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Rab8 Mediates TRPV4 Vesicle Trafficking to the Plasma Membrane in HGF-Stimulated MDCK Cells

Hillary Jean Haws

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

Marc D. Hansen, Chair
Juan A. Arroyo
Jeffery R. Barrow
Joshua L. Andersen

Department of Physiology and Developmental Biology

Brigham Young University

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ABSTRACT

Rab8 Mediates TRPV4 Vesicle Trafficking to the Plasma Membrane in
HGF-stimulated MDCK Cells

Hillary Jean Haws
Department of Physiology and Developmental Biology, BYU
Master of Science

Epithelial to mesenchymal transition (EMT) is a process whereby epithelial cells, which act collectively through robust cell-cell interactions, take on mesenchymal characteristics, breaking cell-cell junctions to become solitary, invasive and motile. Our previous results show that a transient increase in calcium influxes through TRP channels at the plasma membrane is required for hepatocyte growth factor (HGF)- stimulated EMT. Since this transient increase requires an intact microtubule cytoskeleton, we propose that HGF stimulation results in the mobilization of calcium channels to the plasma membrane from an intracellular compartment via microtubule-dependent vesicle trafficking. Through immunofluorescence, we show that prior to HGF treatment, TRPV4 localizes to a perinuclear compartment that stains for rab11. After HGF stimulation, this colocalization is reduced and TRPV4 localizes more precisely to fibrous structures. Similarly, rab8 staining is seen throughout the cytoplasm prior to HGF treatment, but localizes primarily to tubular structures after HGF stimulation. This is indicative of endocytic recycling of TRPV4 via rab8. MDCK cells null for rab8 activator, rabin8, were developed using the CRISPR system and then analyzed for changes in epithelial scattering and trafficking of ion channels to the plasma membrane following HGF stimulation. Rabin8 KO cells had a decrease in TRPV4 vesicle trafficking. While rabin8 KO cells did undergo HGF-induced spreading and some disassembly of cell-cell junctions, they lost all motility. Also, HGF-treated rabin8 KO cells had similar calcium levels to untreated WT cells, which had fewer calcium spikes than HGF-treated WT cells. ERK1/2, a known downstream effector of HGF stimulation, has been shown to activate rabin8, and so we tested the effect of an ERK1/2 inhibitor on HGF-induced WT cells as well. These cells had decreased TRPV4 vesicle trafficking and loss of motility, similar to rabin8 KO cells, indicating that ERK1/2 may act upstream of rabin8 and rab8 in this pathway. Our results indicate that TRPV4 undergoes endocytic recycling via rab8 to the cell surface to allow a necessary calcium influx within one hour of HGF stimulation in MDCK cells, leading to EMT.

Keywords: Rab8, calcium, TRPV4

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CHAPTER 1: Introduction

EMT

An epithelial-mesenchymal transition (EMT) is the process whereby an epithelial cell changes its phenotypical properties to detach from the tissue and become more invasive and migratory (Kalluri & Weinberg, 2009). EMT occurs in development, as seen in neural crest cell migration through the mesenchyme to form the peripheral nervous system, bone and cartilage, and melanocytes, among other structures (Kerosuo & Bronner-Fraser, 2012). It also occurs in cancer during metastasis, where cells dissociate from surrounding tissues and from the basement membrane to enter the bloodstream and travel to other parts of the body (Kalluri & Weinberg, 2009).

In tissue culture, epithelial cells can be induced to undergo a process, often termed epithelial scattering, which is similar to EMT. Epithelial scattering was first observed in cultured epithelial MDCK cells treated with so-called scatter factor (Stoker & Perryman, 1985), a growth factor that later turned out to be hepatocyte growth factor, or HGF (Furlong, Takehara, Taylor, Nakamura, & Rubin, 1991; Montesano, Matsumoto, Nakamura, & Orci; Naldini et al., 1991; K. M. Weidner et al., 1991). HGF stimulation of MDCK cells results in a dramatic change in cell morphology and cell behavior that ultimately results in cells wrenching apart their cell-cell adhesions in dramatic fashion and "scattering" about the culture surface (LI et al., 1992; Sperry et al., 2010; Stoker & Perryman, 1985).

Two hypotheses have been proposed for how epithelial cells scatter. In the first, cell-cell junctions are actively disassembled, which allows for rupturing of adhesions between cells. This is supported by the observation that during EMT cadherin expression changes (Grotegut, von Schweinitz, Christofori, & Lehembre, 2006) and actin and actin-regulatory protein organization

is altered at cell-cell adhesions (Sperry et al., 2010). In the second hypothesis, increased contractility causes tearing at the cell-cell junctions and cells physically pull apart from one another (de Rooij, Kerstens, Danuser, Schwartz, & Waterman-Storer, 2005).

Previously, our lab reported that transient treatment of MDCK cells with blebbistatin leads to cell dissociations identical to those observed in MDCK cells treated with HGF (Hoj et al., 2014). This indicates that a loss and recovery of cell contractility, dependent on myosin II activation, is sufficient to drive cell scattering; that is, cellular tension forces cause rupturing of cell-cell adhesion, and the latter theory is correct. However, the reality is that molecular changes to the architecture of cell-cell junctions accompany force-driven detachment of cell-cell adhesion. In fact, it may be impossible to distinguish these events, as linkage of cadherin-based adhesions to actin appears to be tension dependent (Borghi et al., 2012; Yonemura, Wada, Watanabe, Nagafuchi, & Shibata, 2010).

Cell scattering in HGF-stimulated MDCK cells is characterized by several events. Colonies spread and roughly double in size in the first two hours after HGF stimulation (Sperry et al., 2010), and then begin undergoing several changes in morphology, not always in the same order, but with increasing frequency as time goes on. Cell shape changes from cobblestone to crescent, intracolony spaces form as cell-cell junctions rupture, and retraction fibers form as cells separate (Sperry et al., 2010). Initiation of EMT in WT MDCK cells occurs approximately three hours after HGF stimulation (Sperry et al., 2010).

Because of the connection between EMT and cancer metastasis, it has been the subject of extensive study over the past several years. Researchers have identified several EMT-inducing signals in the stroma of tumors, including HGF, EGF, and TGF-β (Kalluri & Weinberg, 2009), and characterized the role of a number of downstream signaling effectors. However, there is still

much researchers do not understand about the biochemical pathways that occur during EMT. If down-stream processes of EMT-inducing signals could be mapped, it would be possible to identify possible therapeutic targets which would halt metastasis and improve outcomes for cancer patients.

Calcium Influx Occurs After Growth Factor Stimulation and is Necessary for EMT

In 1992, a report showed hepatocyte growth factor (HGF) could induce a rapid increase in cytosolic free calcium in hepatocyte cells. This increase in intracellular calcium was dependent on the presence of extracellular calcium, as well as tyrosine kinase activity (Baffy, Yang, Michalopoulos, & Williamson, 1992). Another group confirmed that after HGF stimulation, intracellular calcium waves originated from a region near the cell membrane and propagated across the cell (Kawanishi et al., 1995). Due to the role HGF plays in EMT and cancer, researchers have since attempted to discover the necessity of this calcium influx to growth factor signaling and EMT.

Our lab's recent study reports a highly reproducible calcium influx pattern in epithelial cells after growth factor stimulation but prior to EMT. In MDCK cells treated with hepatocyte growth factor, two peaks of high frequency calcium influxes can be detected at the plasma membrane via electrophysiological recording (Langford, Keyes, & Hansen, 2012). Although the exact timing of the first peak after stimulation varies to some degree, as does the extent of increase in calcium channel opening events, the timing of the second peak compared to the first and the relative amplitude of the peaks are highly reproducible (Langford et al., 2012).

The research above by Langford et al follows a small cohort of prior studies assessing the role of calcium channels and of calcium signaling in both EMT and cancer progression. In MDA-MB-468 cells, cytosolic calcium levels quickly rise after epidermal growth factor (EGF)

stimulation, then gradually level off in under 800 seconds (Davis et al., 2014). This results in increased vimentin expression (Davis et al., 2014), a widely used marker of EMT. Treatment of cells with a cytosolic calcium chelator, BAPTA-AM, prior to EGF stimulation prevented a rise in vimentin expression (Davis et al., 2014), indicating calcium is necessary for EGF-induced EMT in the cells.

EMT in embryonic development also seems to depend on a rise in cytosolic calcium levels. In Xenopus embryos, Wnt11-R expression in the neural crest and certain somite cell populations is essential for EMT, allowing the cells to migrate into the dorsal fin matrix. Loss of Wnt11-R results in dorsal fin defects and fewer fin core cells (Garriock & Krieg, 2007). However, inducing calcium signaling with thapsigargin, an agonist of calcium influxes, alleviates these defects, and increases fin core cells to near-control levels (Garriock & Krieg, 2007).

