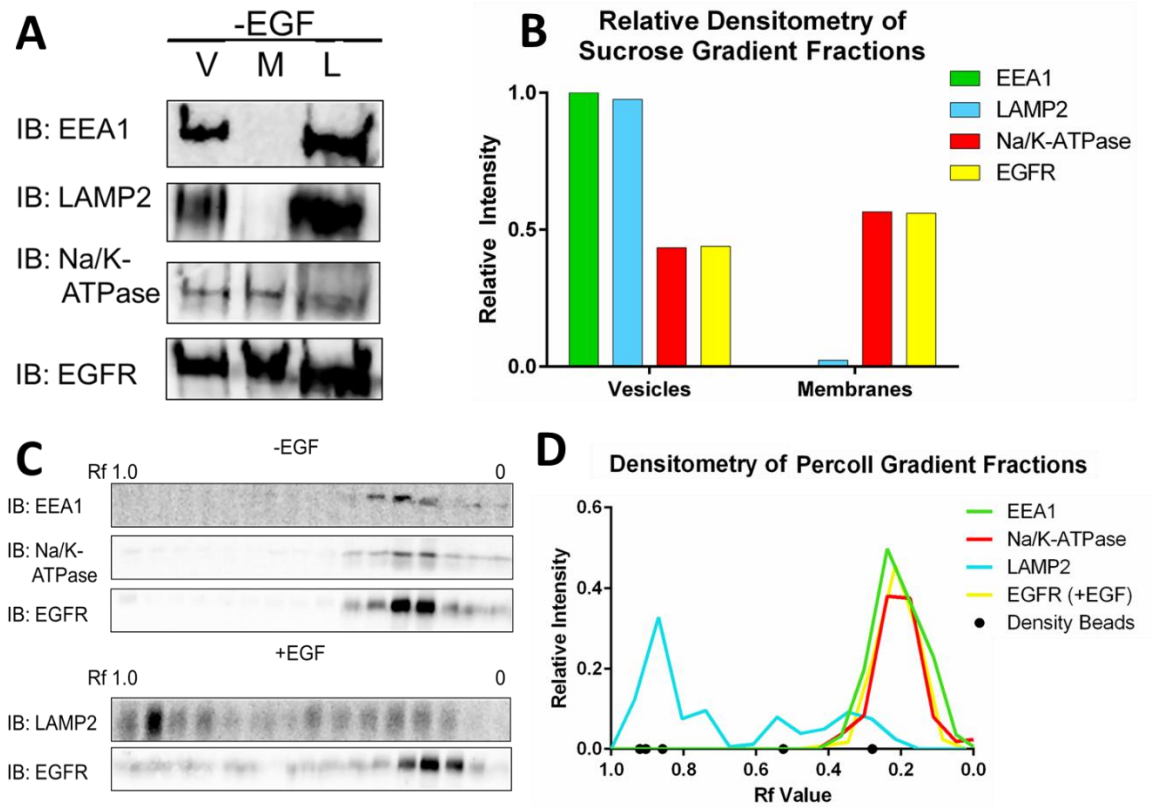

Figure 13. Sucrose gradient removal of plasma membrane reduces Percoll gradient fraction contamination and improves early endosome enrichment. HeLa cells were serum starved at 37°C for 2 hours and incubated with ±10ng/mL EGF for 15' immediately prior to harvest. The PNS was loaded onto a discontinuous sucrose gradient as outlined in the Materials and Methods. Collected "vesicles" fractions were subsequently loaded onto an isotonic 17% Percoll gradient as outlined in the Materials and Methods. **A.** Immunoblots of sucrose gradient fractions from –EGF treated HeLa cells. Samples were loaded 2% of total sample volume and resolved on a 7.5% SDS-PAGE, followed by immunoblotting with indicated primary antibodies. V=vesicles fraction, M=membranes fraction, L=lysates. **B.** Relative intensity of the immunoblots in **A**, estimated using Image J software. Measurements were normalized by setting

the total band intensity of the V and M fractions equal to one for each immunoblot. **C.** Immunoblots of sucrose gradient “vesicles” fractions fractionated on a Percoll gradient. Every other fraction was loaded (approximately 25% of the total volume per fraction) and resolved on a 7.5% SDS-PAGE and immunoblotted using the indicated primary antibodies. **D.** Relative intensity of the immunoblots in **C**, estimated using Image J software. Circles on the x-axis represent density bead migration (Rf ~0.92=1.109g/mL, ~0.90=1.070g/mL, ~0.86=1.057g/mL, ~0.53=1.049g/mL, ~0.28=1.042g/mL).

