Retromer maintains basolateral distribution of the type II TGF-β receptor via the recycling endosome

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ABSTRACT **Transforming growth factor β (TGF-β) is critical for the development and maintenance of epithelial structures. Because receptor localization and trafficking affect the cellular and organismal response to TGF-β, the present study was designed to address how such homeostatic control is regulated. To that end, we identify a new role for the mammalian retromer complex in maintaining basolateral plasma membrane expression of the type II TGF-β receptor (TβRII). Retromer and TβRII associate in the presence or absence of TGF-β ligand. After retromer knockdown, although TβRII internalization and trafficking to a Rab5-positive compartment occur as in wild-type cells, receptor recycling is inhibited. This results in TβRII mislocalization from the basolateral to both the basolateral and apical plasma membranes independent of Golgi transit and the Rab11-positive apical recycling endosome. The data support a model in which, after initial basolateral TβRII delivery, steady-state polarized TβRII expression is maintained by retromer/TβRII binding and delivery to the common recycling endosome.**

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INTRODUCTION

Plasma membrane receptors are regulated, in part, through the action of *cis* regulatory motifs interfacing with the transport machinery. Because a number of diseases result from defects in the ability to sort or transport proteins to their appropriate cellular destination (Stein *et al.*, 2002; Verges, 2007; Mellman and Nelson, 2008) and transforming growth factor β (TGF-β) regulates a variety of cellular processes critical for normal homeostasis (Roberts, 1992; Roberts and Wakefield, 2003; ten Dijke *et al.*, 1996; Akhurst and Derynck, 2001; Elliott and Blobe, 2005), we initiated studies to define and characterize the mechanisms controlling the spatial distribution of the TGF-β receptor (TGF-βR) complex.

The response to TGF-β usually depends on the cell type involved, with effects as diverse as growth and growth inhibition (Moses and Serra, 1996; Serini and Gabbiani, 1999; Bissell, 2001; Yue and Mulder, 2001). In general, the majority of mammalian cells express three TGF-β–binding species, referred to as type I (TβRI), type II (TβRII), and type III (β-glycan) receptors. The pivotal role that TGF-β plays in modulating a number of biological activities makes it essential to identify the regulatory mechanisms through which appropriate type I and type II receptor expression is maintained.

Although there is an enormous body of literature documenting the biological relevance and signaling activity of the TGF-βR complex, there is a paucity of information on the trafficking itinerary of this receptor family and essentially no literature investigating this question in polarized epithelial cell models. To address that issue, we generated a chimeric receptor model consisting of the extracellular domain of the granulocyte-macrophage colony-stimulating factor (GM-CSF) α or β receptor fused to the transmembrane and cytoplasmic domains of TβRI or TβRII (Anders and Leof, 1996; Anders *et al.*, 1997; see *Methods and Materials*, *Cell culture*, for discussion of chimeric receptors). This system provides a technically

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Abbreviations used: αI, chimeric type I transforming growth factor β receptor; ARE, apical recycling endosome; β2AR, β2-adrenergic receptor; BEE, basolateral early endosome; βII, chimeric type II transforming growth factor β receptor; CI-MPR, cation-independent mannose 6-phosphate receptor; CPZ, chlorpromazine; CRE, common recycling endosome; NYS, nystatin; shRNA, short hairpin RNA; SNX, sorting nexin; Tfn, transferrin; TfnR, transferrin receptor; TGF-β, transforming growth factor β; TGF-βR, transforming growth factor β receptor; TβRI, type I transforming growth factor β receptor; TβRII, type II transforming growth factor β receptor; WT, wild type.

facile approach to evaluate the trafficking itinerary of various TGF-βR complexes. Using native as well as chimeric type I (αI) and type II (βII) TGF-βRs, we found that both receptors independently traffic to the basolateral surface in polarized Madin–Darby canine kidney (MDCK) and NMuMg cells adjacent to the zonula adherens complex (Murphy *et al.*, 2004, 2007).

Although the targeting of many basolateral proteins is regulated by tyrosine- or dileucine-based motifs (Matter *et al.*, 1992; Aroeti *et al.*, 1993; Hunziker and Fumey, 1994; Stein *et al.*, 2002; Rodriguez-Boulan *et al.*, 2005; Mellman and Nelson, 2008), analysis of TβRII showed that a unique element (referred to as the LTA motif) between residues 529 and 538 was necessary and sufficient for basolateral delivery (Murphy *et al.*, 2007). Because 1) the cellular activities/factors that maintain appropriate TGF-βR membrane expression have not been identified and 2) these reflect fundamental questions that affect a number of TGF-β-directed phenotypes, as well as the homeostatic mechanisms by which cellular integrity is maintained, we used a terminal 84–amino acid cytoplasmic fragment (residues 484–567) as "bait" in a tandem-affinity tag pull down. Proteins were visualized by silver stain and identified by mass spectrometry. One protein of interest, due to its role in a variety of trafficking functions, was the retromer vacuolar protein sorting protein 26 (Vps26) subunit.

The retromer was initially identified in yeast as a multimeric protein complex that mediates intracellular sorting of Vps10, a receptor that transports vacuolar (i.e., lysosomal) enzymes (Seaman *et al.*, 1998). It consists of two subcomplexes: Vps35, Vps29, and Vps26, which function in cargo selection, and Vps5 and Vps17, which are proposed to sense membrane curvature and induce the formation of tubules. Human orthologues have been found for each of the yeast Vps proteins (the one exception being Vps17), with sorting nexin-1 and/or -2 (SNX1, SNX2) functioning as the Vps5 homologue and Vps17 function in mammalian cells, being mediated by either SNX5 or 6 (Wassmer *et al.*, 2007, 2009). Subsequently the retromer was found to perform an analogous activity in mammalian cells: the endosome-to-Golgi retrieval of the cation-independent mannose 6-phosphate receptor (CI-MPR; Arighi *et al.*, 2004; Seaman, 2005). Retromer subunits have additional regulatory roles, including transcytosis of the polymeric immunoglobulin receptor, Wnt gradient formation, processing of the amyloid precursor protein, and, of most relevance to the present study, apoptotic cell clearance by phagocytic receptor recycling and Rab4-dependent β2 adrenergic receptor (β2AR) recycling (Verges *et al.*, 2004; Verges, 2008; Seaman, 2005; Hierro *et al.*, 2007; Chen *et al.*, 2010; McGough and Cullen, 2011; Cullen and Korswagen, 2012; Temkin *et al.*, 2011; Seaman, 2012).

Because the retromer had not been reported to differentially regulate apical versus basolateral delivery of any cargo in polarized epithelia, we investigated whether this TβRII/retromer association might indicate an additional role for this enigmatic complex. Surprisingly, although retromer knockdown has no significant effect on TGF-βR internalization, Smad2/3 phosphorylation, or initial basolateral targeting via the LTA motif, it provides an obligate function in the maintenance of TGF-βR basolateral membrane expression by promoting TβRII transit from the Rab5-positive early endosome to the common recycling endosome (CRE). In the absence of retromer, however, TβRII becomes mislocalized such that both apical and basolateral expression is observed.