Taken together, these studies indicate that EMT depends on higher cytosolic calcium levels in many cell types, including cancerous and non-cancerous tissues. To date, no tests have been done to show whether calcium is sufficient to drive EMT. Further, little is known about the molecular mechanism whereby calcium influxes are enhanced during EMT.

Transient receptor potential (TRP) channels are permeable to cations, and some are highly calcium selective (Nilius & Owsianik, 2011). Various TRP channels are involved in cancer progression that recapitulates EMT. TRPC6 has been shown to have a role in HGF-induced cellular processes. HGF normally stimulates proliferation of adenocarcinoma cells, but knocking down TRPC6 abolishes this proliferation (Song et al., 2013; Y. Wang et al., 2010). TRPC6 plays the same role in prostate cancer cells, and allows an HGF-induced calcium influx (Y. Wang et al., 2010). TRPC6 inhibitors prevent HGF-induced proliferation, migration, and morphogenesis of HK2 cells (Rampino et al., 2007). In DU145 and PC3 prostate cancer cell lines

(Y. Wang et al., 2010) and in the ACHN renal adenocarcinoma cell line (Song et al., 2013), TRPC6 seems necessary for the G2/M phase transition. Additionally, Langford et al concluded that TRPC6 is specifically required for HGF-induced scattering in MDCK cells, though calcium fluxes through TRPV4 and other channels are all increased at the plasma membrane following HGF stimulation (Langford et al., 2012).

Other growth factors and ligands have been shown to act through other TRP channels. In MDA-MB-468 cells, EGF normally induces a calcium influx resulting in an increase in the EMT marker, vimentin. However, knocking out TRPM7 in these cells decreases vimentin levels after EGF stimulation (Davis et al., 2014). In BTEC cells, activation of TRPV4 channels with its agonist, 4aPDD, promotes cell migration. Furthermore, silencing TRPV4 abolishes arachidonic acid-induced migration in these same cells (Fiorio Pla et al., 2012). Expressing polycystin-1 (PC1), another calcium-conducting channel, in MDCK cells leads to cytoskeletal rearrangement, cell migration, and scattering (Boca et al., 2007), all of which are associated with EMT.

Contribution of Vesicle Trafficking

The patch clamp experiment by Langford et al gives evidence that the rise in calcium after HGF stimulation is most likely due to calcium influx at the cell membrane rather than a release of calcium from the endoplasmic reticulum. How increases in channel openings at the plasma membrane are achieved could be due to several factors: first, calcium-conducting channels could stay open longer; second, calcium-conducting channels could open more often; third, vesicles with calcium-conducting channels could be trafficked to the cell surface, increasing the number of channels inserted in the plasma membrane; or, finally, vesicle trafficking of channels away from the cell surface could decrease, resulting in a build-up of channels at the plasma membrane. Experiments have shown that the most likely cause is due to

an increase of calcium channels at the plasma membrane, as described below.

Conductance magnitude and duration are not affected by HGF treatment, only the frequency of ion influxes (Langford et al., 2012). Either the number of channels in the membrane is dramatically increased or each channel is opening with far greater frequency. Since calcium influxes increase simultaneously through multiple channel types (both TRPC6 and TRPV4 in MDCK cells), a mechanism whereby channels of all types are increased in number at the plasma membrane, possibly by delivery in vesicles, is simpler to fathom. TRP ion channels can be transported to the cell surface via microtubule-dependent vesicle trafficking (Kennedy et al. 2010), and a microtubule polymerization inhibitor effectively blocks HGF-stimulated calcium influxes (Langford et al., 2012). EGF has been shown to induce PI(3)K-mediated translocation of TRPC5 channels to the cell surface (Bezzerides, Ramsey, Kotecha, Greka, & Clapham, 2004). EGF also induces Src family tyrosine kinase-mediated phosphorylation of TRPC4, leading to increased translocation and surface expression of TRPC4, as well as calcium influx (Odell, Scott, & Van Helden, 2005). This indicates that growth factors stimulate vesicle trafficking of TRP channels to the plasma membrane to allow a calcium influx.

Vesicle trafficking is an essential part of cellular signaling. Vesicles carry cargoes from one membrane to another throughout the cell and each specific vesicle transport route uses its own specific and particular machinery. Generally speaking, deposition of cargo proteins in the plasma membrane, such as calcium channels, can occur through exocytosis of newly synthesized proteins or through recycling of proteins between the plasma membrane and internal storage compartments (endosomes). Given the immediacy with which calcium fluxes are observed after initiating EMT, our hypothesis is that vesicle recycling pathways control cell surface expression of calcium channels. We reason that it would simply take too long to initiate gene transcription

and protein synthesis, and then deliver proteins to the plasma membrane so rapidly after HGF stimulation.

Identification of the proteins involved in vesicle trafficking of TRP channels and their activators could explain the pathway through which growth factors induce calcium influx. Rab GTPases, which aid in cargo sorting, uncoating, motility, and docking of vesicles (Stenmark, 2009), must play some role in TRP channel trafficking. However, the relationship between TRP channels and rab GTPases has not been extensively studied. One report shows that rab activation in epithelial cells leads to an increase in TRPV5 and TRPV6 expression at the cell membrane (van de Graaf, Chang, Mensenkamp, Hoenderop, & Bindels, 2006). Rab11 interacts with TRPV5 and TRPV6 while in its GDP-bound form, yet the GTP-bound form is necessary to recycle these channels to the cell membrane (van de Graaf et al., 2006). It is possible rab11 binds to the two calcium channels when inactive, then, once docked on the vesicle, releases the cargo as it activates and then transports the vesicle to the plasma membrane. However, another possibility is that rab11 activates rab8 through rabin8, and then passes the vesicle to rab8 for transportation. Rab11 is known to activate rab8 through the guanine-nucleotide exchange factor, rabin8. Rabin8 has been shown to release GDP from rab8 at a much higher rate when rab11 is present than when it is not (Knödler et al., 2010), and in lumenal cells the three colocalize when rab11 is active (Bryant et al., 2010). Rab11 is a marker for the endocytic recycling compartment (Grant & Donaldson, 2009; Sonnichsen 2000), while rab8 transports vesicles and tubules from this compartment to the cell membrane (Grant & Donaldson, 2009; Weigert 2004). It seems probable, therefore, that the rab11-rabin8-rab8 cascade allows for a direct turnover of TRPV5 and TRPV6 from the rab11-defined endocytic recycling compartment to rab8-dependent tubules once rab11 is activated.

Once TRP channels are endocytosed from the plasma membrane, there are two paths they can take back to the plasma membrane. They could undergo fast endocytic recycling, wherein TRP channels would return immediately to the cell surface in vesicles decorated with rab4, or they could undergo slow endocytic recycling and the TRP channels would first move to the endocytic recycling compartment (ERC), marked by the presence of rab11. The fact that TRP channels have been shown to interact with this GTPase strengthens the already likely hypothesis that TRP channels are transitioning through this compartment as part of their trafficking through the cell. Rab8 transports vesicles and tubules from this compartment to the cell membrane (Grant & Donaldson, 2009). Thus, internalized TRP channels likely accumulate in the rab11 endocytic recycling compartment and then return to the plasma membrane via a rab8-dependent trafficking step.

Rab11, rab8, and rab6 have been shown to specifically immunoprecipitate with PC1 (Ward et al., 2011), which, as discussed above, also conducts calcium ions. Rab6 controls the budding and motility of exocytic vesicles (Peranen, 2011), and mediates the recruitment of rab8, which controls docking and fusion of the vesicles (Grigoriev et al.). Thus rab11, rab8, and rab6 may pass off the duties of trafficking this calcium channel. Silencing rab8 also prevents trafficking of PC1 to the cell surface (Ward et al., 2011).

Rab8 is further implicated as a participant in EMT due to evidence that it is activated by ERK1/2. Researchers have found that active ERK1/2 causes phosphorylation of rabin8, thereby taking it out of an autoinhibitory conformation. Rabin8 then dissociates from rab11 and activates rab8 (J. Wang et al., 2015). This mechanism is slightly different from the traditional rab11-rabin8-rab8 pathway. As ERK1/2 is a known downstream effector of many growth factors (Frémin & Meloche, 2010), including HGF, this discovery shows that rab8 may be activated by

growth factor stimulation. This strengthens the argument that growth factor receptor activation may induce slow-endocytic recycling, controlled by rab8, which might then bring TRP channels to the cell surface for subsequent calcium influx and EMT.

