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# Regulation of protein trafficking by Ral GTPases and Exocyst in epithelial cells

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# REGULATION OF PROTEIN TRAFFICKING BY RAL GTPASES AND EXOCYST IN EPITHELIAL CELLS

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

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To my family and my friends, the people that stay with me during this period of my life.

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# ABSTRACT

In polarized epithelial cells, vectorial protein trafficking is important for transporting specific membrane proteins to generate distinct apical and basolateral membrane protein compositions. The Exocyst is a conserved hetero-octameric protein complex, which regulates different aspects of protein trafficking, including tethering of the Golgi-derived vesicles to target membranes. Two of the Exocyst subunits, Sec5 and Exo84, competitively bind to the small GTPases, RalA and RalB, in a GTP-dependent manner. Although Ral GTPases have been proposed to mediate assembly of Exocyst holocomplexes, we hypothesize that they actually serve to allosterically regulate Exocyst functions by promoting association or disassociation of additional factors. Previous studies have shown that active RalA, but not RalB, accelerated basolateral exocytosis of E-cadherin. In contrast, knockdown of RalB, but not RalA, disrupts endocytosis of E-cadherin. However, mechanisms by which association of Ral GTPases with Sec5 and Exo84 regulate basolateral protein trafficking remain unclear.

Here we investigate roles of Ral GTPases and the Exocyst in regulating basolateral protein trafficking using Madin Darby canine kidney (MDCK) cells and RNA interference (RNAi) technology. We show that RalA, but not RalB, is required for basolateral exocytosis of vesicular stomatitis virus glycoprotein (VSV-G) in the MDCK cells. We combined immunofluorescent labeling and surface biotinylation assays to demonstrate that RalA regulates VSV-G trafficking through the distinct interactions with Sec5 and Exo84. We also show that a Ral-uncoupled Sec5 mutant, but not a Ral-uncoupled Exo84 mutant, inhibits E-cadherin exocytosis. These results suggested that RalA and the Exocyst are required for basolateral exocytosis, and that RalA-Sec5 and

RalA-Exo84 interactions play different roles during this process. Our study may provide new insights into mechanisms regulating protein trafficking in epithelial cells, and potentially lead to development of new therapeutic targets for the treatment of diseases in which exocytosis is impaired, such as Polycystic kidney disease and diabetes.

# PUBLIC ABSTRACT

Protein trafficking entails the transport of biochemical signals through membrane-bound vesicles to a specific cellular destination. Transport of such signals can involve the release (exocytosis) or internalization (endocytosis) of the message outside or inside of the cell respectively. Protein trafficking is important to transport specific membrane proteins to generate distinct apical and basolateral plasma membrane protein compositions to maintain epithelial cell morphology. Exocyst is a protein complex for spatial targeting of the transported vesicles to the plasma membrane. Two of the Exocyst subunits, Sec5 and Exo84, competitively bind to the GTPases, RalA and RalB. Previous studies showed that RalA, but not RalB, accelerated basolateral exocytosis of E-cadherin. In contrast, depletion of RalB, but not RalA, disrupts endocytosis of E-cadherin. However, mechanisms by which association of Ral GTPases with the Exocyst regulate basolateral protein trafficking remain unclear.

Here we show that RalA, but not RalB, is required for basolateral exocytosis of vesicular stomatitis virus glycoprotein (VSV-G) in the MDCK cells. Also, RalA regulates VSV-G trafficking through the distinct interactions with Sec5 and Exo84. Ral-Sec5 interactions, but not Ral-Exo84 interactions, are required for basolateral exocytosis of E-cadherin. These results suggested that RalA and the Exocyst are required for basolateral exocytosis, and that RalA-Sec5 and RalA-Exo84 interactions play different roles during this process. Our study indicates new insights into mechanisms regulating protein trafficking in epithelial cells and may potentially lead to development of new therapeutic targets for the treatment of diseases in which exocytosis is impaired, such as Polycystic kidney disease and diabetes.

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# CHAPTER I: INTRODUCTION

## Epithelial cell polarity

Epithelial cells distribute in many human organs such as kidney, liver, and intestine, where they provide biological barriers that separate two topologically distinct compartments (ie. “outside” and “inside” the body) and regulate the exchange of materials between these compartments (*1*). To maintain their morphology and functions, epithelial cells form strongly cohesive sheets with distinct apical and basolateral plasma membrane domains with unique protein and lipid compositions; such cells are said to be polarized (*2*, *3*). For example, renal epithelial cell polarity determines cell orientation and enables vectorial transport of ions and other solutes in the kidney (*4*). Epithelial polarity establishment is dependent on two events: cell-cell interactions and cell-extracellular matrix (ECM) interactions. When epithelial cells contact the ECM, an integrin-dependent signaling pathway is activated and the attached (ventral) membrane is identified as the basal cell surface. This is a key event in establishing an apical-basal axis of polarity (*5*). In addition, contact between neighboring cells activates a cadherin-dependent signaling pathway that results in segregation of apical proteins to the non-contacting (free) surface and enrichment of basolateral proteins within the contacting surface; tight junctions (TJs) form at the interface between apical and basolateral domains at a site determined by the actions of polarity complexes (*6*). Subsequently, the cytoskeleton is remodeled around these newly forming membrane domains. Microtubules form parallel bundles to vertically extend basolateral membranes and also grow from basal bodies anchored at the apical membrane to form the primary cilium, while actin forms apical microvilli and a

cortical belt that is associated with intercellular junctions (*7*). Cytoplasmic organelles are also re-positioned around polarity cues, with the Golgi complex and centrosomes becoming reoriented between the nucleus and the apical surface, and recycling endosomes moving towards both the apical and basolateral surfaces in order to facilitate transport of apical and basolateral membrane proteins and lipids to their appropriate destinations (*2*). To study development and maintainace of epithelial cell polarity, the Madin Darby canine kidney (MDCK) cell line from *Canis familiaris* is the most extensively studied system (*8*). MDCK cells retain many differentiated properties characteristic of kidney epithelial cells, including asymmetric distribution of membrane proteins and lipids, formation of an Apical Junctional Complex consisting of TJs, Adherens Junctions (AJs) and desmosomes and the ability to vectorially transport ions and water across the epithelium (*9*).

## Vectorial protein trafficking

As described above, to absorb nutrients, communicate with the environment and each other, and maintain functional shapes, epithelial cells have evolved protein sorting and trafficking machinery to transport membrane-bounded materials between endo-membrane compartments and apical or basolateral membranes. Vectorial protein trafficking pathways comprise the endoplasmic reticulum (ER), the Golgi complex, the *trans*-Golgi network (TGN) and endosomal compartments (*10*). Apical and basolateral membrane proteins are synthesized at the ER. They are then transferred to the Golgi and sorted at the TGN or recycling endosomes into specific carriers that are targeted to different plasma membrane domains (*11*). Polarity of membrane proteins is maintained

by reiterative sorting within the endocytic system (*12*). The Exocyst complex has been defined as the tethering factor for the initial recognition and attachment of secretory vesicles with the target membrane (*13*). This protein complex is necessary for targeting post-Golgi secretory vesicles to the plasma membrane or for targeting plasma membrane cargo vesicles to recycling endosomes (*14*).

## The Exocyst complex

### *Exocyst is a conserved hetero-octameric protein complex*

The Exocyst is a conserved hetero-octameric protein complex consisting of subunits Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (*15*). It primarily regulates different aspects of vesicle trafficking including tethering, docking and fusion of Golgi-derived vesicles with specific plasma membrane sites (*13, 15*). In polarized epithelial cells, the Exocyst is required for delivery of newly synthesized proteins from Golgi to the basolateral domain (*14*). Lack of any single Exocyst subunit doesn't affect the assembly of the remaining seven subunits (*16*). Exocyst may represent the assembled or disassembled complex when it functions in vesicle targeting, suggesting that the Exocyst complex might undergo dynamic conformational changes (*13*). Defects in the functions of the Exocyst in renal epithelial cells result in defective basolateral exocytic trafficking and cause diseases, including Autosomal dominant polycystic kidney disease (ADPKD) (*2, 17*). However, the exact subunit organization and structure of the holo-exocyst complex is still unclear.

*Exocyst is the regulator of polarized membrane trafficking*

The Exocyst complex was originally discovered in the late 1970s following isolation of temperature-sensitive secretory ("sec") mutants of the yeast *Saccharomyces cerevisiae (15).* The Exocyst complex has now been identified in many other organisms, including mammals, Drosophila and plants (*13*). The Exocyst has been shown to be involved in variety of cellular processes associated with cell polarity, including yeast budding, epithelial polarity establishment, neurite outgrowth, and ciliogenesis (*18*). In yeast, the Exocyst localizes at sites of daughter bud tips and at mother-daughter cell connections, functioning in membrane expansion, in exocytosis, and in cytokinesis (*19*). In mammalian cells, the Exocyst localizes to sites of membrane expansion (*20*). In epithelial cells, the Exocyst in recruited from cytosol to sites of cell-cell contact, such as cell-cell adhesion induced by calcium-dependent E-cadherin mediated (*21*). In addition, epithelial Exocyst complex also localizes at the trans-Golgi network, and was initially observed to regulate basolateral and apical vesicular trafficking (*22*). In MDCK cells, the Exocyst complex localizes at several endosomal populations and regulates apical recycling and basolateral-apical transcytosis (*23*), suggesting that the Exocyst was the regulator of vectorial vesicle trafficking. In addition to its established role in membrane trafficking, the Exocyst has been implicated in other diverse activities, such as actin regulation during cell migration (*24*), autophagy (*25*) and tumor cell survival (*26*).

*Exocyst may regulate vesicle fusion with the plasma membrane*

Recently, F. Morgera *et al* have identified that in yeast cells the exocyst subunit Sec6 regulates exocytosis through binding of the Sec1 (*27*). Sec1 belong to the

Sec1/Munc18-like (SM) protein family, which regulates soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE)-mediated membrane fusion (*28*). In addition, certain exocyst mutants inhibit assembly of plasma membrane SNARE complexes in yeast (*29*). This suggests that the exocyst is required for assembly of SNARE complexes and fusion of vesicles with the plasma membrane.

### *Exocyst is regulated by small GTPases*

Various small GTPases have been shown to regulate the Exocyst (*18*). For example, Sec15 is a downstream effector of the small GTPase Rab11, which recruits the Exocyst to recycling endosome to regulate membrane trafficking (*30*). Analogously, yeast Sec15 is recruited to vesicles by a yeast ortholog of Rab11, namely Sec4 (*31*). Sec10 is a downstream effector of the small GTPase Arf6, which recruits the Exocyst from the recycling endosomes to the plasma membrane and induce membrane recycling (*32*). Several other small GTPases inlcuding Ral, Rho1, Cdc42, and TC10 also regulate the Exocyst through engagement of other subunits (*33*), indicating that the Exocyst function as a hub to integrate regulatory information from different signaling transduction.