RESULTS

Binding of type II TGF-βR to the mammalian retromer

Trafficking to the appropriate membrane domain is the initial event necessary for regulated epithelial cell growth (Drubin and Nelson, 1996) and is altered in a variety of disease states (Stein *et al.*, 2002; Verges, 2007; Mellman and Nelson, 2008). Because the retromer Vps26 subunit was coprecipitated with a terminal 84–amino acid fragment of the type II TGF-βR (unpublished observations), to determine whether this novel interaction was specific and biologically important, we addressed the following questions. First, was the retromer/TβRII association ligand dependent and/or independent? Second, would retromer loss specifically prevent basolateral TβRII localization and/or TGF-βR signaling? Third, if TβRII trafficking was affected, what intracellular pathways and organelles were affected?

Retromer/TβRII association was examined in MDCK cells transiently transfected with epitope-tagged native TβRI or TβRII (Figure 1A), as well as an MDCK cell line (MD-1) expressing chimeric type I (αI) and type II (βII) TGF-βRs (Figure 1B; Anders and Leof, 1996; Anders *et al.*, 1997; Mitchell *et al.*, 2004; Murphy *et al.*, 2004, 2007). Although both native and chimeric type II TGF-βRs could be coimmunoprecipitated with the retromer Vps26 subunit, this occurred independent of ligand, and no association was detected with TβRI or αI (Figure 1, A and B). Because retromer/type II TGF-βR binding was unexpected, we further verified this interaction by 1) demonstrating binding in another cell line, 2) documenting βII/retromer association regardless of the antibody order used for precipitation and blotting, and 3) showing that the complex could be immunoprecipitated with either Vps26 or Vps35 sera (Supplemental Figure S1). Finally, given that cargo binding is associated with Vps26, Vps29, and/or Vps35 retromer subunits, glutathione *S*-transferase (GST) fusion proteins demonstrated that in the absence of an intact retromer complex, βII binds Vps35 (Figure 1C).

Loss of retromer affects polarized TβRII localization independent of overall junctional integrity, Smad phosphorylation, or TβRI localization

Identification of the retromer complex as a new interacting partner specific for TβRII generates a number of questions relating to TGF-βR regulation, trafficking, and/or signaling. To obtain information concerning the biological significance of TβRII/retromer binding, we knocked down the Vps35 subunit in MD-1 cells. Three independent clones from two short hairpin RNAs (shRNAs) were isolated and subsequently characterized (Figure 2A and Supplemental Figure S2). Consistent with previous publications (Arighi *et al.*, 2004; Gullapalli *et al.*, 2006), functional retromer loss was documented by observing decreased stability of the CI-MPR, as well as diminished levels of other retromer subunits (Figure 2B and Supplemental Figure S2B). Whereas CI-MPR levels depended on retromer expression (i.e., documenting effective retromer knockdown), similar to those reported for epidermal growth factor and transferrin receptors (Arighi *et al.*, 2004), chimeric type I or type II TGF-βR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein expression was unaffected (Figure 2B). Most important, cellular polarity was independent of a functional retromer complex, as there was no apical-to-basal inulin flux in any of the retromer knockdown clones (Figure 2C). The inability of retromer knockdown to affect polarity per se in mammalian epithelial cells is in contrast to its role in the *Drosophila* follicle epithelium, where retromer controls epithelial cell polarity via the lysosomal degradation of the apical determinant Crumbs (Pocha *et al.*, 2011).

In addition to regulating the trafficking itinerary of various cargo, retromer has been implicated in tumorigenesis through interaction with the Golgi-associated oncoprotein GOLPH3 and subsequent recycling of key components required for mTOR action (Scott *et al.*, 2009). Because TGF-β signaling has a critical role in maintaining normal epithelial cell homeostasis, which is lost during tumor