Rab4 is involved in the fast recycling pathway. Although there is not much evidence that rab4 aids in the vesicle trafficking of calcium channels, it does seem to be involved in EMT processes. For example, Furin4 is recycled to the cell surface via rab4 in HT-1080 cells under hypoxic conditions (Arsenault, Lucien, & Dubois, 2012). Furin processes and activates MT1-MMP in cancer cells to allow for cell invasion and breaking through basement membranes, which is important for cancer metastasis.

Rab5a promotes rab4-mediated rapid recycling of MT1-MMP and HGF-induced extracellular matrix degradation (Frittoli et al., 2014). Formation of invadosomes also requires rab4-mediated rapid recycling of MT1-MMP (Frittoli et al., 2014). Interestingly, researchers have also rab8a has also been shown to specifically facilitate MT1-MMP trafficking in macrophages and breast cancer cells and facilitate matrix degradation and invasion in a rab11-independent manner (Bravo-Cordero et al., 2007; Wiesner, El Azzouzi, & Linder, 2013). Thus, both rab4 and rab8 seem to be involved in trafficking MT1-MMP, and may traffic other EMT-essential proteins in parallel in HGF-induced cells.

However, the evidence that rab8 aids in the growth factor-induced trafficking of TRP channels is still greater. My hypothesis is that HGF triggers the activation of rab8, which in turn mediates vesicular transport of calcium channels such as TRPV4 and TRPC6 to the cell surface. The calcium channels allow calcium influxes that are required for EMT.

CHAPTER 2: Results

Rab8 and Rab4 Localization After HGF Stimulation

Our hypothesis is that TRPV4 is recycled through the slow endocytic recycling pathway, and rab8 is activated by HGF stimulation. Due to previous reports, we did not think it likely that TRPV4 undergoes fast recycling through activation of rab4. To prove this, localization of both rab-GTPases before and after HGF stimulation was examined first to check for any indications that vesicle trafficking of either had increased or decreased.

Cell scattering was first observed in MDCK cells (Stoker & Perryman, 1985; K. B. Weidner, J; Vandekerckhove, J; Birchmeier, W., 1990), and these epithelial cells can be induced with HGF to undergo EMT (K. M. Weidner et al., 1991). For this reason, and because cell scattering of MDCK cells has already been well characterized in our lab, this became the experimental cell line.

MDCK cells were seeded onto glass coverslips and treated with HGF. They were stained with anti-rab8 and anti-rab4 antibodies prior to immunofluorescence imaging. Rab8 localization in MDCK cells has a dramatic change from being diffuse in the cytosol before HGF stimulation to becoming organized in precise tracks after stimulation (Figure 2.1B). Ten to thirty minutes after treatment with HGF, rab8-positive vesicles move out of the cytosol and are localized to filamentous tracks that appear to be situated along cell-cell junctions, reminiscent of cytoskeletal elements. This continues for at least an hour after addition of HGF, and the tracks also form transcellular networks after forty-five minutes of stimulation. This indicated that HGF causes a change in the localization of rab8.

Rab4, however, shows a much subtler change after HGF stimulation (Figure 2.1A). Diffuse, cytosolic localization continues for an hour after stimulation, but at about thirty minutes

there is a slight increase in localization at the edges of lamellipodia, which continues until at least an hour after stimulation. While the localization is very different from that of rab8 vesicles, this data indicates that HGF may also cause a change in rab4 vesicle localization.

TRPV4 Localization After HGF Stimulation

We next sought to demonstrate that HGF stimulation induces calcium channels to undergo rab8-mediated trafficking. We previously showed (Langford et al., 2012) that HGF stimulation induces trafficking of several TRP channels, including TRPC6 and TRPV4. Given availability of antibodies, we examined trafficking of TRPV4.

Based on our hypothesis that HGF stimulates rab8 to deliver TRPV4 to the cell surface, we would expect that TRPV4 and rab8 will colocalize in cells following HGF treatment. To test this, TRPV4 localization was examined before and after HGF stimulation. Costaining with rab11 antibodies reveals that TRPV4 colocalizes precisely with rab11 before HGF stimulation, but less colocalization is observed immediately after HGF treatment (Figure 2.2). Prior to HGF stimulation, rab11 stains a large reticular perinuclear compartment that also contains TRPV4. This structure is likely the ERC (Grant & Donaldson, 2009). After HGF treatment, only partial TRPV4 staining of the ERC remains; regions of the ERC stain only with rab11 and not TRPV4, suggesting depletion of TRPV4 from this compartment. TRPV4 also appears on cytosolic vesicles that do not stain with rab11 and appear to be organized on tracks that are reminiscent of cytoskeletal elements. This indicates that HGF stimulation causes the rab11-decorated ERC to be emptied of TRPV4, and causes a change in the patterning of TRPV4.

Interestingly, the cellular distribution of TRPV4 vesicles after HGF treatment is similar to that of rab8, but not rab4 (Figure 2.2). While TRPV4 staining is somewhat diffuse and cytosolic with perinuclear patches in unstimulated MDCK cells, after HGF stimulation TRPV4

vesicles organize into filamentous structures which were particularly pronounced at the 10, 30, and 45 minute marks, including at cell-cell junctions (Figure 2.2). This indicates that TRPV4 localization is similar to rab8 and may be on the same vesicles. Also, TRPV4 does not localize similarly to rab4 at lamellipodia edges, and does not appear to be cargo of rab4-positive vesicles.

In order to demonstrate that TRPV4 is loaded into rab8 vesicles, we costained cells with antibodies recognizing TRPV4 and Myo5b. Myo5b is known to interact with rab8 and rab11 and participate in rab8-mediated vesicle trafficking in the slow recycling pathway (Roland et al., 2011). Cells were seeded onto collagen-coated coverslips and stimulated with HGF for different amounts of time prior to processing for fluorescence.

Costaining with TRPV4 and Myo5b shows precise colocalization of these proteins before and after HGF treatment (Figure 2.3). Prior to HGF stimulation, TRPV4 and Myo5b colocalize precisely in a perinuclear compartment that appears to be the ERC, based on our earlier rab11 staining. Following HGF stimulation, TRPV4 and Myo5b precisely colocalize in cytosolic vesicles that are organized on what appear to be filamentous tracks, particularly 30 to 60 minutes after HGF stimulation (Figure 2.3). Our earlier data demonstrated that TRPV4 localizes with rab11 without HGF stimulation, and this association decreases with HGF stimulation. It is most likely that the colocalization of TRPV4 with Myo5b in the perinuclear regions is due to TRPV4 localizing to the ERC with the rab11-Myo5b complex, and the colocalization of TRPV4 with Myo5b in the filamentous structures from 30 to 60 minutes is due to TRPV4 localizing to vesicles marked by the rab8-Myo5b complex (Roland et al., 2011). This indicates that TRPV4 is a cargo of rab8-mediated vesicle recycling immediately following HGF simulation.

Trafficking in Rabin8 KO and ERK-inhibited Cells

We next asked whether activation of rab8 was necessary for trafficking of TRPV4. Because two isoforms of rab8 exist, rab8a and rab8b, we opted to simplify the genetic cell lines by knocking out the GEF for both isoforms, rabin8 (Hattula, Furuhjelm, Arffman, & Peränen, 2002). The rabin8 gene was mutated using CRISPR. PCR using one primer upstream of the cut site and one primer over the cut site verified that the rabin8 gene was mutated in rabin8 KO cells. This was due to the presence of multiple bands in the rabin8 KO cells compared to the single band in the WT cells. These bands in the rabin8 KO cell line were also higher on the gel than the WT band, and the single band in the WT line was absent in the rabin8 KO line (Figure 2.4B). A section of DNA with a sequence matching the cut site was most likely duplicated and inserted several times into the site where the gene was cut by Cas9, effectively altering the gene.

Rabin8 KO cells were seeded onto cover slips and stimulated with HGF. They were then stained with anti-TRPV4 antibodies. Rabin8 KO cells show diffuse cytosolic staining for TRPV4 before and during HGF stimulation (Figure 2.4A). TRPV4-positive vesicles never organize into filamentous tracks at any time point. This indicates that a lack of active rab8 halts the HGF-induced trafficking of TRPV4.

A recent report showed that ERK1/2 activates rabin8, leading to rab8 activation (J. Wang et al., 2015). We asked whether ERK1/2 facilitates HGF-dependent activation of rab8-mediated trafficking of TRPV4. We treated WT MDCK cells with an ERK inhibitor prior to HGF stimulation, then stained with anti-TRPV4 antibodies. TRPV4 localization remains diffuse and cytosolic before and after HGF stimulation when cells were pre-treated with an ERK1/2 inhibitor, FR 180204 (Figure 2.4A). No TRPV4-positive vesicles become organized into tracks following HGF stimulation of these cells. This indicates that ERK1/2 is necessary for HGF-

induced TRPV4 vesicle trafficking. This result, combined with our finding that rabin8 knockout also prevents HGF-induced TRPV4 recycling, reinforces our conclusion that HGF induces rab8 activation and subsequent TRPV4 vesicle trafficking to the plasma membrane via ERK1/2.