etc.—are associated with early endosomes that do and do not contain EGFR. In order to determine if our affinity purification process is robust and sensitive enough for mass spectrometry analysis, I performed an affinity purification using EEA1 mAb on PNS extracted from HeLa cells treated with and without EGF.

Serum starved HeLa cells were treated with or without 10ng/mL EGF for 15' immediately prior to harvest. Cells were harvested using the harvest method in the sucrose gradient protocol as outlined in Materials and Methods section with minor modifications. Briefly, a PNS was created by passaging cells through a syringe and 22G needle, followed by centrifugation at 800 x g to pellet nuclei and debris. The PNS from each sample was incubated with Protein G Dynabeads pre-conjugated with EEA1 mAb as outlined in the Materials and Methods. The beads elution and pass through were resolved on a 12% SDS-PAGE and the gel was Coomassie stained for protein detection. The elution, pass through, and PNS were also resolved on a separate 7.5% SDS-PAGE and immunoblotted for EEA1, LAMP2, Na/K-ATPase, and EGFR (**Figure 14**).

There was strong Coomassie protein staining in the pass through of both samples, and greatly reduced staining in the elution of both samples (**Figure 14A**). Although faint, there appears to be distinct protein bands in the +EGF elution that are not in the –EGF elution at molecular weights of approximately 200kD, 110kD, and 60kD. The majority of EEA1 is present in the elution of both –EGF and +EGF samples, and there is virtually no LAMP2 or Na/K-ATPase present in the elutions (**Figure 14B**). There is also an increase in EGFR in the elution of the EGF treated sample compared to the EGF untreated sample.