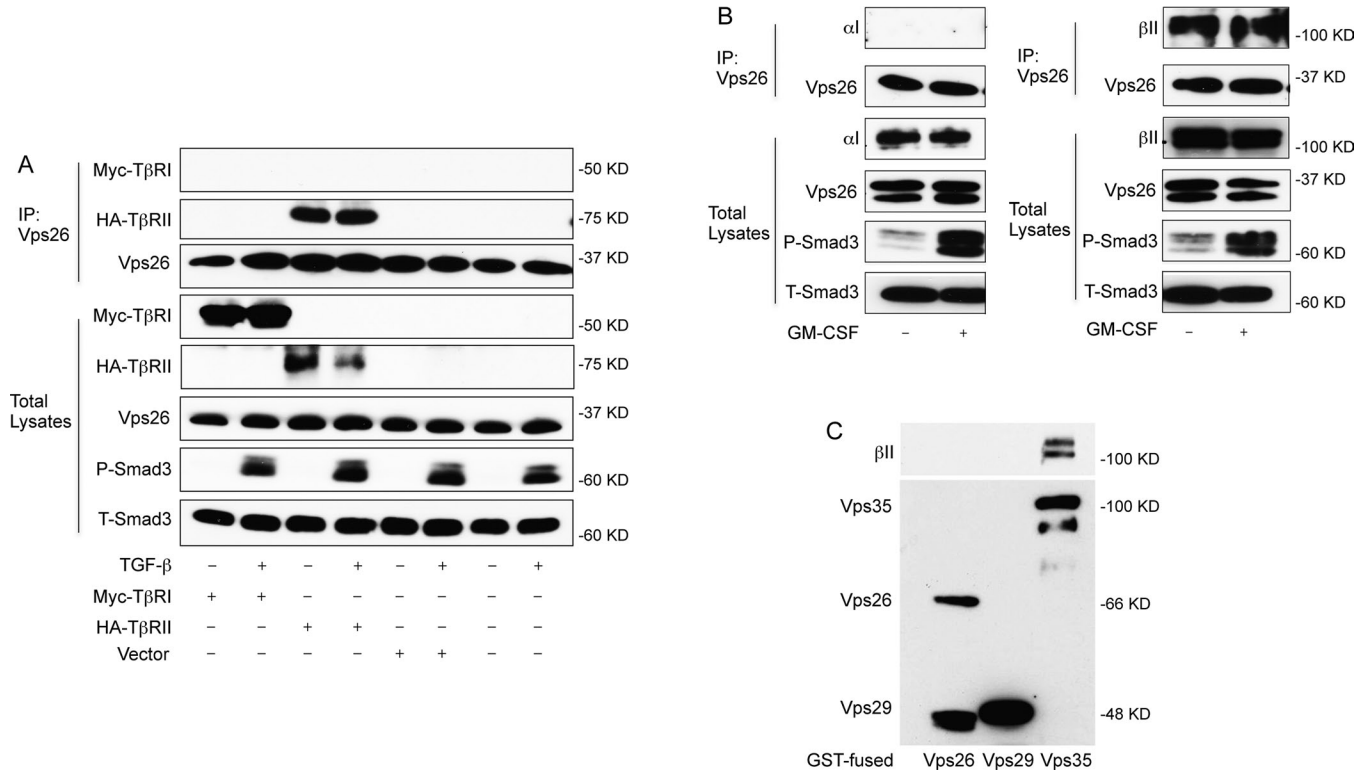

FIGURE 1: The type II TGF-βR binds retromer independent of ligand. (A) MDCK cells were transiently transfected (+) with Myc-TβRI, hemagglutinin (HA)-TβRII, or pcDNA3.1 (vector) as described in *Materials and Methods*. Cultures were left untreated (–) or stimulated (+) with TGF-β (5 ng/ml) for 1 h at 37°C. For coimmunoprecipitation (three top panels), after NP-40 buffer lysis, 1 mg of protein was incubated with anti-Vps26 antibody, followed by Western blotting with antibodies to Myc (TβRI), HA (TβRII), or Vps26. Bottom five panels, expression of the indicated proteins (Myc-TβRI, HA-TβRII, Vps26, phospho-Smad3 [P-Smad3], or total Smad3 [T-Smad3]) in 50 μg of lysate. (B) MD-1 cells were left untreated (–) or treated (+) with GM-CSF (50 ng/ml) for 1 h at 37°C. Equivalent protein (1 mg) was immunoprecipitated with antibodies to Vps26 (left and right, top two panels) and subsequently Western blotted for chimeric type I receptors (αI, top left), chimeric type II receptors (βII, top right) or Vps26 (left and right second panels). Equivalent Vps26 immunoprecipitation for each of the chimeric receptor Westerns is documented in second panel, and the bottom two panels show expression of the indicated proteins (chimeric TβRI [αI], chimeric TβRII [βII], Vps26, phospho-Smad3 [P-Smad3], or total Smad3 [T-Smad3]) in 50 μg of lysate. The top three panels are from separate blots. (C) MD-1 lysates were incubated with the indicated Vps GST fusion protein, and bound chimeric type II receptors (βII) detected by Western analysis (panel). Bottom. fusion protein expression after GST pull down and anti-GST blotting. Lower band in Vps26 lane is a nonspecific protein.

progression (Elliott and Blobe, 2005; Derynck and Akhurst, 2007; Wu and Hill, 2009), we examined the effect of retromer loss on Smad activation. No appreciable difference was observed on Smad2 or Smad3 phosphorylation stimulated through either native (TGF-β) or chimeric (GM-CSF) receptor activation (Supplemental Figure S2, A and B).

In contrast to the lack of a role for retromer in Smad activation, retromer loss abrogated specific basolateral trafficking such that apical type II receptor expression was also observed. This was demonstrated for both native and chimeric receptors using confocal microscopy (Figure 2, D and E, and Supplemental Figure S2C), as well as by domain-specific membrane biotinylation (Figure 2F and Supplemental Figure S2D). Furthermore, consistent with previous data showing lack of retromer/TβRI binding and retromer knockdown having no effect on junctional integrity (Figures 1 and 2C), TβRI, αI, ZO-1, and E-cadherin localization were all unaffected by retromer loss (Figure 2, D–F, and Supplemental Figure S2, C and D). Because the localization of cytokine receptors to defined membrane domains is critical to appropriately respond to external cues, the present findings document a novel role for the mammalian retromer in localizing TβRII to the basolateral plasma membrane in polarized epithelial cells.

Maintenance of basolateral TβRII expression requires retromer-dependent endocytic trafficking

Although the preceding data clearly document a requirement for the retromer in TβRII polarity, they do not address whether this reflects a "targeting" role to a defined membrane locale (i.e., analogous to the *cis*-acting LTA motif in TβRII; Murphy *et al.*, 2007) and/or a "maintenance" activity by which retromer is critical to preserving steady-state basolateral TβRII protein. To investigate this issue directly, first, we Golgi blocked newly synthesized proteins in Transwell polarized control MD-1 and retromer knockdown cells by 20°C treatment; second, we added a dilute trypsin/phosphate-buffered saline (PBS) solution to the apical and basal chambers during the last 30 min of the Golgi block to remove cell surface proteins; and third, we examined TβRII reexpression at the apical and/or basolateral plasma membranes over the next 3 h after transfer to 37°C. As