Calcium Levels in Rabin8 KO Cells

Next, we sought to determine whether active rab8 is necessary for the calcium influx Langford et al. reported. Thus, utilizing live cell calcium imaging with fura-2 dye, we tested the calcium levels in rabin8 KO and WT MDCK cells with and without HGF over a 50 minute time span, with HGF added at the five minute mark.

WT MDCK cells with no HGF added increase in fluorescence levels several minutes into imaging, then slowly decrease over the remaining time (Figure 2.5A). The decrease is most likely due to gradual photobleaching of the fura-2. Whatever the reason for the initial wave, it is not due to HGF induction.

Alternatively, WT MDCK cells treated with HGF demonstrate higher calcium levels on average than untreated cells, and have several sharp spikes of fluorescence beyond the initial wave (Figure 2.5B). While the timing, size, and duration of these spikes can vary, the largest occur most often between 10 and 15 minutes after HGF stimulation. In one colony, there was another distinctive wave just before the 25 minute mark after addition of HGF. This is very similar to Langford's data with the patch clamping experiment.

We were unable to perform this experiment with ERK1/2-inhibited cells because the inhibitor gave off its own fluorescence, distorting our data.

Rabin8 KO colonies induced with HGF demonstrate a slow increase in fura-2 fluorescence, which then gradually falls (Figure 2.5C). There are no spikes at any point, and their average calcium levels are very close to that of untreated WT cells. The collective average

of all rabin8 KO colonies has a fluorescence vs time curve that looks most similar to that of the untreated WT cells, and dissimilar to HGF-treated WT cells (Figure 2.5D). This evidence shows that active rab8 is necessary for HGF-induced calcium influx.

Scattering in Rabin8 KO and ERK-inhibited Cells

Finally, we attempted to verify that active rab8 is necessary for cell scattering. WT Cells were seeded onto glass movie dishes, and then imaged on an upright microscope under a bright light. In HGF-stimulated cells, HGF was added immediately after initiation of imaging.

Untreated WT MDCK cells change very little over the course of eight hours (Figure 2.6). They are non-motile and retain cell-cell junctions in colonies.

When treated with HGF, however, WT cells scatter across the dish (Figure 2.6). Cells undergo spreading within the first two hours, and then change from cobblestone- to crescent-shaped, disassemble cell-cell junctions, and tear away from one another as contractility increases and the cells become motile. These latter changes do not occur in any particular order, but usually initiate around three hours after HGF stimulation.

Similar to HGF-stimulated WT cells, cells treated with ERK1/2 inhibitor spread within the first two hours of HGF stimulation. However, they do not disassemble cell-cell junctions or become motile after three hours, as HGF-stimulated WT cells do (Figure 2.6). This indicates that the regained contractility which occurs after spreading is dependent on ERK1/2.

Rabin8 KO cells treated with HGF also spread but lose all motility (Figure 2.6). Unlike HGF-treated control cells, rabin8 KO cells do not pull away from one another or move throughout the dish. A small degree of cell-cell junction disassembly, seen as holes torn in the middle of the colony, occurs around the eight hour mark, while cell-cell junction disassembly occurs by the third hour after stimulation in WT cells (Figure 2.6). This indicates that active

rab8 is necessary for cell scattering. Our other results imply this is most likely because of its involvement in TRPV4-mediated calcium influx. Furthermore, while ERK1/2 has many downstream effectors, the similarity in these results may mean that active rab8 is at least partially responsible for ERK1/2's role in EMT.

CHAPTER 3: Discussion

Several studies have shown that growth factor-induced calcium influx is necessary for EMT. This is most likely due to vesicle trafficking of calcium channels (Langford et al., 2012). Because of the rapid, periodic nature of the calcium influx in HGF-stimulated MDCK cells that our lab previously noted (Langford et al., 2012), it is likely that endocytic recycling is involved. TRPV4, among other TRP channels, is necessary for the calcium influx (Langford et al., 2012), and so this report sought to elucidate what endocytic recycling pathway might be responsible for trafficking TRPV4; that is, if it were the rapid endocytic recycling pathway, rab4 would be the required rab GTPase, and if the slow, rab8 (Grant & Donaldson, 2009).

The results in the previous chapter indicate that rab8 controls the vesicle trafficking of TRPV4. Not only does TRPV4 have similar localization patterns to that of rab8 before and after HGF stimulation, but knocking out rabin8, the rab8 GEF, prevented TRPV4 trafficking. Also, TRPV4 colocalization with rab11 decreases after HGF stimulation, which indicates that the rab11-marked ERC is being emptied of TRPV4. As rab8 acts downstream of rab11, this further denotes rab8 as the rab GTPase involved in vesicle trafficking. Additionally, Myo5b consistently colocalizes with TRPV4; Myo5b is known to associate with rab11 and rab8, and with our other data, this implies that TRPV4 initially colocalizes with rab11 and Myo5b at the ERC, and after HGF stimulation is moved into rab8- and Myo5b-labeled vesicles. This confirms the hypothesis that active rab8 controls trafficking of TRPV4 through the slow endocytic recycling pathway.

Active rab8 is also necessary for increased calcium levels in HGF-stimulated MDCK cells. Although it is impossible to account for internal release of calcium from the endoplasmic reticulum using the fura-2 fluorescence technique, the distinctive peaks of calcium seen in HGF-

stimulated cells occur in similar timing to those that Langford et al observed. In any case, calcium levels in rabin8 KO cells treated with HGF are similar to those of untreated WT cells, and dissimilar to those of HGF-stimulated WT cells. Finally, active rab8 is necessary for cell scattering, as motility was reduced and disassembly of cell-cell junctions occurred later in rabin8 KO cells. This brings the data full circle, implying that active rab8 contributes to a rise in calcium levels in HGF-induced cells, possibly due to influx through TRPV4 channels, as the other data indicates, leading to cell scattering and EMT.

Our results also imply that ERK1/2 acts upstream of rab8. Both cell scattering and TRPV4 vesicle trafficking were prevented in ERK1/2-inhibited cells. This confirms the report that ERK1/2 causes activation of rab8 through phosphorylation of rabin8 (J. Wang et al., 2015). This provides a rough signaling pathway: HGF activates the c-met receptor, which leads to activation of ERK1/2. ERK1/2 phosphorylates and activates rabin8, thereby activating rab8, which aids in endocytic recycling of TRPV4 to the plasma membrane. Increased levels of TRPV4 at the cell surface causes a calcium influx and downstream processes resulting in increased contractility and EMT (Figure 3.1).

Future Directions

While it is obvious that rab8 is important for EMT, rab8 is not an attractive cancer therapy target. Rab8 is necessary for apical-basal polarity in several cell types (Sato et al., 2007; Ang, Fölsch, Koivisto, Pypaert, & Mellman, 2003). Rab8-deficient mice have reduced nutrient absorption in the small intestine due to mislocalization of apical peptidases and transporters, ultimately resulting in death (Sato et al., 2007). Optineurin binding to inactive rab8 is significantly decreased from that of active rab8, and when binding of optineurin and rab8 is impaired, glaucoma and other retinal degenerative diseases result in mice (Chi et al., 2010).

Thus, a rab8 small molecule inhibitor may have greater risks than rewards for cancer patients. With this in mind, the downstream effectors of rab8 as well as its activators, specifically those which mediate growth factor induction, may be of greater interest for cancer metastasis therapy targets.

However, the implications for developmental research are abundant. EMT occurs many times in the developing embryo, including during gastrulation, and during neural crest cell migration (Hay, 2005). The formation of neural crest-derived structures, including the peripheral nervous system, cranial bone and cartilage, melanocytes and parts of the adrenal medulla (Kerosuo & Bronner-Fraser, 2012), requires EMT to occur correctly. The data in this study shows that rab8 is important not only for HGF-induced calcium influx, but also for EMT. Further study into rab8 in the context of developmental EMT may reveal more about the formation of neural crest-derived structures and the embryo in general.

CHAPTER 4: Materials and Methods

Cell Culture

All MDCK cell lines were cultured in low-glucose DMEM containing 10% FBS and 1% PSK. The stable, monoclonal rabin8 KO cell line was generated by first transforming plasmids (sgRNA cloned into pSpCas9(BB)-2A-Puro) into Single-Use BL21(DE3)pLysS Competent Cells (Promega) selected with 100ug/ml ampicillin and subsequently transformed into MDCK cells using Effectene reagent (Qiagen) selected with 4 ug/ml puromycin, and finally subcloned. The plasmid was cloned with the following sgRNA sequences that match the canine rabin8 gene and contain appropriate overhanging nucleotides for plasmid insertion: CACCGTGTGGCCGATAGATGACACT, AAACAGTGTCATCTATCGGCCACAC.