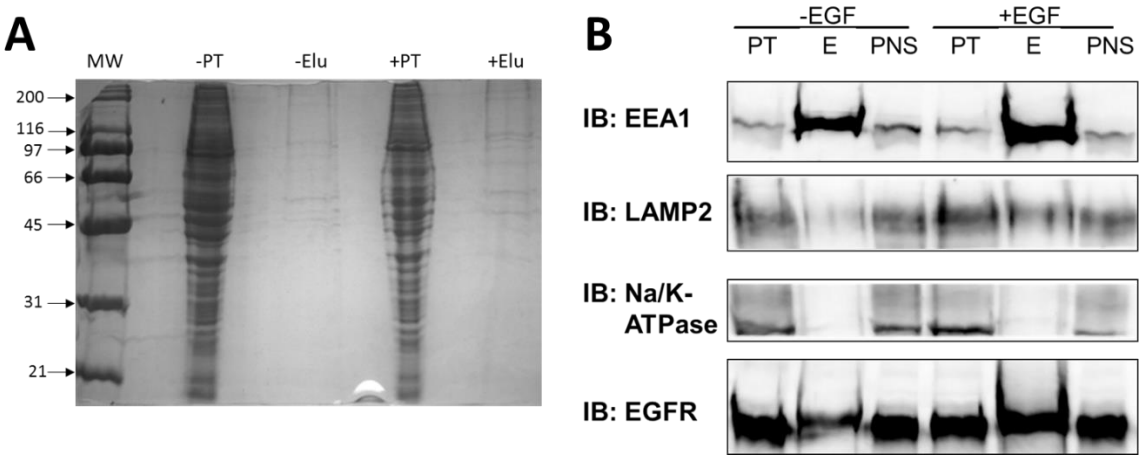

Figure 14. Affinity purification of early endosomes from HeLa cell PNS. Serum starved HeLa cells were treated ±10ng/mL EGF for 15' and harvested with hypotonic lysis in 1mM Tris, 2mM EDTA followed by scraping. Cells were passaged through a 22G needle 20 times and a PNS was created via centrifugation at 800 x g for 3'. The PNS was incubated with EEA1-bound Protein G Dynabeads for 1 hour at 4°C with rotation. The beads elution and pass through were collected and diluted in sample buffer for analysis. **A.** Samples were loaded by volume (2.5% PT, 50% E) and resolved on a 12% SDS-PAGE. The 12% gel was stained with Coomassie and imaged using a Fotodyne imaging system. Molecular weight marker proteins are labeled with their respective molecular weight in kilodaltons. –=no EGF, +=10ng/mL EGF treatment, PT=pass through, E=elution. **B.** Samples were loaded by volume (2.5% PT, 50% E, 1.25% PNS) and resolved on a 7.5% SDS-PAGE, followed by immunoblotting with the indicated primary antibodies.

The coomassie stained gel in Figure 14A was digested and proteins were extracted from both of the elution lanes for liquid chromatography/mass spectrometry (LCMS) analysis, as outlined in detail in the Materials and Methods. A very brief description of these results is depicted in Table 1. A total of 269 proteins were detected in the –EGF sample, and a total of 559 proteins were detected in the +EGF sample. Of the two samples, there was a convergence of 221 proteins present in both samples. As such, there were only 48 unique proteins in the –EGF sample, and 338 unique proteins in the +EGF sample (**Table 1**). The most abundant protein detected was TfnR, and the second most abundant protein detected was EEA1 in both samples.

I performed an initial analysis of the mass spectrometry data by searching for EGFR and its known signaling effectors detected within the +EGF sample. EGFR was detected in very low levels in this sample, however, none of the major effectors (direct interacting proteins as well as downstream effectors) of EGFR that have been previously shown to associate with EGFR in endosomes were detected in the sample (e.g. Shc, Grb2, mSOS, MEK, Src, etc.) (data not shown). However, there were notable differences in the total number of proteins involved in important cellular processes including proteasomal degradation, translation, signaling, and trafficking (**Tables 1 & 2**). Overall, there was an increase in the number of proteins detected that are associated with each of these processes in the +EGF sample compared to the –EGF sample. There were also several candidate proteins that were detected in the +EGF sample that were not detected in the –EGF sample that have been associated with EGFR signaling, including:

	-EGF Early Endosomes	+EGF Early Endosomes
Total # of Proteins	269	559
Total # of Unique Proteins	48	338
Kinases	7	11
Translation-Associated Proteins	29	59
Proteasome-Related Proteins	6	28

Table 1. Number of proteins detected by mass spectrometry of early endosomes isolated from HeLa cells treated with and without EGF.

-EGF	Σ# PSMs	+EGF	Σ# PSMs
RAB1a	11	RAB1a	32
		RAB1b	14
		RAB2a	18
RAB4a	4	RAB4a	39
RAB5a	27	RAB5a	52
RAB5b	12	RAB5b	26
RAB5c	59	RAB5c	86
		RAB6a	6
		RAB7a	25
RAB8a	21	RAB8a	39
RAB9a	4	RAB9a	25
RAB10	6	RAB10	29
RAB11a	12	RAB11a	27
		RAB13	21
RAB14	50	RAB14	104
RAB21	8	RAB21	26
		RAB34	4

- Golgi trafficking
- Recycling endosomes
- Early endosomes
- Late endosomes
- Assembly of tight junctions
- Integrin endocytosis
- Macropinocytosis

Table 2. RAB proteins detected by mass spectrometry of early endosomes isolated from HeLa cells treated with and without EGF.

Σ#PSMs=the total number of identified peptide sequences for the given protein

Protein Kinase C-delta (PKCΔ), Rac1, Cdc42, and CSN6 & 7.

There was an increase in expression of all of the RAB family proteins in the +EGF sample compared to the –EGF sample (**Table 2**). Several new RAB family members were detected in the +EGF sample. RAB6, RAB7, RAB13, and RAB34 were all present in the +EGF but not the –EGF samples, and are involved in Golgi to ER transport, late endosome maturation, the assembly of tight junctions, and macropinocytosis, respectively [69]. The number of proteins were counted in each group by searching for key words within the complete list of proteins detected per sample.