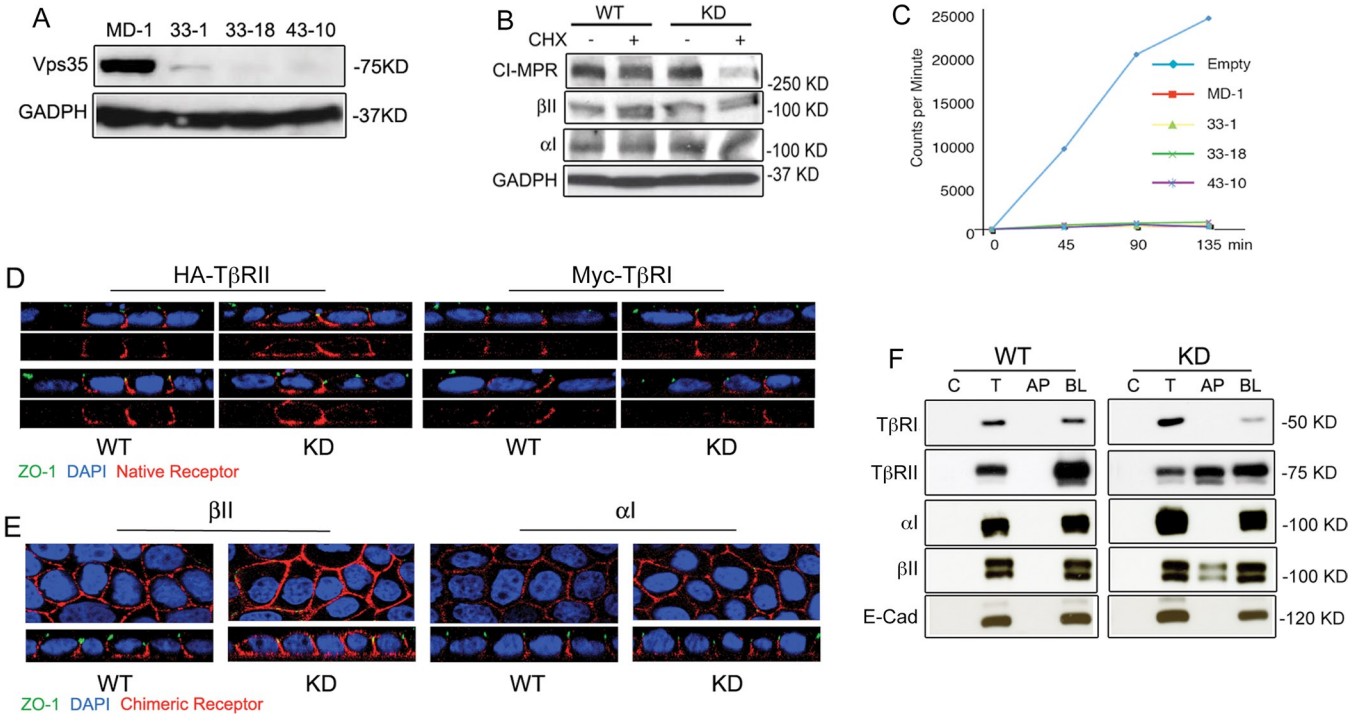

FIGURE 2: Retromer is required for plasma membrane domain–specific type II TGF-βR expression. (A) Western blot expression of Vps35 and GAPDH (50 μg of total protein) in parental MD-1 cells or clones (33-1, 33-18, and 43-10) expressing shRNA to Vps35. (B) Lysates were prepared from MD-1 (wild type [WT]) and Vps35 knockdown (KD) 43-10 cells either untreated (–) or after 20 h treatment with 10 μM cycloheximide (CHX). Immunoblot analysis was performed for CI-MPR, chimeric type II TGF-βR (βII), chimeric type I TGF-βR (αI), or GAPDH. (C) MD-1 and Vps35-silenced 33-1, 33-18, and 43-10 clones were Transwell polarized before inulin flux from the apical to basal chambers was determined as described in *Materials and Methods*. Empty reflects inulin flux in the absence of plated cells. One representative result from at least three repeats is shown. (D) MD-1 (WT) and retromer silenced 43-10 (KD) polarized cells on Transwells were transiently transfected with epitope-tagged native type II (HA-TβRII [red; left two panels]) or type I (Myc-TβRI [red; right two panels]) TGF-βRs. Images are represented as the horizontal, *XY* flat sections above lower, perpendicular *XZ* cross sections. Cultures were costained for ZO-1 (green) and nuclei (blue) stained with DAPI. The second and fourth vertical panels show TGF-βR expression in the absence of DAPI to enhance receptor visualization. (E) Polarized MD-1 (WT) and Vps35 knockdown clone 43-10 (KD) stained for the chimeric type II (βII [red; left two panels]) or type I (αI [red; right two panels]) TGF-βRs. Confocal *XZ* images were visualized for receptor, ZO-1, and nuclei as in D. (F) Transwell polarized MD-1 (WT) and 43-10 (KD) cells were selectively biotinylated at the apical (AP) or basolateral (BL) surface or as a nonpolarized monolayer for total (T) labeling. After streptavidin immunoprecipitation, Western blotting for the indicated chimeric (αI; βII) or endogenous (TβRI; TβRII; E-cadherin [E-Cad]) proteins was performed. Lane C reflects indicated protein immunoprecipitated in the absence of biotinylation.

shown in Figure 3A, although reexpression of TβRII at the plasma membrane became detectable within the first 40–60 min after trypsinization, it was initially localized to the basolateral membranes in both control and knockdown lines. In contrast to parental MD-1 cells, however, in the absence of retromer, apical TβRII localization also became apparent in the Vps35 knockdown cultures between 60 and 120 min. Because this was observed for both native and chimeric type II receptors (Figure 3, A and B, and C and D, respectively), the data indicate that although retromer does not direct membrane targeting of newly synthesized TGF-βRs, it has a fundamental and unique role in maintenance of type II TGF-βRs at the basolateral plasma membrane.

Given that the findings of Figure 3 were somewhat surprising, we further documented this conclusion and investigated the underlying mechanism. For retromer to provide a "maintenance" function, two important criteria would have to be fulfilled. First, apically localized TβRIIs would have to arise from preexisting basolateral receptors; and second, given that junctional integrity is maintained in the absence of retromer (Figure 2C), apical delivery would require the internalization and subsequent intracellular trafficking of basolateral TβRIIs. The first of these issues was addressed (Figure 4) using two distinct methods. For the first approach, polarized MD-1 and retromer knockdown cells were basolaterally treated at 10°C with antibodies to the extracellular domain of the chimeric type II TGF-βR (βII), and their transcytosis to the apical membrane was monitored after 37°C chase (Thompson *et al.*, 2007). Consistent with previous data documenting that TGF-βRs localize to the basolateral plasma membrane (Murphy *et al.*, 2004, 2007), no appreciable apical βII staining was detected in wild-type MD-1 cultures. However, when βII transcytosis was examined in the absence of retromer, significant apical βII expression was observed (Figure 4, A and B). This finding was independently confirmed using cycloheximide to block de novo protein synthesis (inhibited ~95%; unpublished observations), apically treating with trypsin to remove cell surface receptors (time 0), and then examining by confocal microscopy for βII reexpression at the apical and basolateral plasma membrane domains over the next hour (Figure 4, C and D). As expected, control MD-1 cells showed basolateral βII expression at all times. In contrast to MD-1 cultures,