### Ral GTPases

Two of the Exocyst subunits, Sec5 and Exo84, have been identified as the downstream effectors of Ras-like (Ral) GTPases (*34*). The Ral GTPases belong to the Ras small GTPases superfamily, and are expressed only in animal cells (*35*). There are two isoforms of Ral GTPases in mammals: RalA and RalB (*36*). Both of them are geranylated at the carboxy terminus allowing for membrane association required for

proper function (*34*). RalA and RalB are 80% identical in sequence, but RalA has a higher affinity to Sec5 and Exo84 than RalB does (*34*). In biochemical studies, Sec5 and Exo84 have distinct but overlapping binding sites on the RalA (*37*). Therefore, a single Ral GTPase (RalA or RalB) can bind only one Exocyst subunit (Sec5 or Exo84) in a GTP-dependent manner (*24*). Ral GTPases function downstream of Ras to regulate many cellular processes including exocytosis, endocytosis, cell proliferation, cell migration and tight junction formation of epithelial cells (*6, 38*). Like other GTPases, Ral GTPases are activated by a set of guanine nucleotide exchange factors (GEFs), which promote conversion of Ral from a GDP-bound state (inactive form) to GTP-bound state (active form), and deactivated by GTPase-activating proteins (GAPs), which promote GTP-to-GDP exchange (*39*). Although RalA and RalB are 80% identical in sequence, they play different roles in many cellular pathways. For example, they appear to regulate exocyst activities during cell migration differently (*6*). In one study, RalB activation and the RalB–Sec5 interaction were shown to be induced by cell migration (*40*), while RalA activation was neither induced by cell motility nor needed for recruiting the Exocyst to the leading edge during cell migration (*40*). However, an independent study showed that both GTPases collaborated to direct cell motility, but consequences of silencing either through RNAi were different (*41*).

## Ral-Exocyst interactions

Ral GTPases can direct the Exocyst to mediate secretory vesicles tethering (*13*). It has been shown that depletion of RalA leads to dissociation of the exocyst complex and defects in polarized membrane delivery (*18*). Recent research has suggested that RalA

and RalB function differently during basolateral protein trafficking. Constitutively active RalA, but not RalB, accelerated basolateral exocytosis of E-cadherin (*39*). Our previous data showed that knockdown of RalB, but not RalA, disrupts endocytosis of E-cadherin (*6*). In addition, loss of Ral (A or B) function resulted in mislocation of several basolateral proteins (*18*, *42*). These results suggest that 1) both Ral GTPases and the Exocyst are involved in controlling basolateral trafficking; and 2) RalA and RalB may play different roles in basolateral protein trafficking. However, specific mechanisms by which Ral GTPases and their different effectors in the Exocyst complex (Sec5 and Exo84) regulate membrane trafficking are still not known.

Crystallographic studies of the RalA effector domain in complex with the Ral-binding domains of either Sec5 or Exo84 have defined amino acid residues on these proteins that are crucial for their binding. For example, a crystal structure of the RalA-Sec5 complex identified Sec5 Thr11 and Arg27, and RalA Glu38 residues as being essential for binding. The specific interactions unique to the RalA-Sec5 complex were confirmed by isothermal titration calorimetry (*37*). The key residues of Exo84 that are responsible for specific binding to RalA were also identified. PSIBLAST searches showed that Exo84 is highly conserved across different species (*43*). A highly conserved motif of Exo84, $^{228}$AxxNx(K/R)D$^{234}$, where x refers to a small hydrophobic residue (typically Val or Ile) has been discovered. Two downstream residues of the Exo84-Ral binding domain locate on this conserved motif (*44*). In addition, four residues in this region (Ala228, Val230, Asn231 and Lys233) are involved in RalA binding and the intermolecular interactions of RalA-Exo84 complex. The interaction was also confirmed

by site-specific mutagenesis in this region (Exo84 A228W or K233W), which reduced the Exo84–RalA binding affinity in biochemical assay (*44*).

*Different cellular localizations between Sec5- and Exo84- uncoupled RalA mutants*

Active RalA localizes to both plasma membrane and perinuclear recycling endosomes, while active RalB localizes to only plasma membrane (*39*). Recent work from our lab has shown that two exocyst-uncoupled RalA mutants localize to different subcellular localizations. Compared to the localization of a constitutively-active RalA mutant ($RalA^{72L}$, GTP-bound RalA (*24*)), the localization of $RalA^{38R}$ (Sec5-uncoupled mutant (*35*)) shifted to a primarily perinuclear area and the localization of $RalA^{47E}$ (Exo84-uncoupled mutant (*24*)) shifted to a primarily plasma membrane localization. This suggests that RalA may bind Sec5 at the recycling endosome to facilitate trafficking to the plasma membrane. Subsequently, RalA may bind Exo84 at the plasma membrane to mediate late events in exocytosis (*6*).

*Exocyst complex is the scaffold protein*

Several studies have suggested that the Exocyst is a multi-functional scaffold, and that active RalA and RalB bind Sec5 and/or Exo84 to promote recruitment of additional factors, thereby priming it for specific cellular functions (*40, 41, 45*). For instance, RalA-Exocyst binding facilitated recruitment of the polarity protein Par3 to the Exocyst in neuronal cells and this influenced neuronal polarity (*46*). RalA-Exocyst binding also promoted the binding of paxillin to Sec5 and this was shown to regulate cell motility (*41*). Furthermore, binding of RalB and Sec5 induced recruitment and activation

of the Tank-binding kinase1 during host cell defense (*45*). Thus, the Exocyst can be viewed as a protein scaffold whose affinity for additional factors is allosterically regulated by Ral GTPases.

## Thesis Rationale

As described above, Ral GTPases and the Exocyst complex work together to regulate a variety of cellular processes, including cell migration and vesicle trafficking. Many of these cellular events include recruitment of additional factors in response to Ral binding Sec5 or Exo84 in a GTP-dependent manner. Some of these factors have been identified, but a detailed mechanistic understanding of how Ral GTPases, the Exocyst and associated factors regulate membrane trafficking has not yet been described clearly. In this study, we used RNAi technology to identify specific and different roles for RalA and RalB in basolateral protein exocytosis: 1) RalA, but not RalB, is required for basolateral exocytosis of VSV-G in the MDCK cells; 2) RalA regulates VSV-G trafficking through interactions with both Sec5 and Exo84; 3) the Ral-uncoupled $Sec5^{T11A}$ mutant inhibits E-cadherin exocytic trafficking; 4) RalA-Sec5 and RalA-Exo84 interactions might differently mediate basolateral exocytosis. These observations, combined with other studies, help to define the roles of the Exocyst and Ral GTPases in basolateral protein exocytosis.

# CHAPTER II: EXPERIMENTAL RESULTS

## Reduction of RalA activity reduces basolateral exocytosis of VSV-G in MDCK cells

Prior work in our laboratory showed that RalA and RalB play distinct roles in regulating tight junction (TJ) barrier formation. Reduction of RalA expression delayed TJ assembly kinetics, likely by decreasing the trafficking of claudins to the plasma membrane. In contrast, reduction of RalB expression promoted excessive accumulation of claudins within newly forming TJs, thereby causing the epithelium to exhibit abnormally high transepithelial electrical resistance (TER). This latter effect was shown to be due to effects on endocytosis, rather than exocytosis of junctional components, because endocytosis of E-cadherin was specifically disrupted by loss of RalB but not RalA (*6*). To further investigate the role of Ral GTPases in basolateral trafficking, we have generated MDCK cell lines in which expression of either RalA or RalB was reduced. Stable clones of MDCK strain II cells were selected for puromycin resistance following transduction with recombinant lentiviral vectors encoding short hairpin RNAs (shRNAs) specific for RalA (shRalA), RalB (shRalB) or a non-targeting sequence (shCtrl) (*6*). We then performed morphological trafficking assays with a well-studied basolateral membrane-associated cargo protein, the vesicular stomatitis virus glycoprotein (VSV-G). Yellow fluorescent protein (YFP)-tagged VSV-G (YFP-VSV-G) is effective for visualizing VSV-G at all stages of its trafficking, from its synthesis in the endoplasmic reticulum (ER), through the Golgi, TGN and recycling endosomes and finally to the basolateral plasma membrane (*47*). Moreover, trafficking can be synchronized by accumulating cargo at each station through a sequence of temperature shifts. Because our

constructs contain the tsO-45 mutation in VSV-G, which prevents correct folding of the protein and causes it to become trapped in the ER at the restrictive temperature of 40°C, it is possible to accumulate cargo in the ER. When cells are shifted to the permissive temperature of 32°C (or 37°C), a wave of VSV-G protein exits the ER and traffics into the Golgi apparatus. At this point, cells are shifted to 20°C and this incubation step serves to trap the cargo wave in the TGN. When cells are then shifted back to 32°C, VSV-G exits the TGN and traffics to the plasma membrane. Therefore, by performing a series of simple temperature shifts it is possible to analyze effects of Ral/Exocyst manipulation on each stage of the membrane trafficking itinerary of VSV-G.

Stable cell lines expressing control (shCtrl), RalA-targeting (shRalA), or RalB-targeting (shRalB) hairpins were transduced with recombinant adenovirus vectors encoding YFP-VSV-G at 37°C for 24hr. Cells were then shifted to 40°C for 16 hr to accumulate misfolded YFP-VSV-G in the ER. Subsequently, cells were shifted to 20°C for 2 hr and the YFP-VSV-G refolded and accumulated in the TGN. During this incubation, 100 μM cycloheximide was added to culture medium to prevent further protein synthesis. Finally, cells were shifted to 32°C to allow transport of YFP-VSV-G from the TGN to the plasma membrane. At indicated times, cells were fixed without permeabilization and incubated with an antibody that recognizes the extracellular domain of YFP-VSV-G, followed by a Texas Red-conjugated secondary antibody. Images revealed that 30 min and 60 min after shifting cells to 32°C, the internal YFP-VSV-G accumulated in the intracellular region in the shRalA cells, suggesting RalA knockdown inhibited transport of YFP-VSV-G from TGN to the cell surface (Fig. 1B). However,

shCtrl and shRalB show normal transport of YFP-VSV-G from the TGN to the cell surface (Fig. 1A and 1C). To quantify this result, we used ImageJ to analyze the relative ratio of YFP-VSV-G at the surface to the total in the cells. Fig. 1D shows results of quantitative analysis of trafficking at the 60 min time point. When the data are normalized to values obtained from shCtrl cells, RalB knockdown did not have a significant impact on the transport of YFP-VSV-G to the cell surface (Fig. 1D). However, we observed that the relative level of YFP-VSV-G at cell surface was inhibited by more than 75% in shRalA cells (Fig. 1D). Compared to control cells, this difference was significant to $p < .0001$. Therefore, we suggest that RalA, but not RalB, is required for basolateral trafficking of VSV-G. These data are consistent with our previously published data showing that RalA, but not RalB, is required for trafficking of proteins to newly forming tight junctions (*6*).