DISCUSSION

The overall purpose of this work was to develop and optimize a protocol for enriching and isolating early endosomes in order to study EGFR signaling from early endosomes in HeLa cells. This was achieved by testing and combining various subcellular fractionation techniques from published methods. To begin, fluorescence microscopy was used to support the biochemical basis of the proposed work: that EGFR internalizes into early endosomes upon ligand stimulation (Figure 4). Our results were consistent with previous reports that EGFR traffics into early endosomes 20' after ligand stimulation, after which the receptor traffics into late endosomes [67, 68].

Next, I wanted to test the ability of a commercially available lipophilic tracer dye, DiI, to label early endosomes. DiI is a fluorescent dye with a high affinity for all lipids that laterally diffuses into plasma membranes, and has been used as a membrane tracer dye for many years [70-72]. Because the process of subcellular fractionation is necessary for isolating endosomes, using a marker for the target organelle was highly desirable. The use of a fluorescent dye would provide a non-invasive and qualitative means for measuring the migration of early endosomes within a density gradient. The fluorescence of gradient fractions can be quickly measured on a plate reader to determine the fluorescence peak. These fractions could theoretically then be subjected to

affinity purification to isolate the DiI-labeled early endosomes. After determining the optimum DiI incubation conditions in HeLa cells (Figures 5 & 6), cells treated with DiI and EGF concomitantly were fractionated on a Percoll gradient and the fluorescence peak was measured (Figure 7). Biochemical assays confirmed that the DiI fluorescence peak in the gradient fractions co-localized with the peaks of EGFR and TfnR protein expression (Figure 8). These protein peaks likely represent early endosomes, as TfnR is constitutively recycled to early endosomes from the plasma membrane and EGFR internalizes with EGF treatment [46].

Unfortunately, EGFR also peaks in the same fractions without EGF treatment (Figure 8). This is consistent with the EGFR peak representing EGFR from the plasma membrane or other organelles. However, because the next step in organelle isolation is immunoisolation, I supposed that low level contaminants could be removed. Rf values of the EGFR peak fractions were calculated to contain vesicles with a density of ~1.045g/mL. According to Kornilova et al., early endosomes have a density of ~1.035-1.042g/mL, and late endosomes have a density of ~1.048-1.060g/mL [45]. While the DiI fluorescence/EGFR peak was not in the appropriate density range for either early or late endosomes, it was presumed possible that the DiI peak represented early endosomes in the gradient.

As stated, the next step of purifying early endosomes from the Percoll gradient peak fractions was to use affinity purification with early endosome specific markers. Three antibodies were tested for their ability to

immunoprecipitate early endosomes (Figure 9). An EEA1 monoclonal antibody was selected from this preliminary screen of antibodies, as its use resulted in the successful precipitation of TfnR and EGFR (only with EGF treatment), but not LAMP2. This strategy was then utilized to enrich early endosomes from the DiI fluorescence peaks of Percoll gradient fractions (Figure 10). This resulted in the immunoprecipitation of TfnR, EEA1, and EGFR (only with EGF treatment), but not LAMP2. However, it was noted that there was a significant amount of EGFR remaining in the pass through of both EGF treated and untreated samples. Although the affinity purification was effective, the majority of EGFR was not eluted with the early endosomes. This again suggests the presence of other organelle contamination within the Percoll gradient peak fractions.

If the use of DiI is a proper measure of early endosomes within the gradient, the pass through/pooled gradient fractions should be enriched in early endosomes. Due to the high efficiency of the affinity purification as measured by EEA1 in the elution, it is unlikely that the EGFR remaining in the pass through is associated with early endosomes. Further, there is a significant amount of EGFR in the –EGF sample pass through as well. The majority of EGFR in unstimulated cells is localized to the plasma membrane [3, 30, 73]. This suggests that there is significant plasma membrane contamination within the Percoll gradient peak fractions. This is also evidenced by the large amount of EGFR in the Percoll gradients of EGF untreated samples in Figure 8A. Because DiI labels all lipid membranes, it is also possible that the DiI peak that co-localizes with EGFR peaks in the immunoblots are representative of plasma membrane fragments in

the gradient, as well as other organelle contamination such as ER, rather than early endosomes. Due to the presence and pull down of early endosome-specific proteins from these peak fractions (Figure 10), it was concluded that early endosomes were present. However, early endosomes were not necessarily enriched in the pooled gradient fractions. Interestingly, TfnR protein levels also peaked in the same fractions as EGFR and DiI fluorescence (Figure 8B). However, TfnR is not an ideal early endosome marker as it is constitutively recycled to and from the plasma membrane. As such, the next step was to determine the extent of plasma membrane and ER contamination within the Percoll gradients, and if it interfered with the use of DiI as a marker of early endosomes within these gradients.