To determine rabin8 gene integrity, genomic DNA from cultured cells was isolated and PCR-amplified at 50-51° C annealing temperature using primers with the following sequences: TCATTCCAAGCATGCACGTC, and TGTGGCCGATAGATGACACTTGG (which matched the section of DNA that was cut by Cas9). The amplified DNA was run on a 1% agarose gel containing TBE and ethidium bromide, and analyzed with UV light.

Immunofluorescence

Cells were cultured on collagen-coated glass cover slips for 1-2 days before induction. Treatment group cells were stimulated for 10, 20, 30, 45, and 60 minutes with HGF at a final concentration of 25 ng/ml. Control cells were not stimulated (0 minutes). Cells were put on ice, washed twice with ice-cold PBS, then fixed with 4% paraformaldehyde. Cells were washed twice more, then blocked with "IF blocking buffer" containing .2% BSA, 2M NH_4Cl, .5% TritonX, and 1M sodium azide in PBS. DAPI was also added to the stock solution. During staining, cells were washed with a buffer containing .2% BSA and .5% Triton X in PBS.

Antibodies were diluted in this buffer as well. Glass cover slips with the fixed cells were stained for approximately 45 minutes with primary antibodies against rab8 (Bioss), rab11 (Santa Cruz Biotechnology), TRPV4 (LifeSpan BioSciences), and Myosin Vb (Santa Cruz Biotechnology). They were stained for 30 minutes with goat anti-mouse, goat anti-rabbit, or donkey anti-goat secondary antibodies conjugated to Alexa-488 or -546. The cover slips were mounted onto glass microscope slides using VectaShield (Vector). Imaging was performed on an EPI-fluorescent compound microscope at optimal exposure times.

Calcium Imaging

Cells were cultured on eight chambered cover glasses (Lab-Tek) for 2-3 days and incubated with fura 2 dye for thirty minutes and washed off thirty minutes prior to imaging. Five minutes after initiation of imaging, 10 ul (final concentration 25 ng/ml) HGF was added. Cells were recorded approximately every 3 seconds for 45- 50 minutes with 1s exposure time on an inverted microscope (Olympus). Fluorescence was measured as a ratio of 340 and 380 wavelengths as a % of the starting ratio.

Live Cell Imaging

Cells were cultured on collagen-coated DeltaT (Bioptechs) imaging dishes in 2 ml low glucose DMEM + 10% FBS + 1% PSK for 1-2 days before induction. Prior to imaging, media was changed to 1.5 ml LCM + 10% FBS + 1% PSK. A heated microscope stage utilizing a Delta T4 Culture Dish Controller (Bioptechs) maintained temperature at 36 °C. Multiple positions were imaged during each experiment. For ERK1/2 inhibitor movies, 3 ul (final concentration 2 uM) FR 180204 (Tocris Bioscience) was added. Immediately prior to initiating imaging, 1.5 ul (final concentration 25 ng/ml) HGF was added. Phase contrast images of cell scattering were

taken using a 10X objective with 100 ms exposure time. These images were taken with a bright light, and were recorded every 2 minutes for 13 hours.

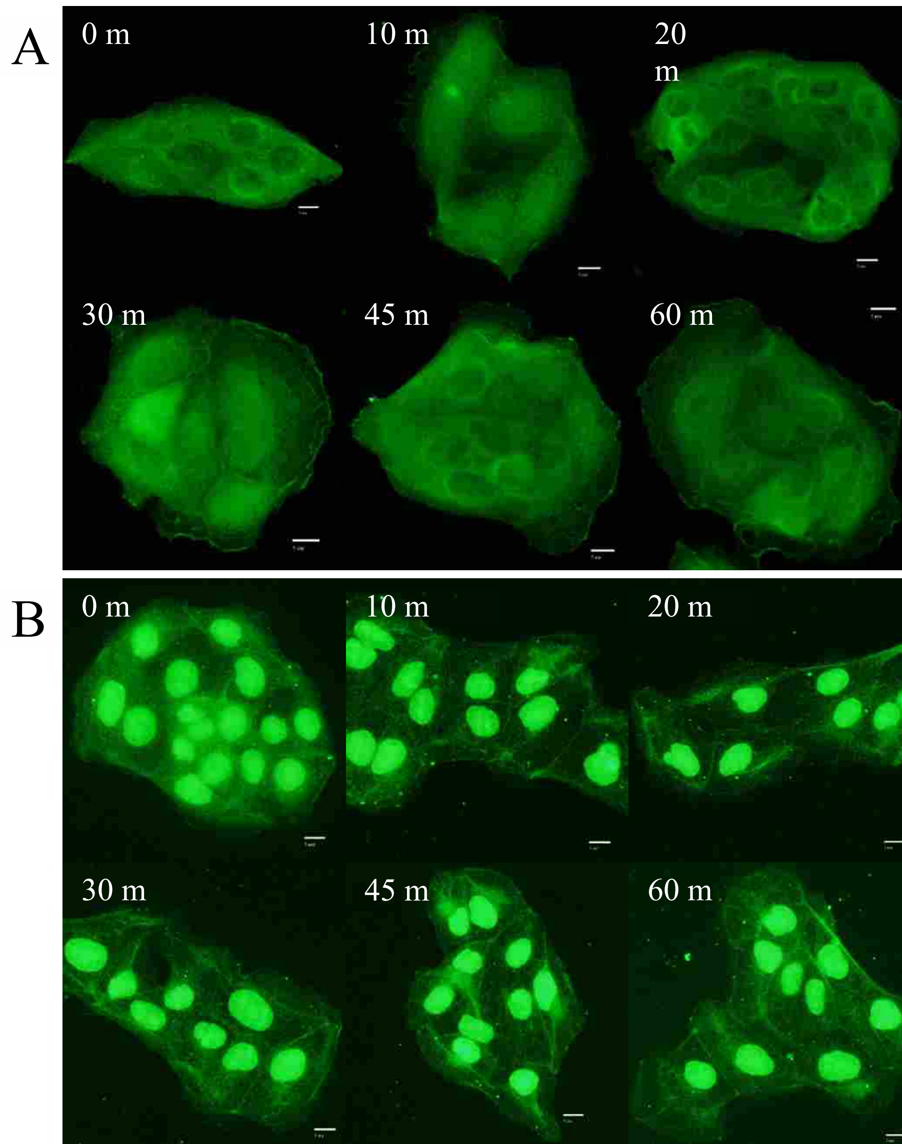

Figure 2.1: Rab4 and Rab8 Immunofluorescence in HGF-treated MDCK Cells.

Cells were treated with HGF for the indicated times, and then stained with anti-rab4 (A) and anti-rab8 (B) antibodies. "0m" cells were untreated. Scale bars are 5 um.