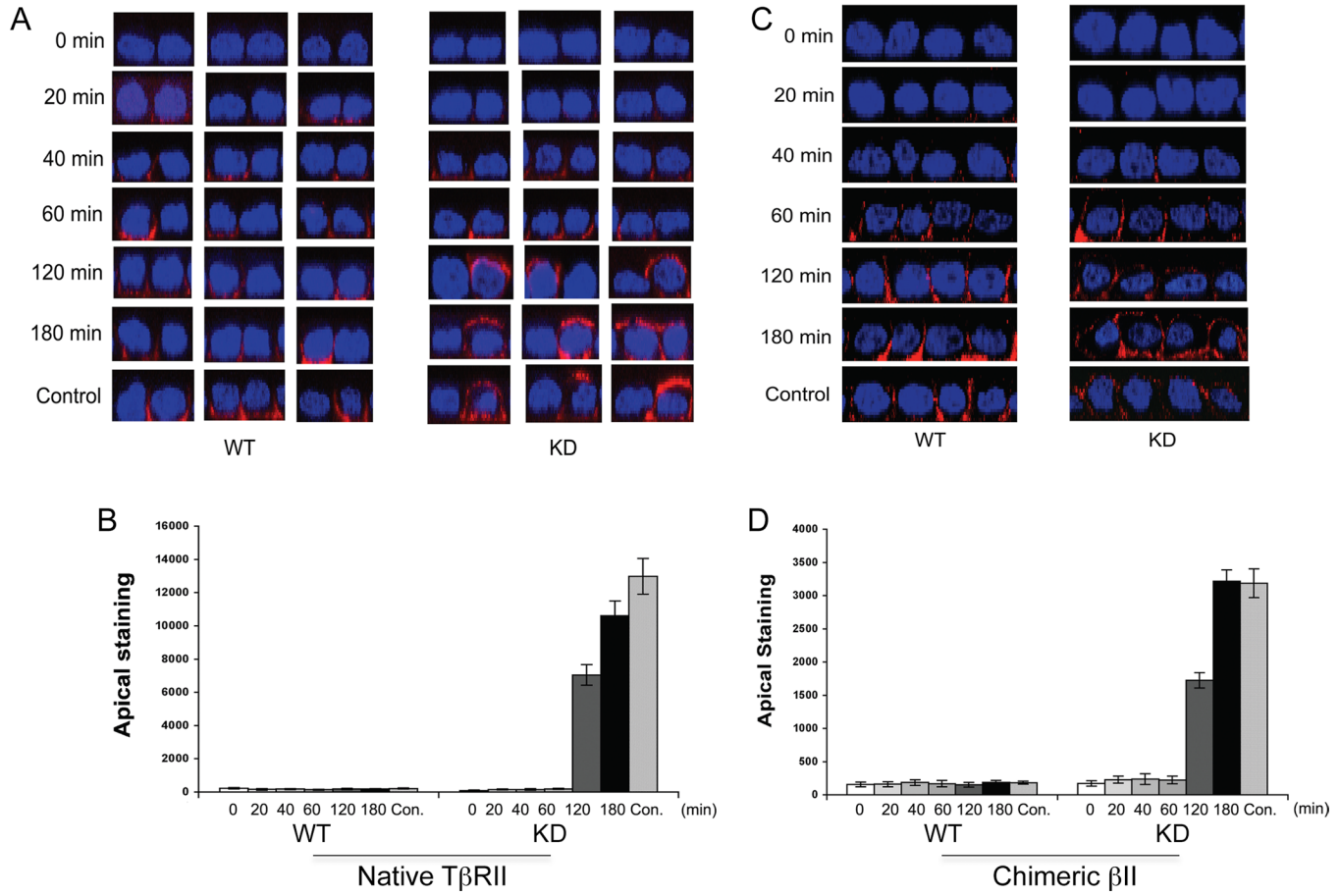

FIGURE 3: Initial targeting of the TβRII to the basolateral membrane occurs independent of retromer. (A) Polarized MD-1 (WT) and 43-10 (KD) cells transiently transfected with HA-tagged native TβRII were Golgi blocked at 20°C for 3 h in serum-free DMEM. The apical and basal chambers were then treated with a dilute (0.025%) trypsin/PBS solution for the last 30 min of the Golgi block to remove cell surface proteins (0 min). After PBS wash (3×), fresh 10% FBS/DMEM was added, the plates were returned to 37°C, and stained for the epitope-tagged native type II receptor at the indicated times after release. (B) Quantification of apical native TβRII expression seen in A defined by MetaMorph as the signal obtained within a square placed on the most apical nuclear surface encompassing the horizontal nuclear dimension and vertical receptor staining. Data represent the mean ± SEM of 83–115 cells taken from three independent experiments. (C) Parental MD-1 (WT) and retromer knockdown 43-10 cells were polarized on Transwell plates. Subsequent to Golgi block and trypsinization as described in A, newly expressed chimeric type II TGF-βRs were visualized by immunofluorescence from 20 to 180 min after release. (D) Apical quantification of chimeric βII as in B from 56–86 cells taken from three independent experiments.

whereas transient trypsinization specifically removed all apically localized type II TGF-βRs from retromer knockdown cells (compare 0 min with control, no trypsin treatment), knockdown cells showed levels of new apical receptor expression approaching control by 60 min.

Figures 3 and 4 are consistent with the hypothesis that in the absence of retromer and new protein synthesis, basolaterally expressed type II TGF-βRs become relocalized to the apical membrane domain. Because this represents a unique role for the mammalian retromer, additional studies were performed to address the operative mechanism and pathway. For instance, if basolateral expressed type II TGF-βRs (i.e., not intracellular or newly synthesized) provide the "receptor pool" that undergoes intracellular trafficking and mislocalization to the apical surface in the absence of retromer, such a process would require endocytic activity and depend on receptor internalization. This is directly examined in Figure 5, A and B. First, the apical membrane of Transwell polarized control and retromer knockdown MDCK cells was treated with a dilute trypsin solution as in Figure 3A to remove cell surface protein; second, cultures were then transfected with wild-type or dominant-negative green fluorescent protein (GFP)–dynamin II (K44A mutant; prevents the scission of endocytic vesicles); and third, apical βII expression was specifically examined in the GFP-positive transfected cells. As shown in Figure 5A and quantitated in Figure 5B, after removal of receptors from the apical surface, dominant-negative dynamin II (but not wild type) prevented subsequent basolateral-to-apical mislocalization of βII in retromer knockdown cells by ~80%. Thus, in the absence of retromer, apical βII expression requires that basolateral receptors undergo dynamin-dependent internalization.

Because numerous endocytic pathways are contingent upon dynamin action (Doherty and McMahon, 2009) and TGF-βR internalization has been reported to use both clathrin- and caveolar-dependent mechanisms (Di Guglielmo *et al.*, 2003; Mitchell *et al.*, 2004), we extended this finding biochemically and determined the specific internalization machinery used for βII basolateral-to-apical mislocalization. As shown in Figure 5C (left), after trypsin removal of