Calnexin was used as a positive control for ER, and Na/K-ATPase was used as a positive control for plasma membrane, as both of these proteins are specifically localized to those organelles, respectively [74, 75]. Diffuse and non-specific ER contamination was detected throughout the Percoll gradients, and a distinct peak of plasma membrane contamination was detected (Figure 11). Fortunately, though these protein markers were present in the pooled Percoll gradient fractions, they were not precipitated upon affinity purification. However, the peak of Na/K-ATPase contamination corresponded to the same fractions in which EGFR, TfnR, and DiI peaked in all previous experiments. Due to the strong plasma membrane contamination within the peak DiI fractions, the DiI staining of the plasma membrane is likely much stronger and masking that of the early endosomes in the gradient. If DiI is to be used as a marker for the fractions

containing early endosomes that will be pooled for affinity purification, plasma membrane contamination must be removed or at least reduced from the samples in order to reveal the true peak of DiI fluorescence from early endosomes.

A discontinuous sucrose gradient was used as a means to separate plasma membrane fragments from intracellular vesicles of HeLa cells. Although not all of the plasma membrane proteins were separated from the vesicles, approximately 25% of the Na/K-ATPase protein concentration was measured in the membranes fractions (Figure 12). This suggests that there is in fact separation of plasma membrane proteins using this protocol, although it is not a 100% enrichment. Further, EEA1 was found exclusively in the vesicles fractions, suggesting that there was no early endosome sample loss into the membranes fraction with this protocol. The DiI fluorescence of the vesicles and membranes fractions was also measured, and it was determined that the DiI fluorescence intensity was more directly correlated to plasma membranes than to early endosomes. As such, DiI was no longer used as a marker of early endosomes.

The next step was to test the use of the sucrose gradient prior to Percoll gradient fractionation to reduce plasma membrane contamination. The results from this experiment revealed a new profile of early endosome and plasma membrane proteins within the fractions compared to previous Percoll gradients (Figure 13). The Rf values associated with both plasma membrane and early endosome marker proteins were ~0.15-0.30, and the calculated densities of these fractions were ~1.040g/mL. A miscalculation resulted in using sucrose at a concentration of ~0.16M. The proper sucrose concentration of a Percoll gradient

should be isotonic, or 0.25M. This discrepancy accounts for the previous density calculations of early endosome protein-containing fractions that did not agree with the known densities of early endosomes [45]. As such, the use of a sucrose step gradient prior to Percoll gradient fractionation yields reduced plasma membrane contamination and a distribution of early endosome proteins that corresponds with proper early endosome density. These results also correlate with the distribution of EGFR in Percoll gradients within the literature [60, 76].

The final test performed in this thesis work was mass spectrometry on affinity purified early endosomes. Before performing mass spectrometry on early endosomes isolated using the finalized protocol, we first wanted to test the specificity and sensitivity of the affinity purification step. Post-nuclear supernatants collected from HeLa cells treated with and without EGF ligand were subjected to affinity purification, followed by LCMS analysis. While the overall goal of this research is to analyze EGFR signaling effectors in early endosomes, this initial experiment was a preliminary test of our abilities to analyze isolated early endosomes.