## RalA regulates basolateral trafficking of YFP-VSV-G via binding to both Sec5 and Exo84

Because loss of RalA expression caused dramatic effects on YFP-VSV-G trafficking, we sought to determine whether the Exocyst cooperates with RalA to mediate the transport of YFP-VSV-G from TGN to cell surface. To answer this question, we generated RalA or RalB rescue MDCK cell lines, which were selected for G418 resistance following transduction of knockdown cells (shRalA or shRalB) with piggybac transposon vectors encoding shRNA-resistant forms of RalA wild-type (RalAWT), RalB wild-type (RalBWT), constitutively active RalA Q72L mutant ($RalA^{72L}$, a RalA GTP-bound-state mutant), Sec5 uncoupled RalA mutant ($RalA^{38R}$), Exo84 uncoupled RalA

mutant (RalA$^{47E}$) (*6*). These MDCK cell lines were then used to assess VSV-G trafficking with the morphological transport assay, as described above.

As shown in Figure 2, 60 min after shifting cells to 32°C YFP-VSV-G was trafficked efficiently to the plasma membrane of shCtrl cells (Fig. 2a-c), but accumulated in the perinuclear region of shRalA cells (Fig. 2d-f), as observed above (Fig. 1). Post-Golgi trafficking of VSV-G was largely rescued by expression of an RNAi-resistant, constitutively active RalA$^{72L}$ (Fig. 2g-i). However, RalA$^{38R}$ completely failed to rescue VSV-G trafficking (Fig. 2j-l), and RalA$^{47E}$ only partially restored VSV-G trafficking to control levels (Fig. 2m-o). To quantify these results, we used ImageJ to analyze the relative ratio of YFP-VSV-G at the surface to the total expressed in cells. Each ratio was then normalized to that of shCtrl cells. Quantitation of surface-to-total YFP-VSV-G in cells following incubation at 32°C for 60min is shown in Fig. 2p. When compared to shCtrl cells, decreases in cell surface expression of YFP-VSV-G observed in shRalA, RalA$^{38R}$-expressing and RalA$^{47E}$-expressing cells were all significant to $p<.0001$.

Unexpectedly, VSV-G trafficking appeared to be inhibited in different ways in cells expressing different Exocyst-uncoupled RalA mutants (Fig. 2j-o). VSV-G was entirely withheld in a perinuclear compartment in cells expressing the Sec5-uncoupled Ral$^{38R}$ mutant, suggesting that RalA engagement of Sec5 is required to transport cargo from a perinuclear compartment to the cell periphery. In contrast, much of the VSV-G was trafficked to the cell periphery in cells expressing the Exo84-uncoupled Ral$^{47E}$ mutant. There, it accumulated within thicker, more diffuse regions than those observed in

control cells, suggesting that RalA binding to Exo84 might be required for a late exocytic event at the plasma membrane. Collectively, these results suggest that Sec5 and Exo84 could participate in distinct steps during RalA-mediated basolateral exocytic trafficking. These data complement other recently published results from our lab: whereas the constitutively active $RalA^{72L}$ mutant was equally distributed between a perinuclear compartment and the plasma membrane, the Sec5-uncoupled $RalA^{38R}$ mutant was largely associated with the perinuclear compartment and the Exo84-uncoupled $RalA^{47E}$ mutant was primarily at the plasma membrane (*6*). Therefore, we suggest that RalA may bind Sec5 at the TGN/recycling endosome to facilitate vesicle budding or trafficking to the plasma membrane, and subsequently bind Exo84 at the plasma membrane to mediate vesicle tethering to sites of fusion. We also assessed VSV-G trafficking in shRalB cells, and shRalB cells in which the GTPase was re-expressed from a hairpin-resistant vector (Fig. 3). As anticipated from our earlier experiments (Fig. 1), we observed that RalB reduction or re-expression had no significant effect on YFP-VSV-G trafficking compared to shCtrl cells (Fig. 3).

To further confirm our cell imaging data of YFP-VSV-G trafficking, delivery of YFP-VSV-G to the plasma membrane was quantified biochemically by cell surface biotinylation (*48*). At various time points after transferring cells to 32°C, surface YFP-VSV-G was biotinylated and recovered by streptavidin precipitation of biotinylated proteins. In shCtrl cells, the surface YFP-VSV-G at different time points indicates that YFP-VSV-G gradually accumulated at the cell surface during a 60-min time course (Fig. 4A and 4B). In RalA knockdown cells, YFP-VSV-G was delivered to the plasma

membrane with reduced kinetics and the maximum level of protein that accumulated there was reduced compared to that in shCtrl cells (Fig. 4A and 4B). However, re-expression of an RNAi-resistant form of RalA restored trafficking of YFP-VSV-G to levels similar to those observed in shCtrl cells. (Fig. 4A and 4B). VSV-G trafficking was also assessed biochemically in shRalB and RalBWT rescue cells (Fig. 5). Again, reduction or re-expression of this GTPase had no observable impact on cell surface expression of YFP-VSV-G compared to shCtrl cells (Fig. 5A and 5B). Therefore, our biochemical analysis is in agreement with our morphological analysis. In both cases, loss of RalA, but not RalB expression is associated with the reduced delivery of YFP-VSV-G to cell surface. In addition, compared to shCtrl cells, delivery of YFP-VSV-G to the surface of cells expressing either $RalA^{38R}$ or $RalA^{47E}$ were reduced to similar extents (Fig. 4C and 4D). These data need to be repeated for statistical significance. However, these data agree with our morphological transport assay and indicate that both Sec5 and Exo84 are required to mediate RalA-dependent basolateral trafficking of YFP-VSV-G.

Ral uncoupled Sec5 mutant inhibits E-cadherin exocytic trafficking

Like VSV-G, E-cadherin is vectorially delivered to basolateral membranes of MDCK cells. Because the Exocyst has been implicated in vesicle tethering to “target patches” on basolateral membranes, we investigated the possibility that Sec5 and Exo84 coupling to Ral GTPases was required for basolateral trafficking of E-cadherin-containing vesicles to the plasma membrane. We first traced the post-Golgi, exocytic transport of RFP-tagged E-cadherin (Ecad-RFP) in non-polarized MDCK cells. We co-transfected MDCK cells with plasmids encoding Ecad-RFP and a myc-tagged Ral-

uncoupled Sec5 mutant (Myc-Sec5$^{T11A}$). Cell images were captured with a Zeiss 700 laser-scanning confocal microscope. We observed that Ecad-RFP accumulated intracellularly in the myc-Sec5$^{T11A}$ expressing cells (Fig.6A, bottom panel). Quantitative data also showed that fewer cells expressed surface Ecad-RFP when they also expressed the Ral-uncoupled Sec5$^{T11A}$ mutant (Fig.6B). In contrast, Ecad-RFP was mostly expressed at the cell surface in control cells (pCMV-myc vector only) and myc-tagged Sec5 Wild-Type (Myc-Sec5 WT) expressing cells (Fig.6A, middle panel).

We also analyzed whether Ral-Exo84 interactions were required for exocytic transport of Ecad-RFP. We co-transfected with plasmids encoding Ecad-RFP and a myc-tagged Ral-uncoupled Exo84 mutant (Exo84$^{A228W}$) in MDCK cells. We observed that Ecad-RFP was correctly expressed on the cell surface in cells expressing the mutant Exo84 construct. Quantitative analysis showed that there was no statistically significant difference in the number of cells expressing Ecad-RFP at the surface between populations expressing myc-Exo84$^{A228W}$, myc-tagged Exo84 Wild-Type (Myc-Exo84 WT), or vector-only transfected cells (Fig. 7). Collectively, these results show that a Ral-Sec5 interaction is essential for basolateral trafficking of Ecad-RFP, but that Exo84 binding to Ral may not be as critical for this event.

# CHAPTER III: MATERIALS AND METHODS

## Cell culture

Madin-Darby Canine Kidney (MDCK) strain II G cells were grown in low glucose Dulbecco's modified Eagle's medium (LG-DMEM) with 1.8 mM $Ca^{2+}$ containing 1 g/l sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS; Cell Generation, Fort Collins, CO), penicillin, streptomycin, and gentamicin (PSG) (HCM; High $Ca^{2+}$ Media) and incubated at 37°C with 5% $CO_2$. shRalA and shRalB cells were generated by stable integration of short hairpin RNAs (shRNAs) targeting canine RalA (sense: 5'-CGAGCTAATGTTGACAAGGTA-3') or RalB (sense: 5'-GAGTTTGTAGAAGACTATGAA-3'). shRNAs were transducted with recombinant lentiviral vectors that were pseudotyped with VSV-G protein. Cells were selected and maintained in medium containing 5 μg/ml puromycin. shCtrl cells were generated by transducing MDCK II cells with lentiviral vectors encoding a non-targeting shRNA (sense: 5'-CCAGACCTTCAAGGAATCCAT-3') and selecting them in puromycin too. RalAWT and RalBWT rescue cell lines were generated as following: We introduced three wobble-base point mutations into the first three codons of the target simian RalA or RalB cDNA sequence. These cDNAs were cloned to piggybac-Ef1α-IRES-NEO vector (System Bioscience) and delivered into shRalA cells using Effectene Transfection Reagent (Qiagen) to selected by 500 μg/ml G418 and assayed by immunofluorescence and immunoblotting (*6*). $RalA^{72L}$, $^{38R}$, and $^{47E}$ point mutations were introduced into the RalA cDNA using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA) and were followed by the same process that generated and screened RalAWT

and RalBWT rescue cells. RalA cDNA was a generous gift from Dr. Larry Feig (Tufts University School of Medicine, Boston, Massachusetts).

## Antibodies and reagents

Mouse monoclonal antibodies (mAbs) against VSV-G (P5D4) or VSV-G extracellular domain (8G5F11, KeraFast) have been described previously (*49*). mAbs against Myc (9E9, Millipore; Billerica, MA) was obtained commercially. YFP-VSV-G and RFP-E-cadherin plasmids were described previously (*50, 51*). Sec5WT and $Sec5^{T11A}$ cDNAs were cloned to pCMV-Myc vector (addgene), as well as Exo84WT and $Exo84^{A228W}$ cDNAs. Fluorescein isothiocyanate (FITC)-goat anti-mouse and TexasRed-goat anti-mouse immunoglobulin IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Horseradish peroxidase-conjugated goat anti-mouse and goat antirabbit antibodies were purchased from Promega (Madison, WI).

## Immunofluorescent staining

MDCK Cell lines incubated on the collagen coated coverslips were fixed at 4℃ with 2% paraformaldehyde for 20 minutes and quenched by Ringer's saline (150 mM NaCl, 1.8 mM $Ca^{2+}$, 7.2 mM KCl, and 10 mM HEPES, pH 7.4) containing 50 mM $NH_4Cl$. Permeabilization was performed with CSK buffer (1% TritonX-100, 10 mM Pipes, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$) for 10 minutes supplemented with protease inhibitors (1 mM pefabloc and 10 μg/ml each of aprotinin, antipain, leupeptin, and pepstatin A). After samples were blocked with 0.2% fish-skin gelatin and goat serum in Ringer's saline (blocking buffer) for 1 hour, primary antibodies

were diluted in blocking buffer and applied for 1 hour at 4°C. After 3 washes with blocking buffer, FITC or Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch) were applied for 1 hour at 4°C and DAPI was performed for 5 min at room temperature. Samples were washed 3 times with blocking buffer, and coverslips were mounted onto slides using Elvanol mounting medium (Waterborne). Cell Images were obtained using Zeiss 700 scanning confocal microscope (Thornwood, NY; 63X objective) equipped with a krypton/argon laser (FITC excitation using 488 nm laser line, or and Texas Red excitation using 543 laser line).