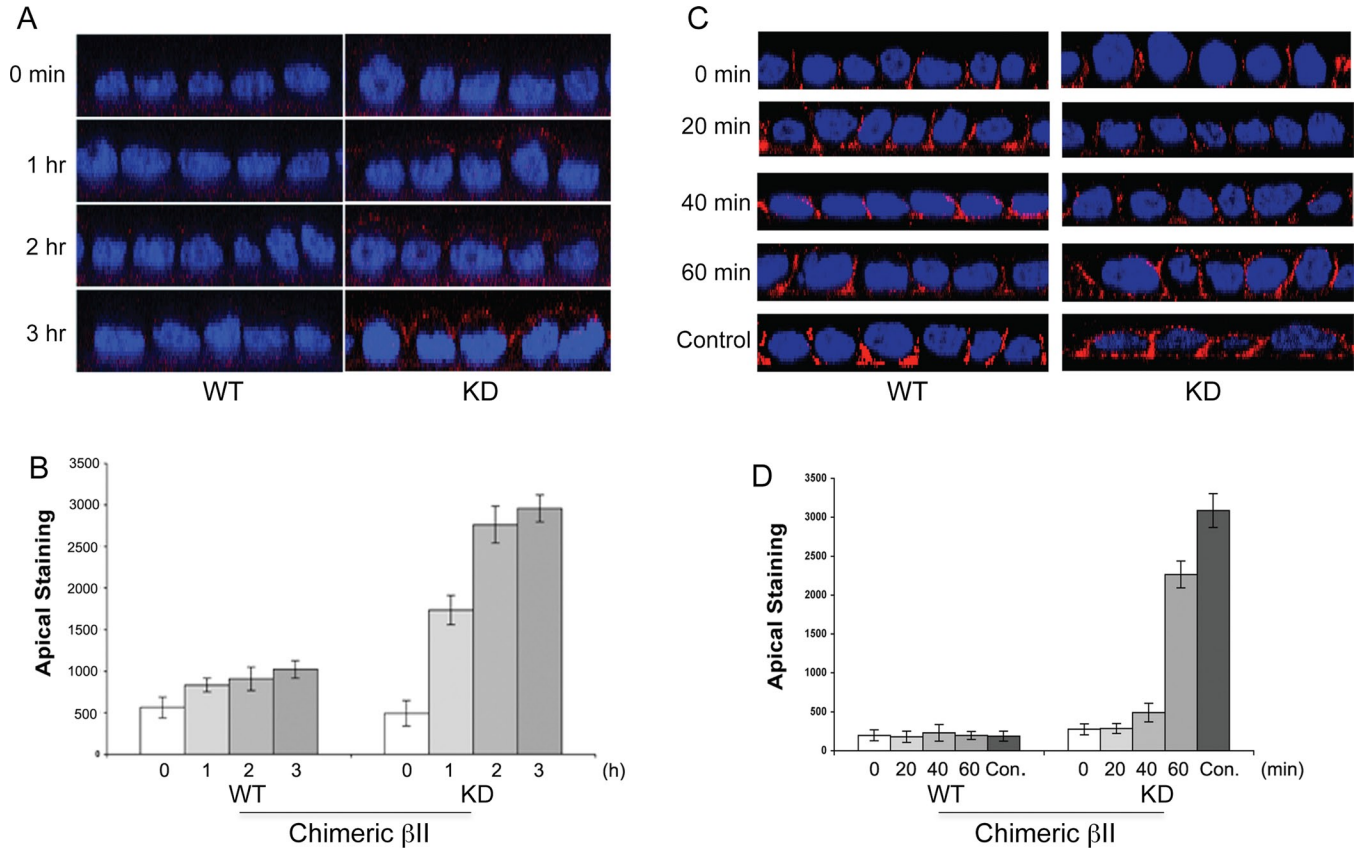

FIGURE 4: Apical type II TGF-βRs arise from preexisting basolateral receptors in retromer knockdown cells. (A) Transwell polarized MD-1 (WT) and 43-10 (KD) cells were washed with ice-cold binding buffer (50 mM HEPES/DMEM, pH 7.2) and then incubated for 1 h at 10°C with 4 μg/ml GM-CSFR-β antibody added to the basolateral chamber. After binding buffer wash (3×), plates were incubated in DMEM at 37°C for 15 min, basolaterally washed with DMEM, pH 3.0 (2×), and reneutralized by additional (2×) DMEM, pH 7.4, washes. Fresh DMEM was added, the cultures placed at 37°C for the indicated times, and subsequent to 0.2% BSA/PBS wash (3×) incubated at 10°C for 1 h with Cy3-conjugated secondary antibody diluted in 5% NDS/0.2% BSA/PBS added only to the apical chamber. (B) MetaMorph quantification of apical chimeric βII seen in A represented as arbitrary units of fluorescence ± SEM of 40 cells from three independent experiments. (C) Polarized MD-1 (WT) and 43-10 (KD) cells on Transwells were pretreated with 71 μM cycloheximide (CHX) for 30 min at 37°C. After apical membrane trypsinization as in Figure 3 (0 min), the plates were placed in fresh 10% FBS/DMEM/CHX, returned to 37°C, and at the indicated times stained for the chimeric type II TGF-βR. Control reflects chimeric βII staining before apical trypsinization. (D) Quantification was performed as in B and represents mean ± SEM of 56—86 cells taken from three independent experiments.

apical proteins, inhibition of clathrin-dependent internalization with chlorpromazine (CPZ) prevented βII apical mislocalization to the same (or greater) degree as dominant-negative dynamin. In contrast, nystatin (Nys) inhibition of caveolar uptake was without effect, in that the reappearance of apically biotinylated type II TGF-βRs was detected with identical kinetics as seen in the absence of drug (control). That basolateral βII was unaffected by either apical trypsinization or CPZ/Nys (Figure 5C, right) further confirms junctional integrity and the absence of drug toxicity, respectively. Specificity for clathrin and caveolar pathway inhibition by CPZ and Nys was determined using transferrin (Tfn) and lactosylceramide, respectively (unpublished observations).

Retromer regulates type II TGF-βR recycling downstream of Rab5

The preceding findings (Figures 2–5 and Supplemental Figure S2, C and D) show that maintenance of the type II TGF-βR at the basolateral plasma membrane in polarized epithelia requires a retromer- and clathrin-regulated endocytic response that is independent of ligand. Because previous work documented constitutive TβRII recycling dependent upon clathrin and Rab11 in nonpolarized monolayers (Di Guglielmo *et al.*, 2003; Mitchell *et al.*, 2004) and retromer has been implicated in regulating similar activity for phagocytic and β2-adrenergic receptors (Chen *et al.*, 2010; Temkin *et al.*, 2011), we examined whether a requirement for retromer in βII recycling might account for its apical mislocalization. Consistent with that hypothesis, when recycling assays were performed on MD-1 wild-type and retromer knockdown cells, the absence of retromer resulted in an ~50–60% decrease in recycling (Figure 6, A and B).

Because recycling can be inhibited either before or after cargo internalization, we next investigated whether retromer acted at a defined site in type II TGF-βR trafficking. To initially address this question, we first performed studies using nonpolarized cultures. As shown in Figure 6, C–E, retromer knockdown had no effect on βII internalization to the Rab5-positive early endosome nor were receptors shunted to an alternative Rab4 recycling compartment.