We were able to successfully enrich early endosomes with minimal contamination from other organelles (Figure 14). The most abundant proteins detected in both samples of endosomes were TfnR and EEA1, both of which were used as positive controls for early endosomes. Na/K-ATPase, Calnexin, and LAMP2 were used as negative controls. Na/K-ATPase was not detected in either sample, however, LAMP2 and Calnexin were detected in the +EGF sample, albeit at very low abundance. This suggests there was no plasma

membrane contamination in the enriched samples, however, there was a small amount of late endosome and ER contamination in the +EGF sample. Encouragingly, EGFR was detected only in the +EGF sample, supporting the fact that EGF treatment leads to EGFR endocytosis into early endosomes. However, the amount of EGFR detected was very low, and specific effector and scaffold proteins that are known to associate with EGFR in early endosomes upon ligand stimulation—Shc, Grb2, mSOS, MEK2, etc.—were not detected in this sample.

While several specific EGFR effectors that are known to associate with EGFR in early endosomes were not detected, several other proteins that are involved in EGFR signaling were detected in the +EGF sample. For example, PKCΔ was present in the EGFR-containing early endosomes. PKC has been shown to inhibit EGFR signaling via MAPK [77]. Rac1—a Rho family GTPase that is downstream of EGFR—and CDC42—a cell cycle regulator that is also downstream of EGFR—were detected in the +EGF sample. Both Rac1 and CDC42 are positive regulators of EGFR signaling that are involved in cell motility and cell cycle progression [78]. CSN6 and CSN7 are subunits of the COP9 signalosome complex, which is involved in the ubiquitin-proteasome pathway. Both of these subunits were also detected in the +EGF sample. CSN6 was recently linked to the progression of colorectal cancer via a CSN6-ERK2 interaction [79].

We detected in the EGF-treated sample an increase in the number of proteins involved in several important cellular processes, including translation, proteasomal degradation, and signaling (Table 1). There was also an increase in

the number and type of RAB proteins detected in the +EGF sample compared to the untreated sample (Table 2). Interestingly, there was an increase in the number and type of RABs associated with late endosomes in the +EGF sample, suggesting that the early endosomes were in the process of maturing into late endosomes [80]. This is consistent with EGFR being trafficked into “dynamic” early endosomes, which are associated with microtubules and rapidly mature into late endosomes [46].

In this thesis work, I was able to modify published protocols to enrich early endosomes. We were also able to successfully isolate endosomes from HeLa cells treated with and without EGF ligand and use mass spectrometry to analyze their protein make-up.

SUMMARY AND CONCLUSIONS

The overarching goal of the work performed in this thesis was to develop an early endosome isolation protocol in order study EGFR effector signaling from this compartment. Current methods for endosome isolation in the literature were utilized as a starting point for this work. Several variations of subcellular fractionation were tested and optimized to achieve a final protocol that results in discrete enrichment of early endosomes with minimal contamination from other organelles. The affinity purification step was also tested and shown to be robust and highly specific for the purification of early endosomes. This step of the procedure was also sensitive enough for proteomics analysis via LCMS of the purified compartments. This analysis revealed distinct differences in the protein composition of endosomes isolated from cells stimulated with and without EGF. In the future, mass spectrometry analysis will be performed on early endosomes isolated using the finalized enrichment protocol in order to study the effects of EGF treatment on early endosome protein composition and EGFR downstream signaling.

Strengths of This Work

A major strength of this work is in its use of HeLa cells. This cell line expresses EGFR at levels similar to what has been measured in normal human epithelial tissues (~50,000 EGFRs per cell), allowing us to correlate findings in these cells with the physiologic activity of EGFR. To this same end, physiologic/

low levels of EGF ligand were used to stimulate EGFR activation (10ng/mL). Further, HeLa cells grow rapidly, permitting the generation of large populations of cells, if they are required for subsequent endosome enrichment.

Another important strength of this work is that we were able to isolate early endosomes without introducing exogenous factors into the cells. While it is generally acceptable to use cell lines that have been genetically altered to enhance detection and capture of the target protein of interest, we were able to enrich early endosomes without the use of epitope tags or transfection reagents. As a result of this, the isolated early endosomes are physiologically relevant. The proteomics data obtained from these compartments can thus be taken at face value, without concerns about non-physiologic changes in their structure or function.