## VSV-G trafficking assay

YFP-VSV-G exocytic trafficking is followed by the method from Julia von Blume *et al* (*49*). YFP-VSV-G were transfected to shCtrl, shRalA, and shRalB cell lines using adenovirus transduction at 37°C for 24hr, respectively. Then, cells were shifted to 40°C for 16 hr and the YFP-VSV-G had the thermo-sensitive-folding defect to accumulate in the endoplasmic reticulum (ER). After that, cells were shifted to 20°C for 2 hr and the YFP-VSV-G refolded to accumulate at the trans-Golgi network (TGN). 100 μM cycloheximide was added to prevent further new-synthesized protein. Then, cells were shifted to 32°C allowing the transport of YFP-VSV-G from the TGN to the plasma membrane. At the indicated times, cells were fixed without permeabilization and incubated with the antibody that recognizes the extracellular domain of YFP-VSV-G and Texas Red-conjugated secondary antibody. Cell images were performed by immunofluorescence microscopy and images were captured with a Zeiss 700 laser-scanning confocal microscope.

Surface biotinylation assay

shCtrl, shRalA, and RalA rescued MDCK cell lines were incubated in 12-mm Transwell 0.45-μm polycarbonate filters at high density to form the monolayer and establish polarity within several days. YFP-VSV-G was transfected using adenovirus transduction on both apical and basolateral side. Following the YFP-VSV-G trafficking assay, cells were placed on ice at indicated time points. Then, Sulfo-NHS-SS-Biotin was applied to both apical and basolateral surfaces to label the cell-surface delivered YFP-VSV-G. Biotinylation reactions were quenched by washing cells with TBS buffer (120 mM NaCl and 10 mM Tris, pH 7.4) containing 50 mM NH4Cl and 0.2% bovine serum albumin at 4°C. Cells were lysed by RIPA buffer. Then, 10% of the lysate was removed and mixed with SDS-PAGE sample buffer for quantitation of total protein expression. The remained 90% lysate were followed by streptavidin precipitation of biotinylated proteins. Samples were analyzed using westernblot (WB) and anti-VSV-G antibody (P5D4, abcam). WBs were performed as described previously (*52*). WBs were analyzed by Odyssey, version 2.0. The relative ratio of streptavidin-precipitated to total lysate at every time point will be calculated.

E-cadherin trafficking assay

MDCK II G cells were seeded on collagen coated coverslips for 24 hr incubation at 37℃. Cells were then trypsinized and co-transfected with RFP-E-cadherin plasmid and pCMV-Myc based Sec5WT or $Sec5^{T11A}$ by Nucleofection method (Lonza). The DNA ratio of RFP-E-cadherin to pCMV-Myc based plasmid is 1 to 3. The same procedure was performed to pCMV-Myc based Exo84WT or $Exo84^{A228W}$. After nucleofection, cells

were reseeded on collagen coated coverslips and incubated for 24 hr at 37℃. Cells were then fixed and permeabilized for Zeiss 700 laser-scanning confocal microscope.

# CHAPTER IV: DISCUSSION

## Summary

In this study, we used RNAi technology to identify specific and different roles for RalA and RalB in basolateral protein exocytosis in MDCK epithelial cells. We determined that RalA, but not RalB, is required for basolateral exocytosis of VSV-G in MDCK cells. RalA regulates VSV-G trafficking through the interactions with both Sec5 and Exo84 subunits of the Exocyst complex. Combined with our previous study (*6*), we suggest that RalA may bind Sec5 at the TGN/recycling endosome to facilitate trafficking to the plasma membrane, and subsequently bind Exo84 to mediate vesicle tethering to sites of fusion. Finally, we showed that overexpression of Ral-uncoupled Sec5 mutant inhibits E-cadherin exocytic trafficking, but that overexpression of Ral-uncoupled Exo84 mutant did not. While this result is open to multiple interpretations, we conclude from our collective studies that RalA and RalB do not perform redundant activities in polarized membrane trafficking, and that the two Exocyst subunits that serve as Ral effectors (namely Sec5 and Exo84) do not have identical functions in basolateral exocytosis.

## Significance

In this study, we determined that RalA regulates basolateral protein trafficking through interactions with Sec5 and Exo84. We used RNAi technology, coupled with rescue or partial rescue studies, which no other lab used before, to create Sec5- or Exo84-uncoupled Ral GTPase mutant constructs. Continued analysis of our cell lines is expected to establish distinct roles for RalA and RalB in epithelial polarity development and

elucidate molecular details concerning how Ral GTPases regulate Exocyst activites during basolateral membrane trafficking. This contribution will be significant because it is expected to provide new insights into molecular mechanisms involved in membrane trafficking. These mechanisms are likely to be fundamental in many cell and tissue types, and likely to be perturbed in various disease states. For example, autosomal dominant polycystic kidney disease (ADPKD), the most common mono-genetic disease in man, is accompanied by defective basolateral trafficking that is caused by defects of the exocyst (*17*). Therefore, it is reasonable to expect that understanding how Ral GTPases and the Exocyst function during basolateral trafficking in renal epithelial cells will reveal details about how trafficking is perturbed in ADPKD. In addition, Ral GTPases and the Exocyst are involved in other trafficking events that are perturbed in other human diseases. This includes GLUT-4 trafficking in adipocytes, which is impacted in Type II diabetes (*53, 54*), and GTP-dependent exocytosis of dense core vesicles in many different cell types such as insulin-secreting cells (*55, 56*), catecholamine-secreting chromaffin cells (*35, 57*) and platelet granule secretion (*58*). Therefore, completion of our studies will likely provide novel insights into the mechanism by which Ral GTPases and the Exocyst regulate exocytosis in multiple cell types, and potentially lead to discovery of new targets for the treatment of major diseases such as ADPKD, diabetes and hypertension.

## Discussion

The objective of our study was to analyze roles of Ral GTPases and the Exocyst in regulating basolateral protein trafficking by using cell lines stably expressing canine-specific shRNAs that target either RalA or RalB. In shRalA and shRalB cell lines,

knockdown efficiency was >70% for RalA and >95% for RalB, as determined by immunoblotting (*6*). Knockdown of RalA was somewhat less efficient than that of RalB. This leaves open the possibility that residual endogenous RalA might continue to work with the Exocyst. To solve the problem, future efforts will be directed to developing cells in which the clustered regularly interspaced short palindromic repeats (CRISPR) system is used to achieve total knockout of RalA and RalB.

### *Interactions between RalA and the Exocyst are necessary for efficient basolateral exocytosis*

We have observed that knockdown of RalA, but not RalB, inhibited basolateral YFP-VSV-G exocytosis (Fig. 1). Cell imaging studies showed that active RalA is localized at both the plasma membrane and perinuclear recycling endosomes, while active RalB is localized only at the plasma membrane (*39*). This difference in localization is consistent with our hypothesis that active RalA is involved in transporting vesicles from perinuclear recycling endosomes to plasma membrane. Also consistent with this interpretation is the finding that constitutively active RalA, but not RalB, accelerated basolateral exocytosis of E-cadherin (*39*). Finally, an earlier study reported that decreasing RalA protein expression interferes with polarized trafficking of multiple basolateral proteins, including gp58 and epidermal growth factor receptor (EGFR) (*18*). Collectively, these results indicate that RalA is as a positive regulator of basolateral exocytosis in epithelial cells.

RalA and RalB have identical effector binding domains. However, how these two

proteins perform distinct functions remains unclear. The reasons might be that 1) active RalA binds to the exocyst complex much more efficiently than active RalB does (*39*); 2) the subcellular localization of RalA and RalB are different. Recent crystal structural analysis has showed that a major binding interaction between RalA and Sec5 might occur at residues 38 to 47 of the switch 1 region of RalA structure (*37*). However, RalB might interact with Sec5 through its switch 2 regions, suggesting that RalA and RalB might bind differently to Sec5 and/or Exo84. On the other hand, the variable region near the C-terminus of Ral GTPases are crucial for the specific sub-cellular localization of active Ral GTPases. This region is different between RalA and RalB. Interestingly, a chimeric RalA containing the variable region of RalB failed to localize at the recycling endosome, even though it still possessed high-affinity binding ability to Sec5 and Exo84 (REF). Thus, the variable region and the Exocyst binding domain are likely working together to promote Ral specificity during basolateral protein delivery (*39*). Our YFP-VSV-G trafficking results (Fig. 1) and surface biotinylation data (Fig. 4 and Fig. 5), together with published results from our lab (*6*) support the conclusion that these two highly related GTPases play different roles in membrane trafficking in epithelial cells.

### *RalA-Sec5 and RalA-Exo84 interactions play different roles in basolateral trafficking*

We have observed that YFP-VSV-G trafficking was arrested in a perinuclear compartment, and that a Sec5-uncoupled $RalA^{38R}$ mutant failed to rescue this trafficking defect (Fig. 2j). We conclude that RalA-Sec5 interaction is crucial to transport YFP-VSV-G from the perinuclear compartment to the cell periphery. In the $RalA^{47E}$ rescue

cell line, we observed that a significant fraction of the YFP-VSV-G was transported to the cell periphery, although some remained arrested in the cytoplasm (Fig. 2m). Since the overall surface expression of YFP-VSV-G was still substantially reduced compared with control cells, we conclude that the RalA-Exo84 interaction is also required for basolateral trafficking of this cargo. Because binding sites for Sec5 and Exo84 overlap on RalA, it is not possible for one GTPase protein to simultaneously bind both Exocyst subunits (*37*). We suggest that correct localization of RalA, and of cargo trafficked in a RalA-dependent manner, may depend on sequential binding of the GTPase to Sec5 and Exo84. Consistent with this prediction, recent work from our lab has shown that $RalA^{38R}$ is primarily localized at the perinuclear compartment, but that $RalA^{47E}$ is primarily localized at the plasma membrane in MDCK cells (*6*), Larry Feig *et al* also have showed that active RalA ($RalA^{72L}$) was enriched at both plasma membranes and recycling endosomes, where it colocalized with another Exocyst subunit (Sec6) (*6*). Other recent studies also support the idea that the Exocyst functions at both the plasma membrane (*21*) and in recycling endosomes (RE) (*59*). We suggest that RalA acts to coordinate the trafficking of cargo-laden transport vesicles from the recycling endosome to the plasma membrane by acting sequentially upon Sec5 and Exo84 at the two sites.