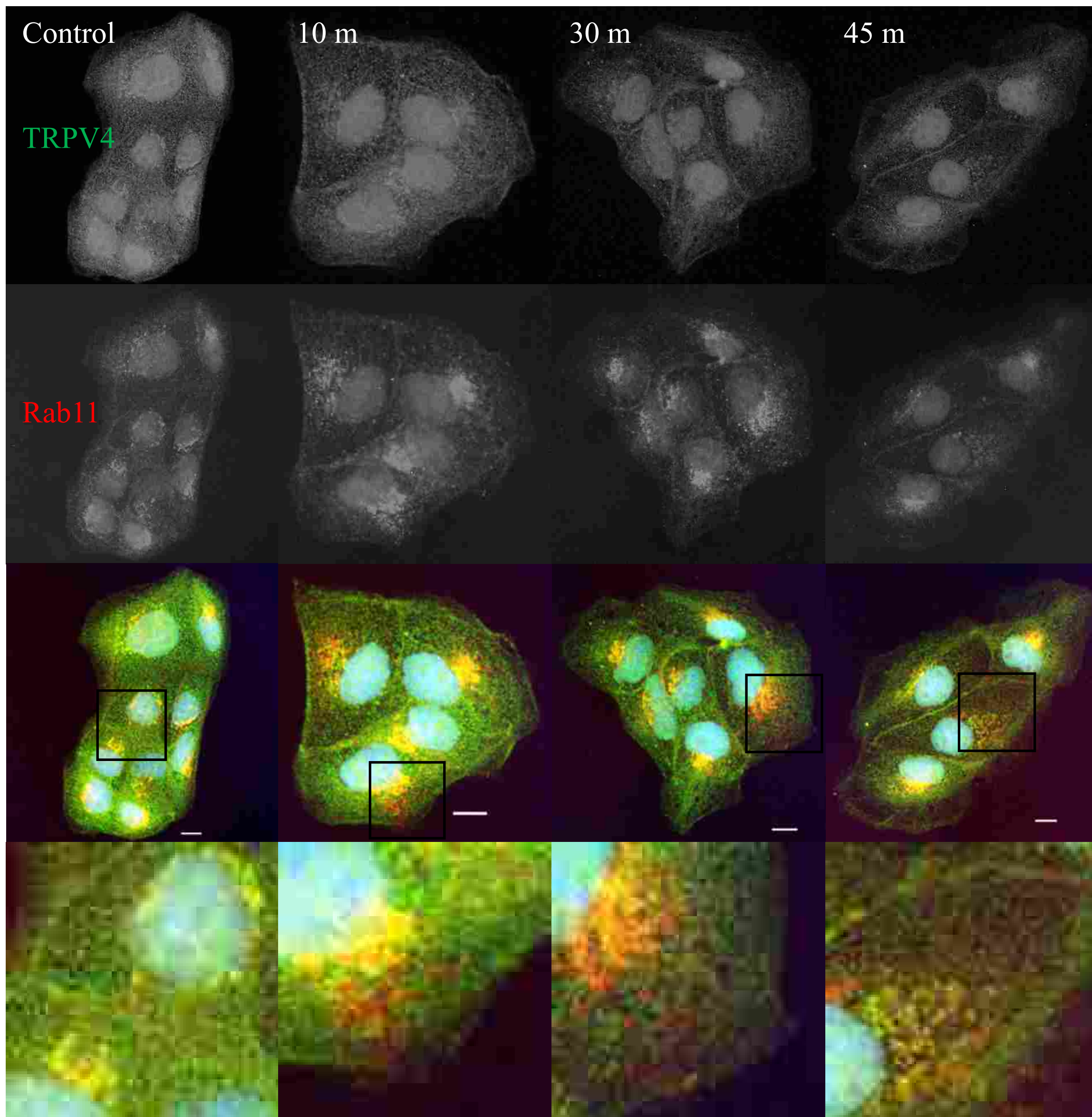

Figure 2.2: TRPV4 and Rab11 Co-localization in HGF-treated MDCK Cells.

MDCK cells were treated with HGF for the indicated times and then imaged with anti-TRPV4 and anti-rab11 antibodies. Dapi staining was also used. Boxes indicate inset boundaries displayed below. TRPV4 staining before HGF stimulation was fairly diffuse in the cytosol, except where it localized to rab11. After HGF stimulation, TRPV4 and rab11 colocalization decreased as shown in the insets, and TRPV4 was localized to filamentous structures. Scale bars = 5 µm.

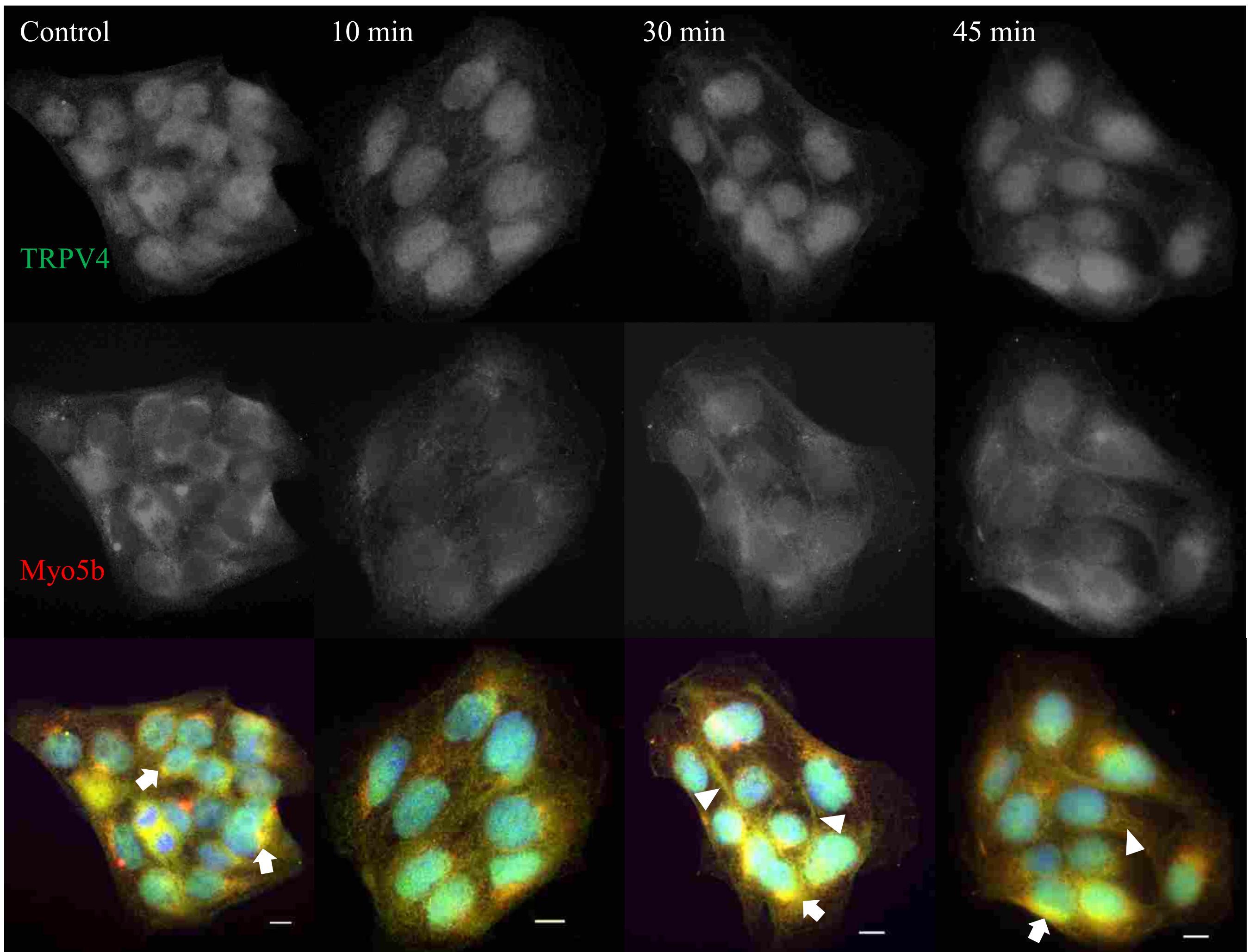

Figure 2.3: TRPV4 and Myo5b Co-localization After HGF Stimulation.

MDCK cells were treated with HGF for the indicated times and then imaged with anti-TRPV4 and anti-Myo5b antibodies. Dapi staining was also used. Arrows indicate perinuclear colocalization while arrowheads indicate tubular localization. Scale bars = 5 µm.

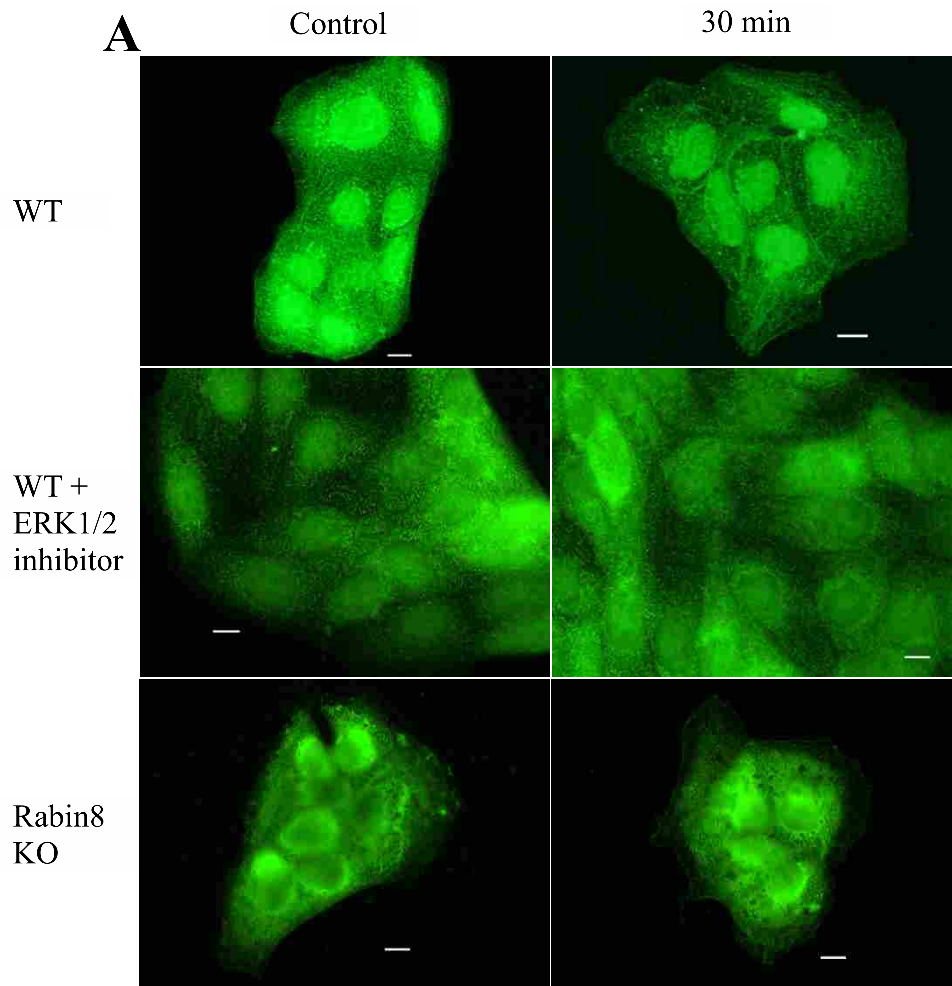

Figure 2.4: Rabin8 and ERK1/2 are Necessary for TRPV4 Vesicle Trafficking.