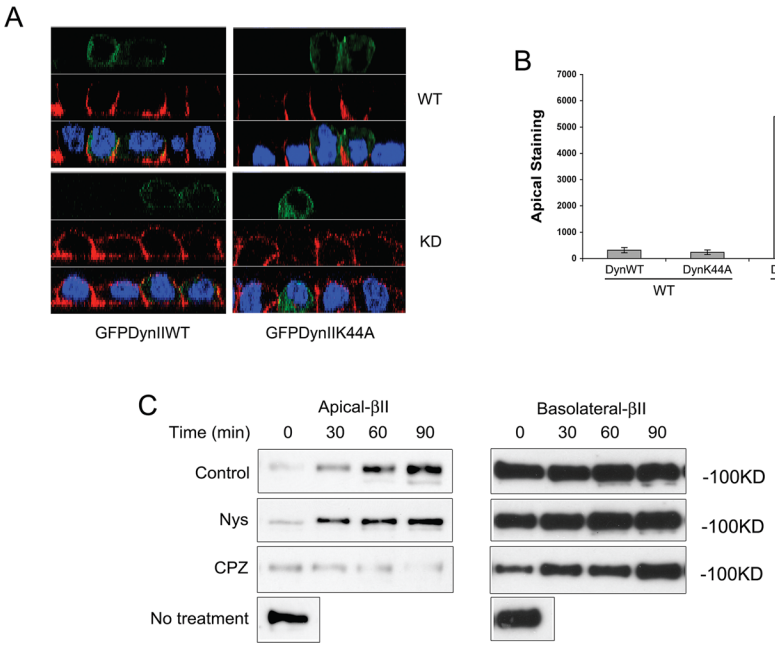

FIGURE 5: Retromer maintains basolateral type II TGF-βR expression via a dynamin- and clathrin-dependent process. (A) Before (48 h after plating) complete polarization on Transwells, MD-1 (WT) and 43-10 retromer knockdown (KD) cells were transfected with GFP-conjugated wild-type (GFPDynIIWT) or K44A mutant (GFPDynIIK44A) dynamin II for 24 h. The apical membrane was trypsinized as in Figure 3A and, after 2 h of incubation at 37°C in 10% FBS/DMEM, chimeric βII expression in GFP-DynIIWT and -K44A–expressing (green) cells was visualized by confocal microscopy. (B) Apical βII expression in the GFP-DynII–transfected cells was quantitated as described in Figure 3B and represents the mean ± SEM apical chimeric βII fluorescence of 60–79 dynamin-expressing cells taken from three independent experiments. (C) Transwell polarized retromer knockdown 43-10 cells were untreated (Control) or pretreated with nystatin (Nys, 25 μg/ml) or chlorpromazine (CPZ, 8 μg/ml) for 30 min at 37°C subsequent to apical trypsinization as in Figure 3A. Cultures were placed in 10% FBS/DMEM supplemented with Nys or CPZ, and at the indicated times chimeric type II TGF-βRs were selectively biotinylated at the apical or basolateral membrane and expression determined by streptavidin immunoprecipitation and Western blotting. "No treatment" reflects βII protein at the indicated membrane surface before apical trypsinization.

Similarly, in agreement with a recent study (Temkin *et al.*, 2011), transferrin recycling via either the rapid Rab4 pathway or the Rab11 recycling endosome (Supplemental Figure S4), as well as Tfn receptor (TfnR) cofractionation with Rab4 (Figure 6E), was unaffected by retromer knockdown. In contrast to these findings, because TGF-βR recycling in monolayer cultures depends on retromer and Rab11 (Figure 6B; Mitchell *et al.*, 2004), as expected, βII/Rab11 colocalization decreased ~40–50% in retromer knockdown cells (Figure 6, F and G). Thus, although initial type II TGF-βR trafficking to a Rab5-positive compartment is unaffected by the absence of retromer, subsequent transit to the Rab11-positive recycling endosome is reduced coincident with decreased recycling.

Apical type II TGF-βR mislocalization in retromer knockdown cells is independent of Golgi transit and the Rab11-positive apical recycling endosome

The preceding data identify a new role for the mammalian retromer in maintaining basolateral expression of the type II TGF-βR. Because retromer was initially characterized for its role in mediating retrograde endosome-to-Golgi trafficking and has been shown to regulate transport of Shiga toxin from the recycling endosome to the Golgi complex (Seaman, 2005; Lieu and Gleeson, 2010), we further investigated the pathways and organelles affected. Initial studies examined the role (if any) of retromer in retrograde Golgi transport by assessing the colocalization of internalized βII or CI-MPR membrane receptors with the *trans*-Golgi network (TGN) marker galactosyltransferase. In agreement with previous work (Arighi *et al.*, 2004; Gomez and Billadeau, 2009), although retromer-dependent Golgi colocalization of internalized CI-MPR was observed, negligible βII/Golgi staining was detected in either monolayer (Figure 7, A and B) or polarized (Figure 7C) cultures.

Given that 1) Transwell polarized retromer knockdown cells show apical plasma membrane type II TGF-βR mislocalization (Figures 2–5 and Supplemental Figure S2, C and D) and 2) retromer is required for Rab11-dependent βII recycling after internalization to the Rab5-positive early endosome in nonpolarized MDCK cells (Figure 6), we investigated whether TβRII recycling in polarized cells both was retromer dependent and might reflect the operative pathway accounting for the apical mislocalization. Supportive of that hypothesis and analogous to what we observed in monolayer, the absence of retromer decreased βII recycling ~40–50% in polarized cultures yet had no effect on chimeric or native type II TGF-βR transit to the Rab5-positive basolateral early endosome (BEE; Figure 8, A–C). Because TβRII trafficking to a Rab5 compartment is retromer independent yet TβRII recycling is retromer dependent, this indicates that retromer functions downstream of Rab5.

Given that we previously reported a role for Rab11 in TβRII monolayer recycling (Mitchell *et al.*, 2004) and Rab11 is believed to function primarily at the apical recycling endosome (ARE) in polarized epithelia (Fölsch *et al.*, 2009; Golachowska *et al.*, 2010), we next determined whether apical βII expression in retromer knockdown polarized cells reflected shunting to the ARE. Although there was no appreciable colocalization (Figure 8D, top) or cofractionation (Figure 8E, lane 3) of type II TGF-βRs and Rab11 in polarized wild-type MDCK cells (as expected, since this would reflect apical transit), contrary to our expectations, a similar lack of association was also seen in retromer knockdown cultures (Figure 8D, bottom; Figure 8E, lane 4) in spite of the apical mislocalization. The latter result in polarized cultures is contrasted by the expected retromer-dependent TβRII/Rab11 association observed in nonpolarized cells (Figure 8E, compare lanes 1 and 2). Thus, whereas apical type II TGF-βR expression is observed in retromer knockdown cells, it does not reflect trafficking through the Rab11-dependent ARE.