An open question is whether the same RalA GTPase binds sequentially to its two effectors within the Exocyst complex, or whether sequential binding involves different RalA protein molecules that bind (and perhaps dissociate) at the two sites. For the former to occur, there must exist a mechanism whereby active RalA can disengage one subunit without hydrolyzing its GTP, and subsequently bind the other subunit. One possibility is

that binding to membrane phosphoinositol lipids could compete for RalA to Exo84, because its Ral-binding domain is also a pleckstrin-homology domain that has affinity for specific lipids (*18*). An alternative mechanism is suggested by recent work from the Saltiel group, who recently demonstrated that Sec5 phosphorylation promotes its disengagement from active RalA prior to membrane fusion (*13*). They suggest that active RalA first engages Sec5 to induce Exocyst recruitment onto transport vesicles. Subsequent phosphorylation of Sec5 by protein kinase C (PKC) causes its dissociation from active RalA. This results in the release of the vesicle from Sec5 so that it can fuse with the plasma membrane (*60*). Dephosphorylation of Sec5 by a phosphatase then occurs for another cycle of vesicle recognition and transport (*60*). However, the fate of the RalA that dissociated from Sec5 was not examined in this paper. Perhaps this RalA protein, still charged with GTP, now binds Exo84 at the plasma membrane to mediate a late stage of exocytosis. Based on our results, and those published by us and others, we propose that RalA binds Sec5 at the donor compartment (either the TGN or recycling endosomes) to promote some event that is required for vesicle release and/or trafficking to the cell periphery. Subsequently, we suggest that either the same RalA or a newly recruited RalA GTPase binds Exo84 at the plasma membrane to promote a late stage in vesicle tethering or docking. This anticipates that uncoupling RalA from Sec5 and Exo84 will have distinct impacts on trafficking, and our analysis of both VSVG and E-cadherin trafficking supports this model. However, additional work will still be required to determine the mechanism by which RalA-Exocyst interactions affect the basolateral exocytosis observed here. In addition, Hierro *et al* have suggested that the Golgi-associated retrograde protein (GARP), a hetero-tetrameric tethering factor, improves

fusion of endosome-derived, retrograde transport carriers to the trans-Golgi network (TGN) (*61*). Since GARP is also structurally related to the Exocyst, this indicates an attractive idea that RalA-Sec5 and RalA-Exo84 interactions may involve in similar mechanism with retrograde transport to dock vesicles from plasma membrane to RE or TGN.

### *RalB may act antagonistically to RalA in protein trafficking*

In our study, we have observed that silencing of RalB had no effect on basolateral exocytosis (Fig. 1C, Fig. 3, and Fig. 5). What, if anything, might be the function of RalB in protein trafficking? RalB has been shown to be involved in in receptor-mediated endocytosis, including tranferrin receptor endocytosis and EGFR endocytosis (*62*). Although RalA and RalB have identical Sec5 or Exo84-binding domains, the two isoforms have different affinities for Exocyst subunits and different subcellular localizations, which allow them to perform distinct functions. C. Hazelett *et al* showed that knockdown of RalB, but not RalA, disrupts the endocytosis of basolateral E-cadherin in MDCK cells, suggesting that RalB regulates tight junction (TJ) assembly via selectively controlling endocytosis of TJ components (*6*). Hazelett *et al* also demonstrated that RalB-dependent endocytosis of TJ components was dependent on interactions with the Exocyst and not other Ral effectors (*6*). Knockdown of RalA generally decreased stability of TJ components, while knockdown of RalB generally increased stability of these same components (*6*). Therefore, we propose that RalA promotes basolateral exocytosis via interactions with Sec5 and Exo84, while RalB regulate endocytosis via interactions with these same effectors. However, the mechanism

by which RalB regulates endocytosis via the Exocyst is not known and is the subject of future studies.

## Future Directions

Identification of novel roles for Ral GTPases and the Exocyst in protein trafficking led to additional questions. We have shown that: 1) RalA, but not RalB, is required for basolateral exocytosis of VSV-G in MDCK cells; 2) RalA regulates VSV-G trafficking through interactions with both Sec5 and Exo84; and 3) the specific contributions of RalA-Sec5 and RalA-Exo84 interactions to basolateral trafficking may be distinct. Major unresolved questions remain concerning the molecular mechanisms by which RalA, RalB, Sec5 and Exo84 regulate membrane trafficking and epithelial cell polarization. Our overall model is that the Exocyst is a multimeric protein scaffold that recruits additional factors to accomplish specific cellular activities. We imagine that the ability of the Exocyst to recruit these accessory factors is allosterically regulated by Ral GTPase binding to Sec5 and Exo84. However, the identification of specific accessory factors that engage the Exocyst during exocytosis and endocytosis of basolateral membrane cargo is not complete. We also lack a detailed understanding of how RalA or RalB binding to Exocyst subunits facilitates recruitment (or disengagement) of these accessory factors. These are the major areas of future study.

### *Determine how RalA and RalB differentially regulate protein trafficking through the Exocyst*

We have shown that RalA, but not RalB, through binding both Sec5 and Exo84 is

required for basolateral VSV-G exocytosis (Fig. 1 and Fig. 2). In addition, C. Hazelett *et al* showed that knockdown of RalB, but not RalA, disrupts the endocytosis of basolateral E-cadherin in MDCK cells (*6*), suggesting that RalA and RalB may have different, even opposing functions in regulating polarized membrane definition. That these appear to involve binding to a common pair of effectors (Sec5 and Exo84) is both fascinating and puzzling.

One resource that will be valuable for further studies will be MDCK cell lines in which expression of either Sec5 or Exo84 is reduced or depleted, and additional cell lines in which Ral-uncoupled mutants of each subunit are expressed. Like the Ral-reduced cell lines used in this study, we will generate Sec5- and Exo84-depleted MDCK cell lines using RNAi technology. ShRNA oligos targeting either canine Sec5 or Exo84 have been cloned into the pSiCoR-PGK-Puro (Addgene) lentivirus system to generate lentivirus with shRNAs for cellular transduction. Sec5 and Exo84 knockdown efficiency will be confirmed using quantitative PCR and WB. Once these cell lines have been generated, we can use them to perform YFP-VSV-G or E-cadherin trafficking assay. We also have generated pCMV-Myc based Sec5 WT, Exo84 WT, $Sec5^{T11A}$ (Ral uncoupled Sec5 mutant) and $Exo84^{A228W}$ (Ral uncoupled Exo84 mutant) mutants for rescue experiments. The rescue experiments above will provide more information to explain Ral-Exocyst interaction in basolateral protein trafficking. It is possible that we might have difficulty in generating stable knockdown of Sec5 and Exo84 via shRNA. This could cause difficulty in interpreting results if residual endogenous Sec5 and Exo84 continue to assemble into Exocyst complexes. To solve the problem, we can use the clustered regularly interspaced

short palindromic repeats (CRISPR) system to achieve knockout of Sec5 and Exo84, and use genome-edited cells that completely lack either protein for our studies.

We have observed that uncoupling RalA from either Sec5 or Exo84 causes defects on basolateral VSV-G exocytosis, like RalA knockdown does (Fig. 2). In addition, because we observe different sub-cellular localizations of $RalA^{38R}$ and $RalA^{47E}$, we suggest that RalA may bind Sec5 at the TGN/RE to facilitate trafficking to the plasma membrane, where RalA-Exo84 subsequently mediates vesicle tethering to sites of fusion (*6*). We hypothesize that RalA can sequentially engage Sec5 at recycling endosome (RE) and Exo84 at plasma membrane (PM) to transport basolateral proteins from the Golgi to basolateral membrane. We will use the approach of shRNA knockdown, immunofluorescent staining, and cellular fractionation to study how RalA sequentially engages Sec5 and Exo84. With the advent of super-resolution microscopy, it will be possible to determine whether cargo-laden vesicles that traffic to the cell periphery in cells with compromised RalA-Exo84 binding are locked in a tethered but non-fused state beneath the plasma membrane, as we imagine them to be.

The Exocyst has also been implicated in endosomal recycling and transcytosis (*14*). Knockdown of RalB resulted in defective endocytosis of E-cadherin (*6*), but knockdown of RalB had no effect on exocytosis of YFP-VSV-G (Fig. 1C). In addition, the evidence that suggests RalA and RalB serve antagonistic functions during such complex events as cell growth and migration (*63, 64*). Furthermore, active RalB localizes to the plasma membrane, where it could participate in endocytosis. We hypothesize that

RalB can engage the Exocyst to regulate endocytosis of basolateral proteins, but whether this is a direct or indirect function is not known. In our previous study, we have used quench biotinylation assay to prove that knockdown of RalB reduces endocytosis of E-cadherin (*6*). We have generated RalBWT, $RalB^{38R}$ (Sec5-uncoupled) and $RalB^{47E}$ (Exo84-uncoupled) rescue and partial resuce cell lines. These will be used to assess whether Sec5 and Exo84 work with RalB in E-cadherin endocytosis using quench biotinylation assay.

*Determine how RalA regulates exocytosis by the Exocyst and accessory factors*

We suggest that Ral GTPases engage the Exocyst complex to prime it for specific functions by recruiting additional factors (*40, 41, 45*). Recent work from our lab has shown that knockdown of RalA reduced the association of the Exocyst subunit Sec6 with Munc18c, while knockdown of RalB had no effect on this association (data not shown). Munc18c belongs to the Sec1/Munc18-like (SM) protein family, which regulates soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE)-mediated plasma membrane fusion (*28*). Munc18c specifically binds to the target SNARE (t-SNARE) Syntaxin4 N-terminus to stabilize it at basolateral membrane (*65*). Also, Munc18c binds to the trans-SNARE complex and strongly accelerates membrane fusion kinetics (*28*). In addition, F. Morgera *et al* also have identified that in yeast cells the exocyst subunit Sec6 regulates exocytosis through binding of the Sec1, a yeast SM protein (*27*). Furthermore, in other experiments in yeast, loss of Exocyst function still allowed secretory vesicles to be delivered to the plasma membrane, but these vesicles could not dock and fuse because assembly of SNARE complexes was compromised (*29,*

*66*). Therefore, the above evidence suggests that the RalA and Exocyst function may be required for the assembly of SNARE complexes and the fusion of the vesicles with the plasma membrane. However, the precise mechanisms underlying how the Exocyst regulates the SNARE complex and plasma membrane fusion is incompletely understood.

Based on the above evidence, we hypothesize that RalA through its interaction with Sec5 and Exo84, may somehow facilitate the interaction of Sec6 with Munc18c, and that this is critical for membrane fusion to occur. We will use cell fractionation and IF to confirm the cellular colocalization of RalA, Sec6 and Munc18c. In addition, we will determine how RalA-mediated exocyst complex bind Munc18c. The difference of Munc18c binding affinity betweeen exocyst-bound Sec6 and non-exocyst-bound Sec6 will be analyzed using RalA-depleted cells, co-immunoprecipitation (Co-IP) and gel filtration, indicating the mechanism by which the Sec6-Munc18c interaction occurs within the Exocyst complex. Furthermore, we will analyze whether RalA and the Exocyst can affect the formation of SNARE complex using Co-IP deceased association between v-SNARE and t-SNARE among RalA-, Sec5-, and Exo84-depleted and controlled MDCK cells. Vesicle fusion events at the plasma membrane will be assessed by transfection of TfR-mCh-SEP, a dual color probe (*65*), into RalA-, RalB-, Sec5-, Exo84-depleted and controlled MDCK cells. We expect that the defect of SNARE complex formation and decreased membrane fusion signal in RalA-depleted cells.