(A) WT cells were either treated with HGF alone or with HGF and an ERK1/2 inhibitor. Rabin8 KO cells were treated with HGF. Cells were imaged with anti-TRPV4 antibody. Scale bars = 5 μm. (B) PCR amplification was performed for DNA isolated from WT and rabin8 KO cells, and then run on a 1% agar gel.

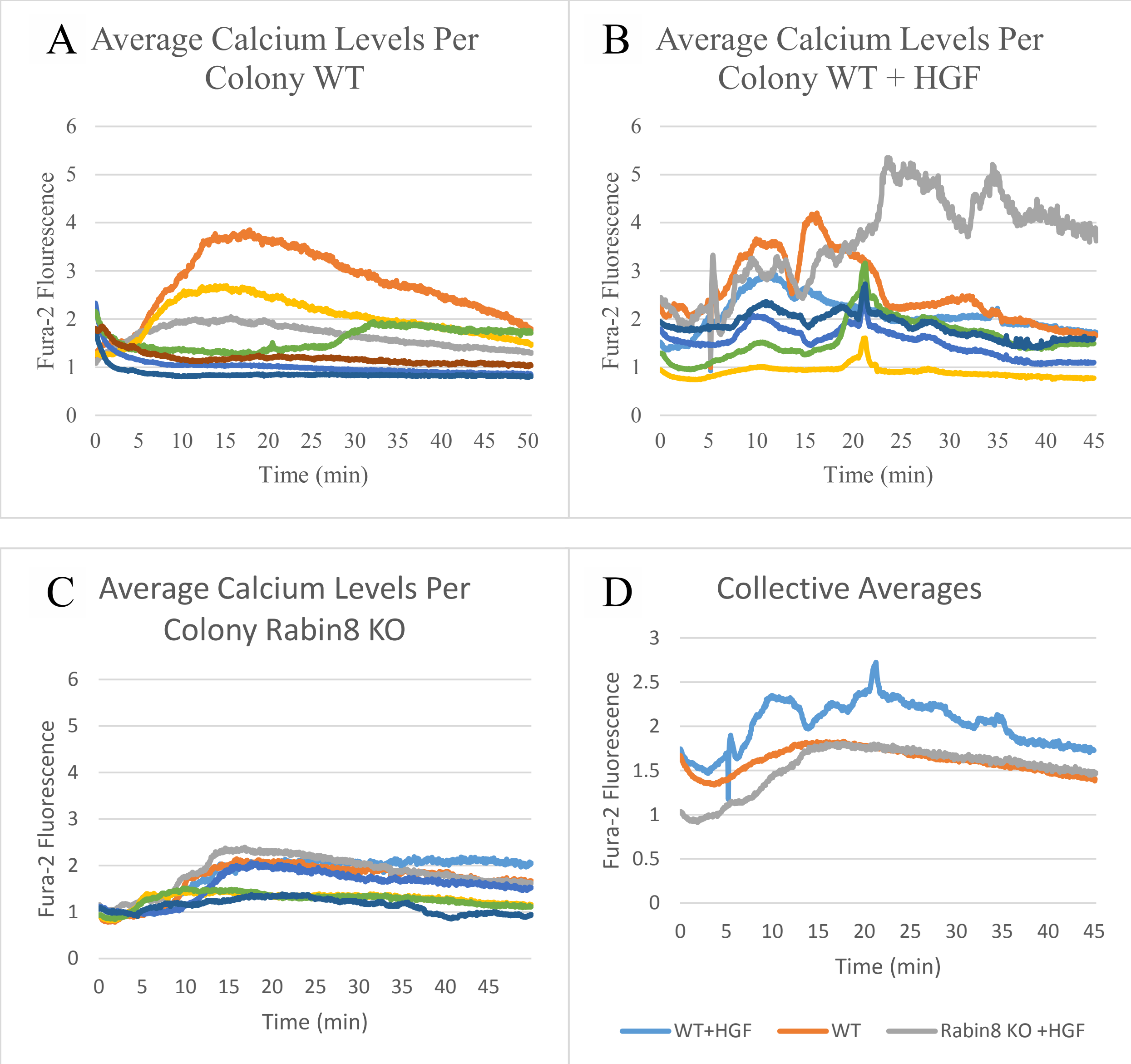

Figure 2.5: Calcium Levels Over Time in WT and Rabin8 KO Cells.

WT and rabin8 KO cells were stained with fura-2 and then imaged for 45-50 minutes. Fura-2 fluorescence is based on the 340/380 wavelength ratio as a percent of the starting ratio. When treated with HGF, HGF was added at five minutes after initiation of imaging. (A-C) Each line represents the fluorescence of an individual colony. (D) Each line represents the average of all colonies for the cell and treatment type.

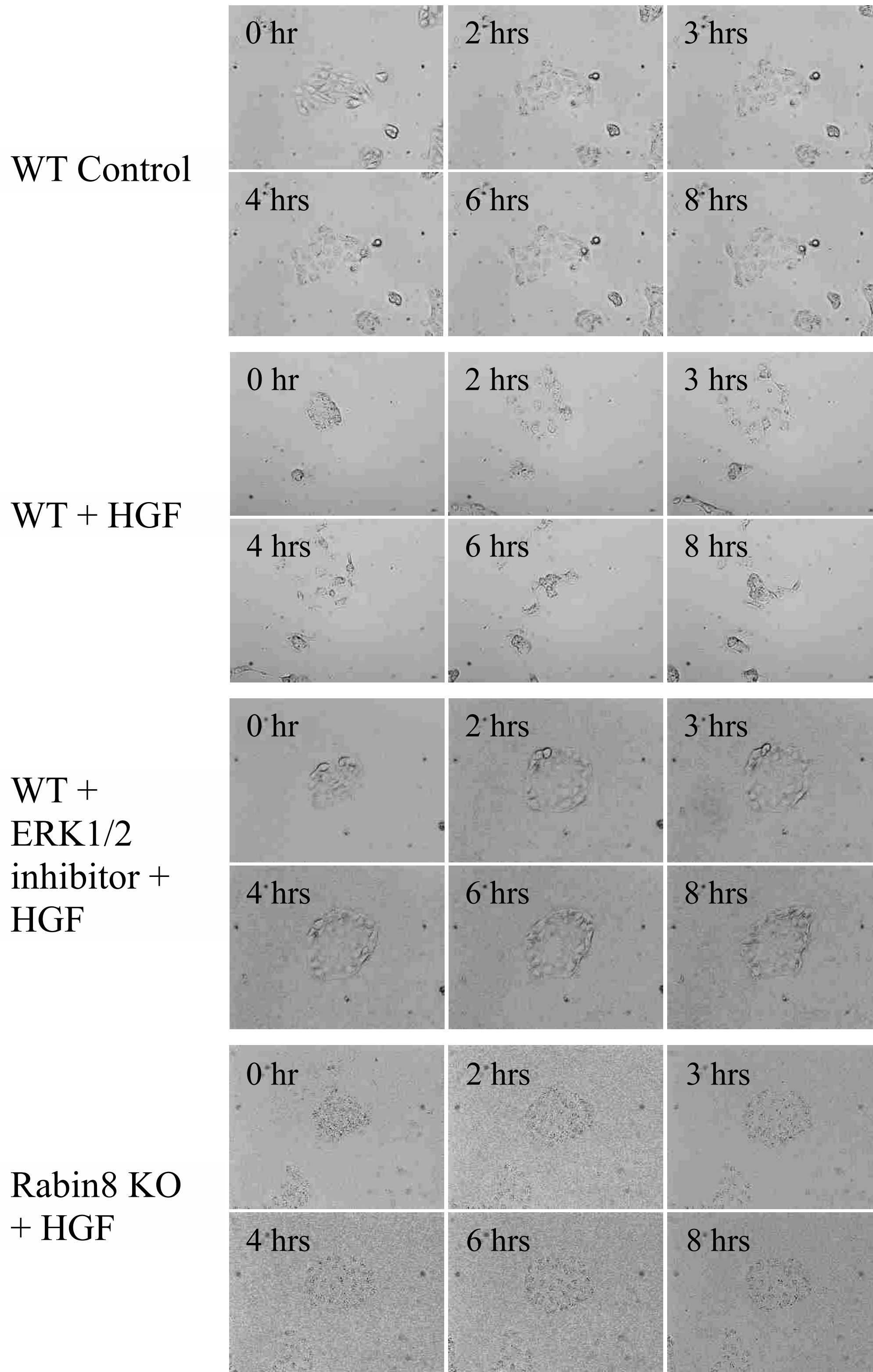

Figure 2.6: Active Rab8 Is Necessary for Cell Scattering.