Limitations of This Work

One of the few limitations of this thesis work is that it was performed using an *in vitro* cell line. A major drawback to using tissue culture rather than animals is that the results cannot be correlated to the whole organism level. *In vitro* cell lines lack the complex interplay of signaling that occurs within organisms and even tissues. Tissue culture models are ideal for preliminary studies, however, isolating early endosomes from an animal would provide an even more physiologically relevant analysis of EGFR signaling from these compartments.

Future Directions

One of the most important experiments to perform moving forward will be to affinity purify early endosomes enriched using the finalized protocol with both

sucrose and Percoll gradients. This should provide a more robust precipitation of early endosomes and increased sensitivity for the detection of EGFR effectors with mass spectrometry. In order to draw conclusions from the mass spectrometry analysis, we would need to repeat these experiments and determine if the results obtained are reproducible. Once this has been completed, we will then be able to determine the extent of the effects of EGFR spatial regulation on downstream signaling. Not only will we be able to determine what effectors co-localize with EGFR in early endosomes upon ligand activation, but we will also use this information to test how the subcellular localization of effectors changes downstream signaling. Using stably transfected cell lines, we will be able to change the cellular localization of EGFR effectors and determine the physiological role that effector localization plays in EGFR signaling outcomes.

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CURRICULUM VITAE

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EDUCATION

University of Louisville, Louisville, KY 2014 - Present
Pharmacology and Toxicology PhD student
GPA: 3.9

- Recipient of IPIBS Fellowship 2014-2016
- Recipient of Graduate Student Council Travel Grant 2015
- Class Representative 2015-2016

Anderson University, Anderson, IN 2008 - 2012
B.A. in Biology & Psychology *(double major)*
GPA: 3.4

- Dean's List 2008-2012
- "Distinguished Student Scholarship" Recipient 2008-2012

PRESENTATIONS

Oral Research Presentation at University of Louisville 03/19/2015
"EGFR Signaling from the Early Endosome"

Poster Presentation at University of Louisville 10/27/2015
Research! Louisville Conference
"A Non-Invasive Strategy for Enriching Early Endosomes to Examine EGFR Signaling"

Poster Presentation at ASCB National Meeting 12/13/2015
"A Non-Invasive Strategy for Enriching Early Endosomes to Examine EGFR Signaling"

RESEARCH EXPERIENCE

University of Louisville, Louisville, KY
Graduate Fellow 08/2014 - Present

- Study the endocytic trafficking and signaling of EGFR
- Designed and optimized a protocol for isolating early endosomes
- Indirect immunofluorescence & fluorescence microscopy used to visualize EGFR trafficking

- Tissue culture of HeLa, hTCEpi, and CHO cells
- Immunoprecipitation/affinity purification of early endosomes
- Western blotting & enhanced chemiluminescence
- Extensive experience with various methods of density gradient centrifugation
- Experience with affinity chromatography, differential centrifugation, flow cytometry, adenovirus infection and transfection

PGXL Laboratories, Louisville, KY 12/2012 - 07/2014
Clinical Laboratory Technician

- DNA isolation, quantification, and Real Time PCR analysis of genes directly involved in drug metabolisms including SNPs, RFLPs, and CNVs
- Developed protocols for training new employees
- Independently operated machines involved in liquid handling, DNA isolation, and Real Time PCR
- Researched genes including COMT, MLH1, MSH2, and PMS2 for new assay development
- Attended seminars and participated in training for NextGen Sequencing
- Assisted in preparations for CAP certification of the laboratory
- Completed continuing education courses as required each year by HIPAA

Anderson University, Anderson, IN
Research Assistant 05/2011 - 08/2011

- Independently assisted Dr. Kimberly Lyle-Ippolito in research on the effects of fat content of diet on the bacterial composition of mouse intestine
- Responsibilities included caring for 100+ mice, weighing and observing mice, charting differences in weights, collecting fecal samples and isolating bacterial DNA, using agarose gel electrophoresis to visualize DNA and PCR to amplify DNA obtained

GRANTS/FELLOWSHIPS

Submitted
NIH (F31) 1F31GM122256-01
Gosney (PI) Project Period: 09/01/2016-TBD
Title: EGFR:Effector Communication from the Early Endosome
Goal: To characterize the effects of EGFR spatial regulation on downstream signaling via the endocytic pathway.

ABSTRACTS

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