*Future expectation*

In the past, the functions of the Exocyst were thought to be confined to only the tethering step of membrane trafficking, and not earlier or later stages of exocytosis. However, our results suggest that disrupting Exocyst activities can result in cargo accumulation in a perinuclear compartment (likely to be the recycling endosome, which is the donor organelle for basolateral membrane-destined transport vesicles). We also observe that interfering with Exocyst functions in other ways can affect cargo delivery at post-tethering stages. Completion of these above studies will provide with a novel mechanism to explain how Ral GTPases and Exocyst regulate basolateral exocytosis in renal epithelial cells. Also, the bridge between membrane fusion and the Exocyst will be established. In the future, several questions is still needed to be asked. For example, are there any other binding partners that facilitate communication between the Exocyst and SNARE complexes to regulate their function? Does the Exocyst coordinate plasma membrane events with activities at other organelles such as the TGN and recycling endosomes? If so, how? What are the roles of Ral-Exocyst interactions in other cellular and developmental processes of renal epithelial cells, such as primary ciliogenesis and cystogenesis? Since we have generated Ral-depleted and Exocyst-depleted cell lines, further studies for above questions can be elucidate by the loss- and partial gain-of-function analysis.

**Figure 1. Knockdown of RalA, but not RalB, inhibits exocytic transport of YFP-VSV-G.** (A) Cell surface delivery of YFP-VSV-G is inhibited in RalA knockdown, not RalB knockdown MDCK cells. shCtrl (A), shRalA (B), or shRalB (C) MDCK cells were transfected with YFP–VSV-G proteins. The arrow head shows intracellular accumulated YFP-VSV-G; The Arrow shows YFP-VSV-G on cell surface. Methods: After 24 h incubation at 37°C, YFP–VSV-G transfected cells were shifted to 40°C for 16 hr, Then, cells were incubated for 2 h at 20°C in the presence of 100 μM cycloheximide to accumulate VSV-G in the TGN. Finally, cells were shifted 32°C to allow delivery of YFP-VSV-G from the TGN to the cell surface. Samples were collected at the indicated time points, and cell surface YFP-VSV-G was recognized by the antibody against the extracellular domain of VSV-G (8G5F11) and Texas Red-conjugated secondary antibody. Scale bar: 20 μm. (D) After 60min shifting to 32°C, the relative ratio of YFP-VSV-G at the cell surface to the inside the cells were determined by ImageJ. Data are means ± SD. (n = 50) ****: $P < 0.0001$ by one-way ANOVA multiple comparisons.

**Figure 1.**

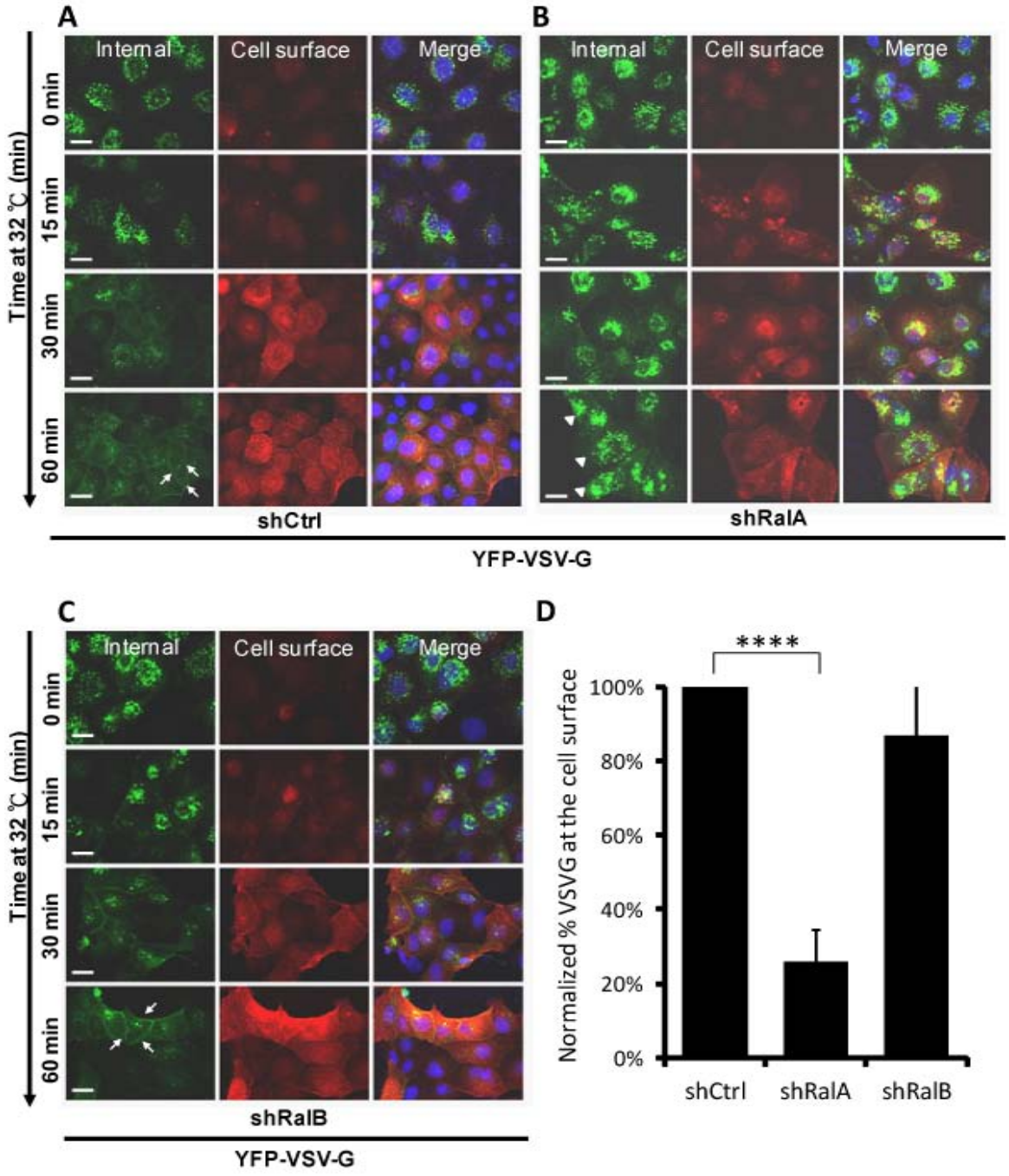

A
B
Internal
Cell surface
Merge
Time at 32 ℃ (min)
0 min
15 min
30 min
60 min
shCtrl
shRalA
YFP-VSV-G
C
D
shRalB
****
Normalized % VSVG at the cell surface
100%
80%
60%
40%
20%
0%
shCtrl
shRalA
shRalB

**Figure 2. Sec5 and Exo84 are required for RalA-mediated basolateral YFP-VSV-G trafficking.** After shifting to 32°C for 60 min, compared to shCtrl cells (a-c), YFP-VSV-G accumulated in the intracellular region in the shRalA cells (d-f). $RalA^{72L}$ can restore the cell surface delivery of YFP-VSV-G (g-i); $RalA^{38R}$ can't restore RalA knockdown phenotype (arrow) (j-l), $RalA^{47E}$ partially restore RalA knockdown phenotype (arrow head: YFP-VSV-G cell surface signal) (m-o). Cell surface YFP-VSV-G was localized using the antibody against the extracellular domain of VSV-G (8G5F11) and Texas Red-conjugated secondary antibody. Scale bar: 20 μm. (p) After 60min shifting to 32°C, the the relative ratio of YFP-VSV-G at the cell surface to inside the cells were determined by ImageJ. Data are means ± SD. (n = 50) ****: $P < 0.0001$; ***: $P < 0.001$; *: $P < 0.1$ by one-way ANOVA multiple comparisons

**Figure 2.**

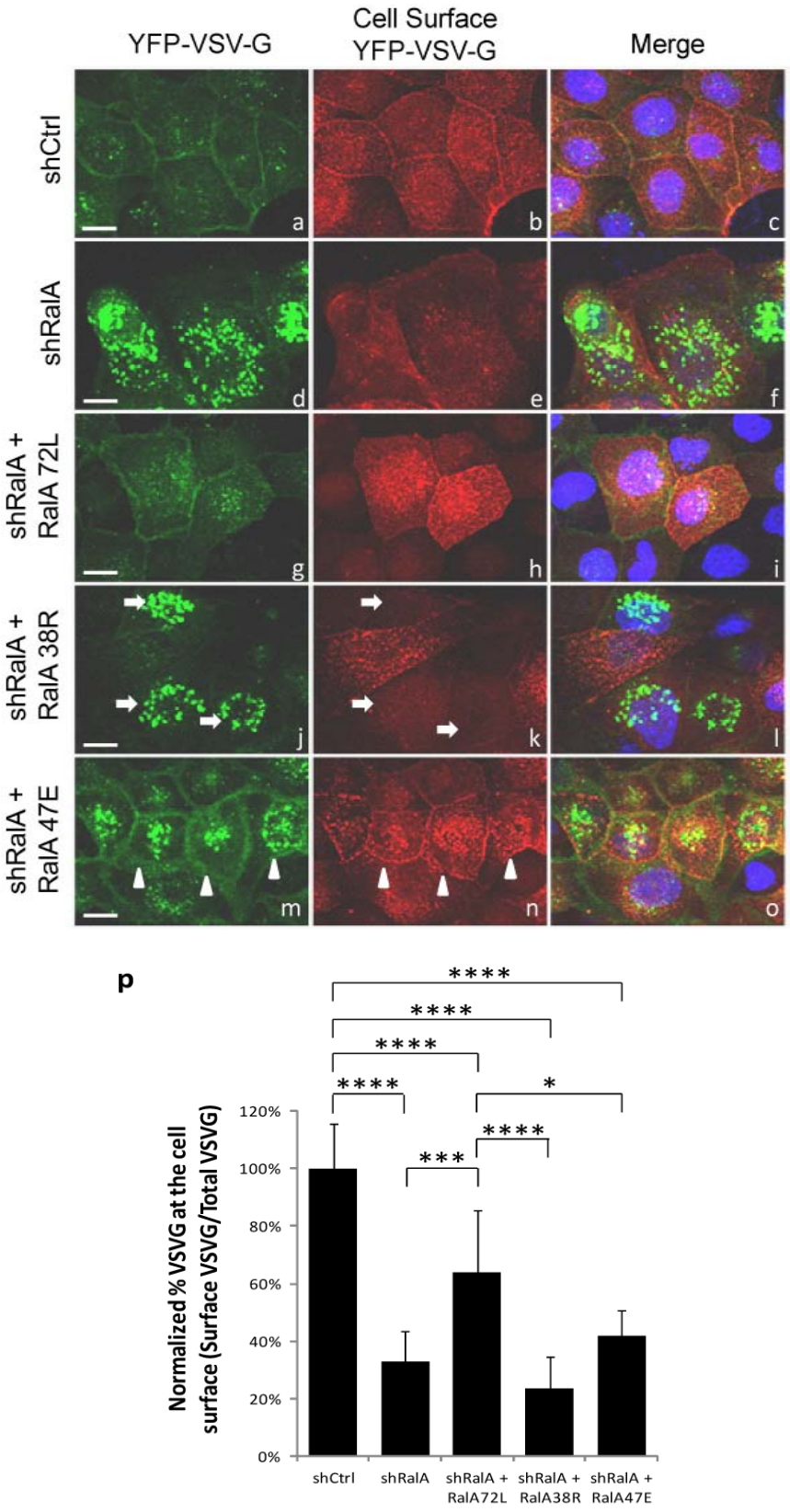

YFP-VSV-G
Cell Surface YFP-VSV-G
Merge
shCtrl
shRalA
shRalA + RalA 72L
shRalA + RalA 38R
shRalA + RalA 47E
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
Normalized % VSVG at the cell surface (Surface VSVG/Total VSVG)
120%
100%
80%
60%
40%
20%
0%
****
****
****
****
*
****
***
shCtrl
shRalA
shRalA + RalA72L
shRalA + RalA38R
shRalA + RalA47E

**Figure 3. RalB knockdown doesn't affect basolateral YFP-VSV-G exocytosis.** (A) After shifting to 32°C for 60min, compared to shCtrl cells, in shRalB and in RalBWT rescued cells, YFP-VSV-G can still be delivered to cell surface. Cell surface YFP-VSV-G was localized using the antibody against the extracellular domain of VSV-G (8G5F11) and Texas Red-conjugated secondary antibody. Scale bar: 20 μm. (B) After 60min shifting to 32°C, the relative ratio of YFP-VSV-G at the cell surface to inside the cells were determined by ImageJ. Data are means ± SD. (n = 50) No significance by one-way ANOVA multiple comparisons.