WT control, WT cells treated with ERK1/2 inhibitor, and Rabin8 KO cells were live imaged for the indicated times. In cells treated with HGF, HGF was added at the 0 hr mark.

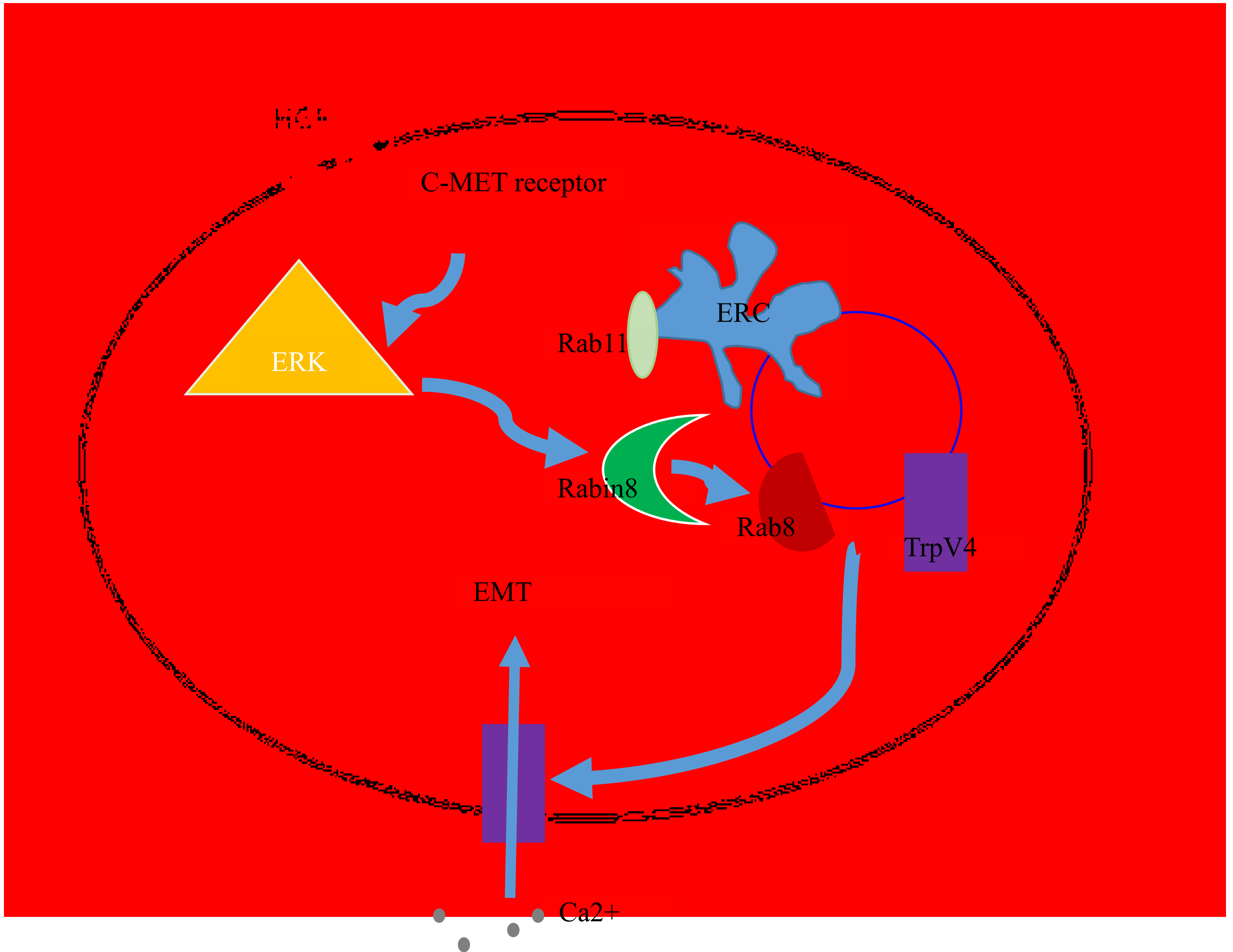

Figure 3.1: Proposed Pathway for HGF-induced Rab8-mediated Vesicle Trafficking of TRPV4.

HGF induces ERK1/2 which activates rabin8. Rabin8 activates rab8 and allows trafficking of TRPV4 to the cell surface from the endocytic recycling compartment (ERC), allowing calcium influx and subsequent EMT

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CURRICULUM VITAE

1080 S. Anne Circle
Orem, UT 84058

Hillary Haws

(801) 860-6250
ulmer.hillary@gmail.com

Education

April 2016
Brigham Young University, Provo, UT
M.S. in Physiology and Developmental Biology

April 2013
Brigham Young University, Provo, UT
B.A. in English
Chemistry Minor
University Honors Graduate

Professional Experience

January 2014-Present
Physiology Lab Instructor
Brigham Young University, Provo, UT
Taught students short lecture about body systems and laboratory experiments; supervised laboratory procedures; ensured safety protocols were followed; answered questions regarding homework; graded homework.

November 2013-January 2014
Plasma Donor Processor
Grifols Biomat Plasma Center, Taylorsville, UT
Took and recorded vitals and hematocrit of each donor prior to donation; certified donor eligibility through a series of questions regarding health behaviors; updated records on the computer and on paper; prepared donation equipment for each donor.

Volunteer Experience

December 2013-June 2014
Same Day Surgery and Mother and Baby Volunteer
Utah Valley Regional Medical Center, Provo, UT
Stocked supplies in rooms and IV trays; assembled information packets for patients; transported patients to cars in wheelchairs; brought supplies to patients; checked patient comfort and brought food and other desired necessities.

September 2012-December 2012
Activities Assistant
Orem Rehab and Nursing, Orem, UT

Coordinated games with groups of 4-6 patients; served food; talked with residents to stimulate cognition; helped quadriplegic patient to write letters.

June 2010-December 2011
Ecclesiastical LDS Missionary
Washington Spokane Mission, Spokane, WA

Taught in English and Spanish languages; compiled and handed out food at a food bank, and translated for Spanish-speaking applicants.

October 2007-April 2010
Tutor/Teacher's Assistant
Amelia Earhart Elementary, Provo, UT

Assisted 4th grade students with Math homework; read to students; compiled student reports; went over English assignments with students to help improve their writing.

Research

January 2014-Present
Department of Physiology and Developmental Biology, Brigham Young University
Rab8 Mediates TRPV4 Vesicle Trafficking to the Plasma Membrane in HGF-stimulated MDCK Cells.

Performed immunoflourescence tests on MDCK cells to show Trpv4 calcium channels are brought to the cell surface in vesicles after induction with HGF due to Rab8 activation, leading to EMT.

February 2010 –April 2010
Department of Psychology, Brigham Young University
PTSD's Effect on Cognitivie Skills Metaresearch

Evaluated articles regarding PTSD's effect on cognition, and excluded articles where participants were alcoholic; extracted and compiled data from previous tests into a datasheet.

Honors and Awards

Teaching Assistantship. 2014-2016.
Simmons Center for Cancer Research Fellowship. May- August 2015.
BYU University Honors Graduate. April, 2013.

Publications

In progress: Control of Cellular Contractility by Calcium Fluxes during Epithelial Scattering. Hillary Haws, Melissa McNeil, and Marc Hansen.

Posters

HJ Haws, MD Hansen. Rab-mediated vesicle trafficking in HGF-induced MDCK cells. Poster B1116/P1246. ASCB annual meeting, San Diego, CA. December, 2015.