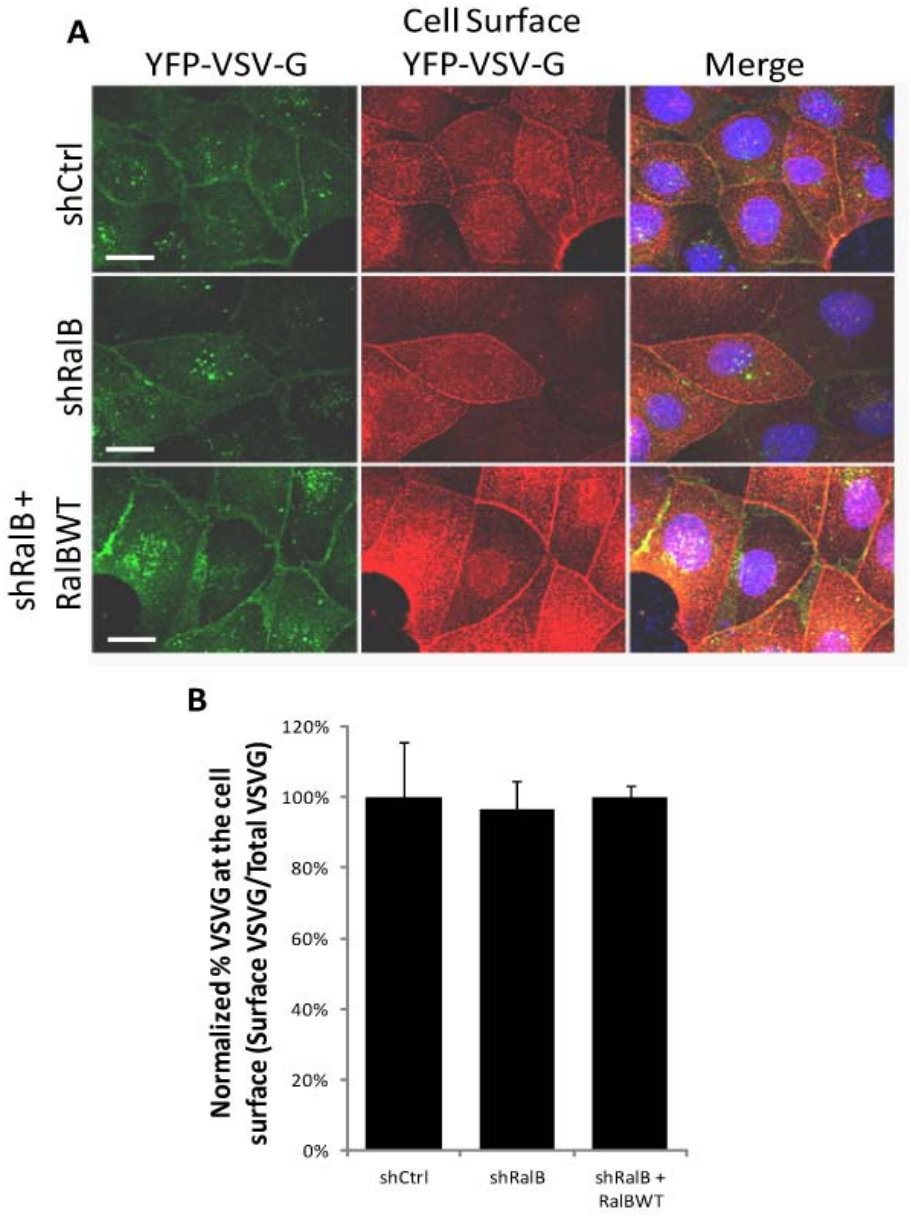


**Figure 4. Both Sec5 and Exo84 are required to VSV-G trafficking.** (A) A surface biotinylation assay was performed in shCtrl, shRalA, and RalA rescued cells. At various time points after shifting to 32°C, surface proteins were biotinylated and precipitated with streptavidin agarose. Samples of total cell lysate (10%) or streptavidin-agarose pull-downs (90%) were analyzed by SDS-PAGE and immunoblotted with anti-VSV-G antibody (P5D4). The intensity of bands on WBs were analyzed by Odyssey. (B) The kinetics with which YFP-VSV-G achieved a steady-state distribution at the plasma membrane were decreased in shRalA cells, relative to shCtrl cells. RalAWT can also rescue RalA knockdown phenotype, (C)(D) Both $RalA^{38R}$ and $RalA^{47E}$ can't rescue RalA knockdown phenotype. However, these data need to be repeated for statistical significance.

**Figure 4.**

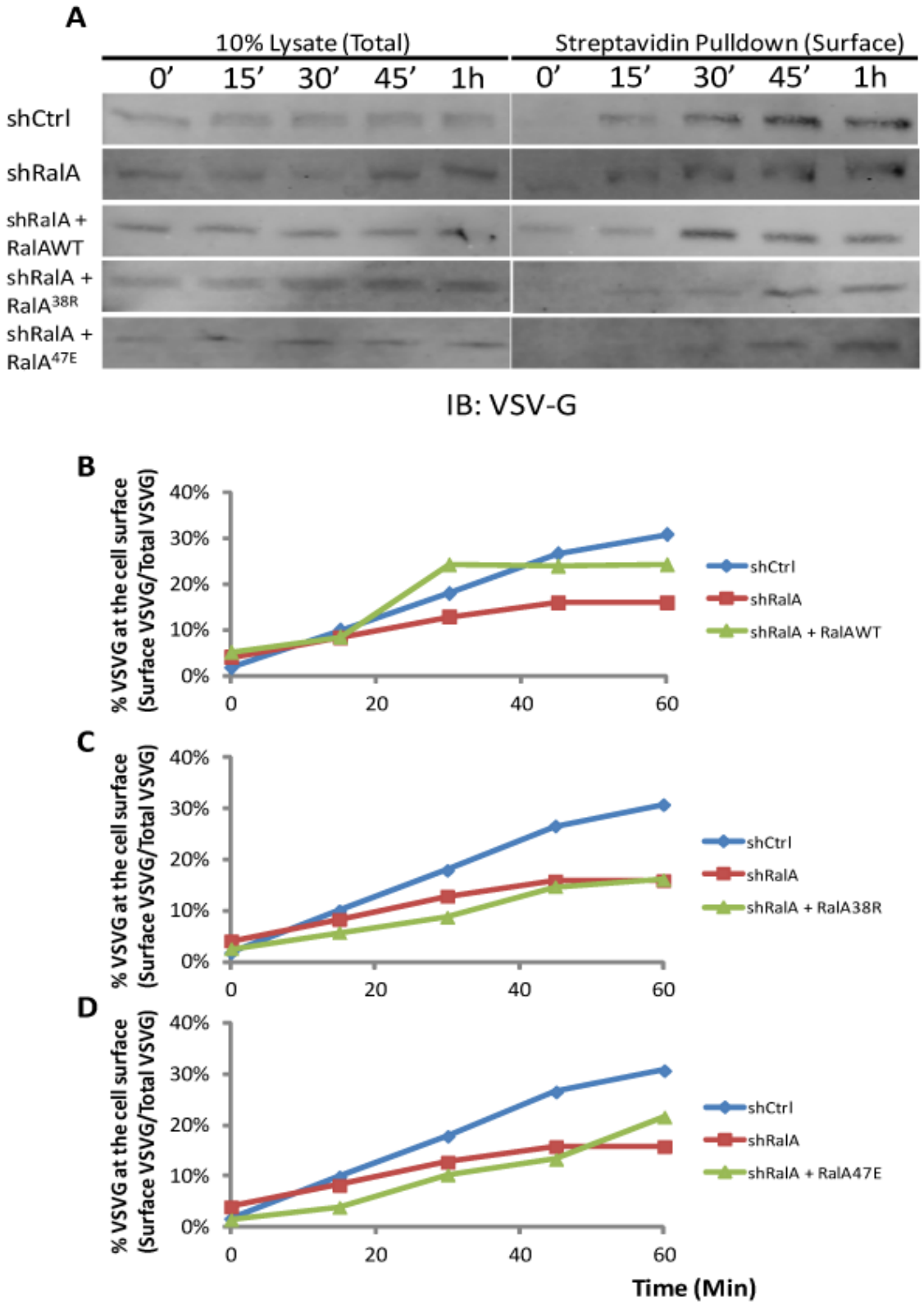
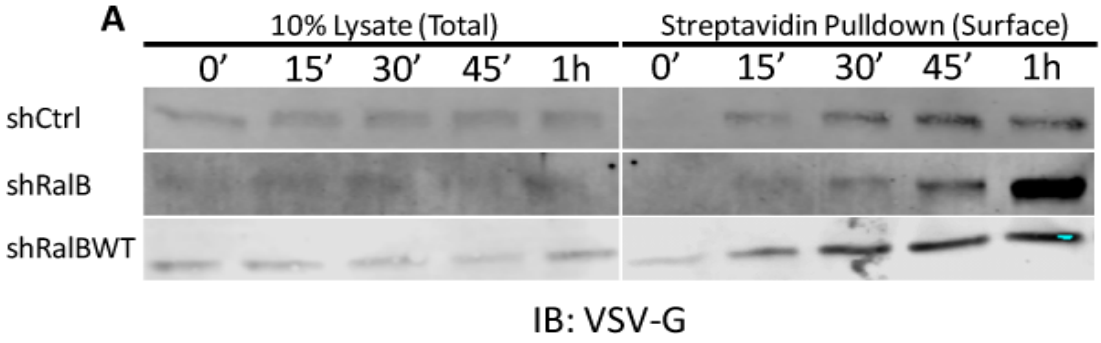

A
10% Lysate (Total)
Streptavidin Pulldown (Surface)
0′ 15′ 30′ 45′ 1h 0′ 15′ 30′ 45′ 1h
shCtrl
shRalA
shRalA + RalAWT
shRalA + RalA38R
shRalA + RalA47E
IB: VSV-G
B
C
D
% VSVG at the cell surface (Surface VSVG/Total VSVG)
40%
30%
20%
10%
0%
0
20
40
60
shCtrl
shRalA
shRalA + RalAWT
shRalA + RalA38R
shRalA + RalA47E
Time (Min)

**Figure 5. RalB knockdown doesn't affect basolateral YFP-VSV-G exocytic trafficking in surface biotinylation assay.** (A) A surface biotinylation assay was performed in shCtrl, shRalB, and RalBWT rescued cells. At various time points after shifting to 32°C, surface proteins were biotinylated and precipitated with streptavidin agarose. Samples of total cell lysate (10%) or streptavidin-agarose pull-downs (90%) were analyzed by SDS-PAGE and immunoblotted with anti-VSV-G antibody (P5D4). The intensity of bands on WBs were analyzed by Odyssey. (B) There is no difference in the kinetic curves observed for shRalB, RalBWT rescue cells and shCtrl cells. However, these data need to be repeated for statistical significance.



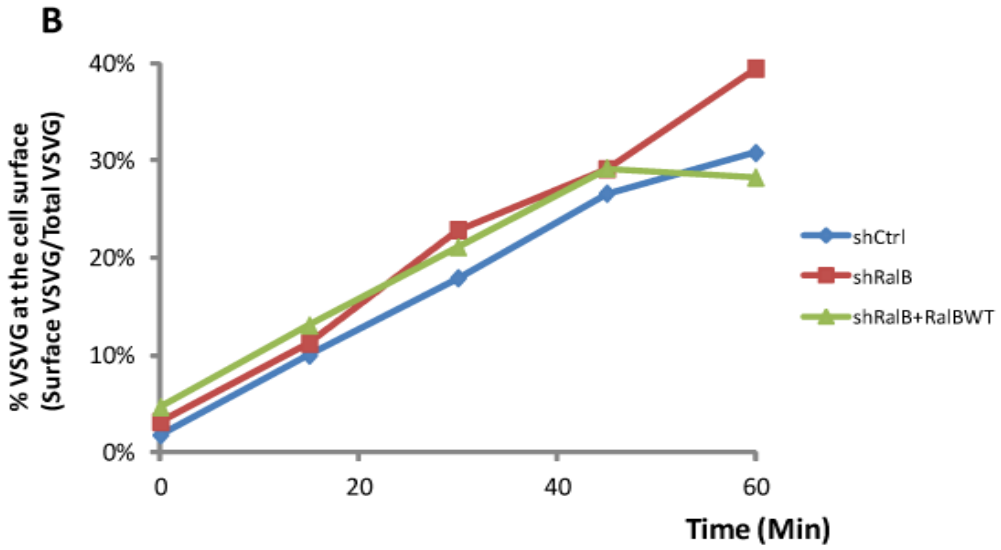



**Figure 6. Ral-Sec5 interaction is required for basolateral E-cadherin exocytosis.** (A) Compared to myc-tagged Sec5WT overexpressed MDCK cells and vector-only cells, myc-tagged $Sec5^{T11A}$ mutant inhibits E-cad-RFP exocytosis; Vector means pCMV-myc. Scale bar: 20 μm. (B) Quantitative analysis was used to measure the percentage number of cells with surface E-cadherin accumulation between three different transfected cells. Data are means ± SD. (n = 50) ****: P < 0.0001 by one-way ANOVA multiple comparisons.

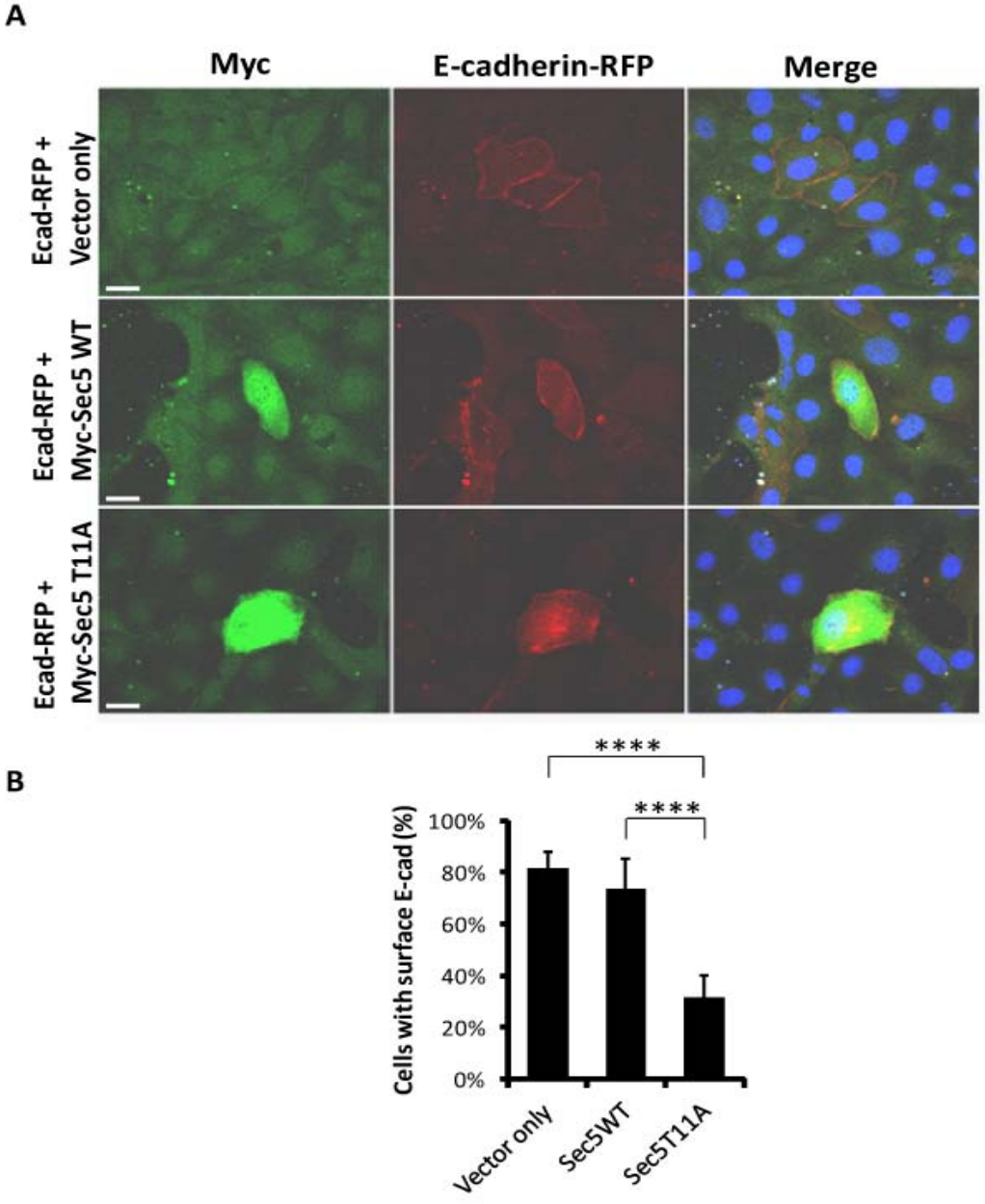
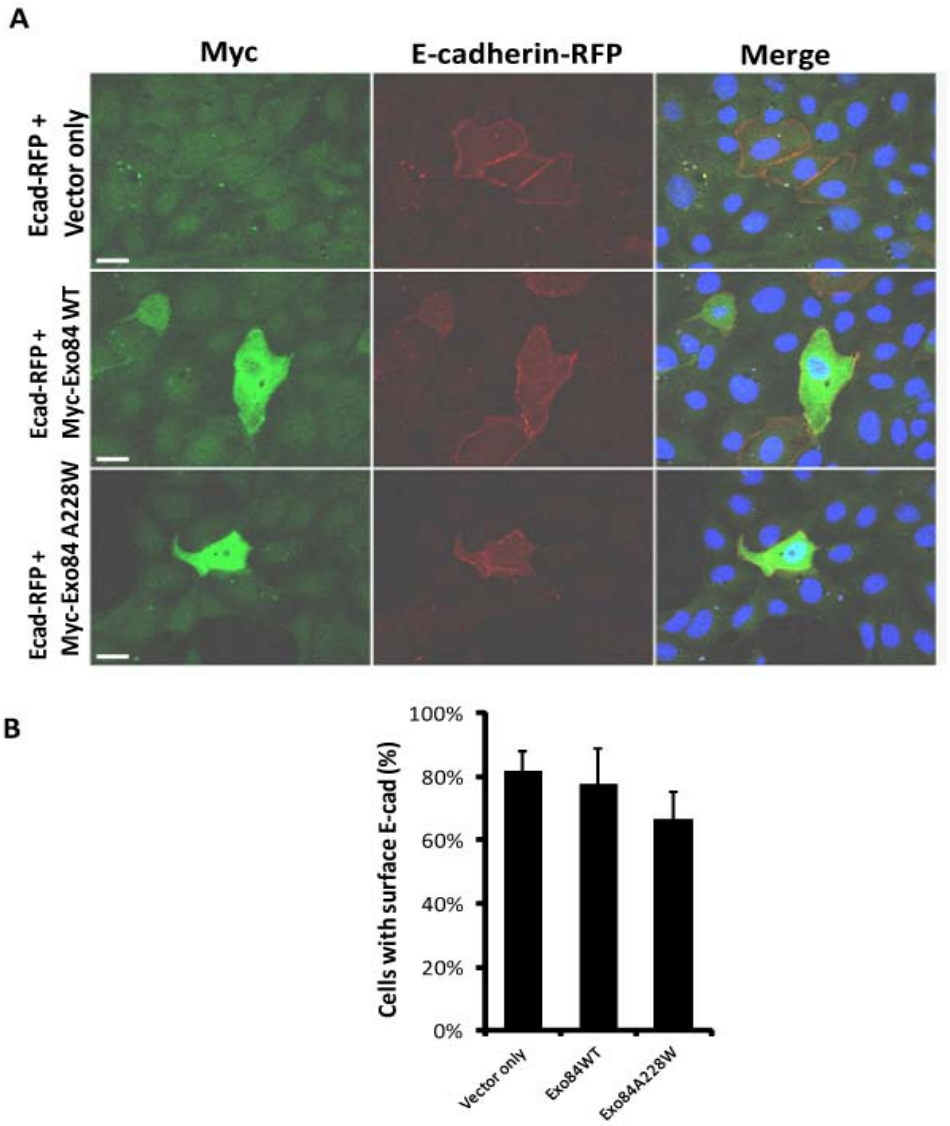


**Figure 7. Ral-Exo84 interaction isn't required for basolateral E-cadherin exocytosis.** (A) Compared to myc-tagged Exo84WT overexpressed MDCK cells and vector only cells, myc-tagged Exo84$^{A228W}$ mutant didn't affect Ecad-RFP exocytosis. Scale bar: 20 μm. (B) Quantitative analysis was used to measure the number of cells with surface E-cadherin accumulation between three different transfected cells. Data are means ± SD. (n = 50) No significance by one-way ANOVA multiple comparisons.



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