Whereas the previous data support a new role for retromer in the homeostatic control of the type II TGF-βR, an important question is whether this is a general or cargo-specific function. To initially address this issue, we further examined the effect of retromer loss on the TfnR in both nonpolarized and polarized epithelia, as it also is basolaterally expressed and undergoes constitutive clathrin-dependent recycling (Sheff *et al.*, 1999; Grant and Donaldson, 2009). Consistent with our previous findings (Figure 6E and Supplemental Figure S4) and those of Temkin *et al.* (2011) showing an absence of

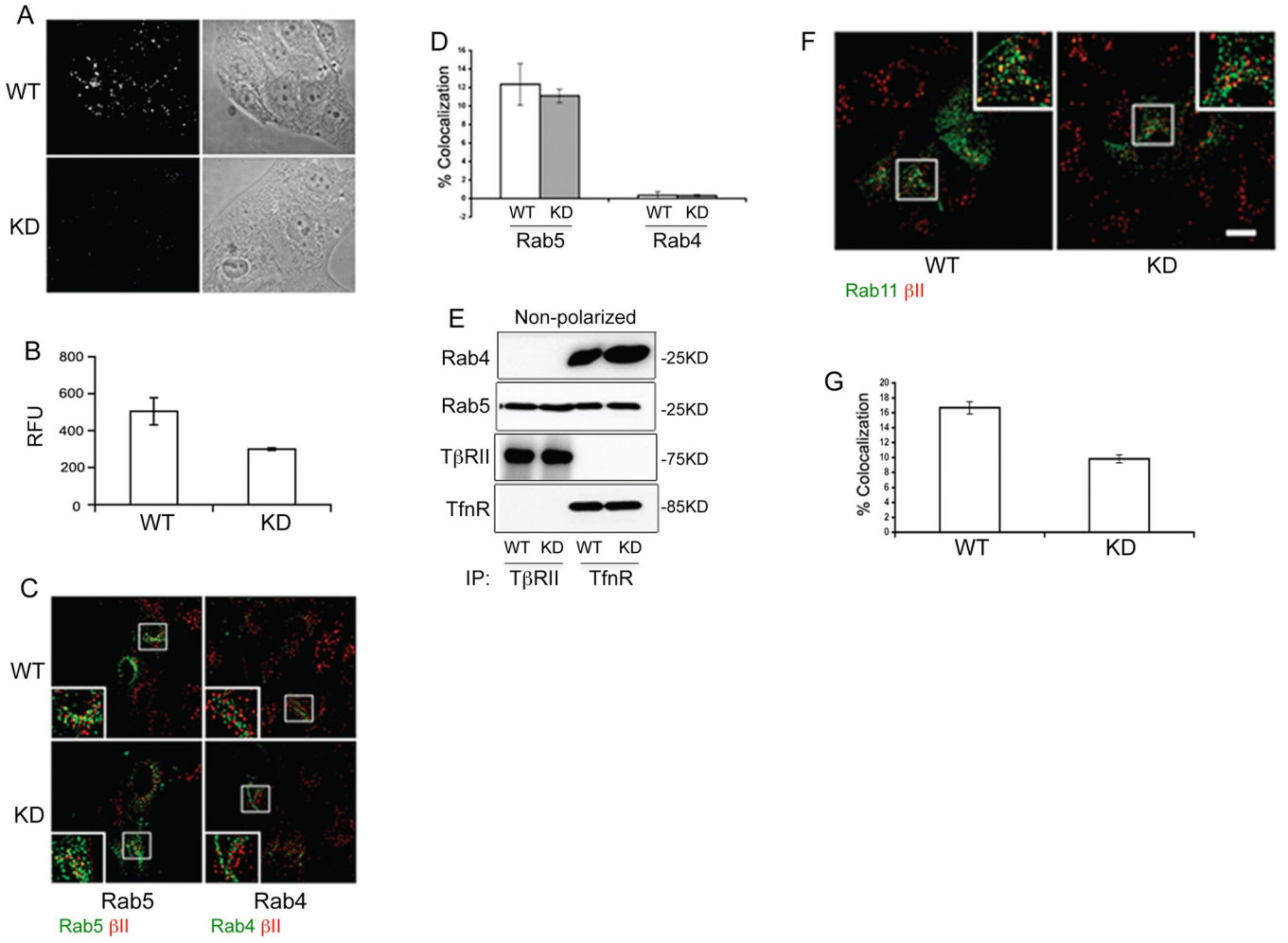

FIGURE 6: TGF-βR recycling to a Rab11-positive compartment in monolayer cultures is dependent upon the mammalian retromer. (A) Recycling of the chimeric type II TGF-βR in nonpolarized MD-1 (WT) and retromer knockdown 43-10 (KD) cells was determined as in *Materials and Methods*. Left, fluorescence; right, phase images. (B) Data represented as arbitrary units of fluorescence ± SD from 30 cells in each of three independent experiments. (C) MD-1 (WT) and 43-10 (KD) cells transiently transfected with GFP-Rab5 (green) or CFP-Rab4 (green) were fixed, permeabilized, and incubated with GM-CSFR-β antibody, followed by incubation with Cy3-conjugated secondary antibodies to detect total chimeric βII (red). (D) Percentage of receptor colocalization with GFP-Rab5 or CFP-Rab4 represented as the mean ± SEM of 30 cells from three independent experiments. (E) Microsomes were purified from nonpolarized MD-1 (WT) or 43-10 (KD) cells as described in *Materials and Methods* and specificity confirmed in Supplemental Figure S3. After immunoprecipitation of TβRII (lanes 1 and 2) or TfnR (lanes 3 and 4), cofractionation of the indicated proteins was determined by Western blotting. Before immunoprecipitation, wild-type and knockdown samples were first normalized by Western blotting to the TβRII or TfnR levels in the microsome lysate to allow direct comparison between conditions. (F) MD-1 (WT) and 43-10 (KD) cells transiently transfected with GFP-Rab11 were incubated with antibody to the chimeric type II TGF-βR and processed as in C. (G) Mean ± SEM colocalization of 75 cells from three independent experiments. Bar, 10 μm.

a retromer requirement in TfnR recycling, TfnR association with Rab11 was unaffected by retromer knockdown in either nonpolarized (i.e., strong association due to role of Rab11 in TfnR recycling) or polarized (i.e., little association, as TfnR is a basolateral protein and Rab11 functions with the ARE) cultures (Figure 8E, lanes 5–8).

The aforementioned results indicate that TfnRs and type II TGF-βRs use both overlapping (i.e., Rab11 dependence in nonpolarized cells) and distinct (i.e., retromer requirement) recycling mechanisms. This was further documented by examining chimeric βII receptor colocalization with pulse-chased TfnR at 25 min (i.e., the CRE as described by Thompson *et al.*, 2007) in polarized control MD-1 and retromer knockdown cells. As expected due to the divergent effect of retromer loss on TβRII and TfnR recycling/locale, although significant βII/TfnR colocalization was observed in MD-1 cells, this was diminished in knockdown cultures (Figure 8, F and G). Thus, after initial targeting to the basolateral plasma membrane in polarized epithelia, native and chimeric type II TGF-βRs, in contrast to TfnRs or the type I TGF-βR, use a retromer-dependent mechanism(s) for delivery to the recycling endosome in the maintenance of basolateral TβRII expression (Figure 9).

DISCUSSION

While the idea of recycling for TGF-β family receptors is a nearly 30-yr-old concept (Massagué and Like, 1985; Sathre *et al.*, 1991), defining the pathways and mechanisms controlling TGF-βR trafficking has significantly lagged from that reported for other plasma

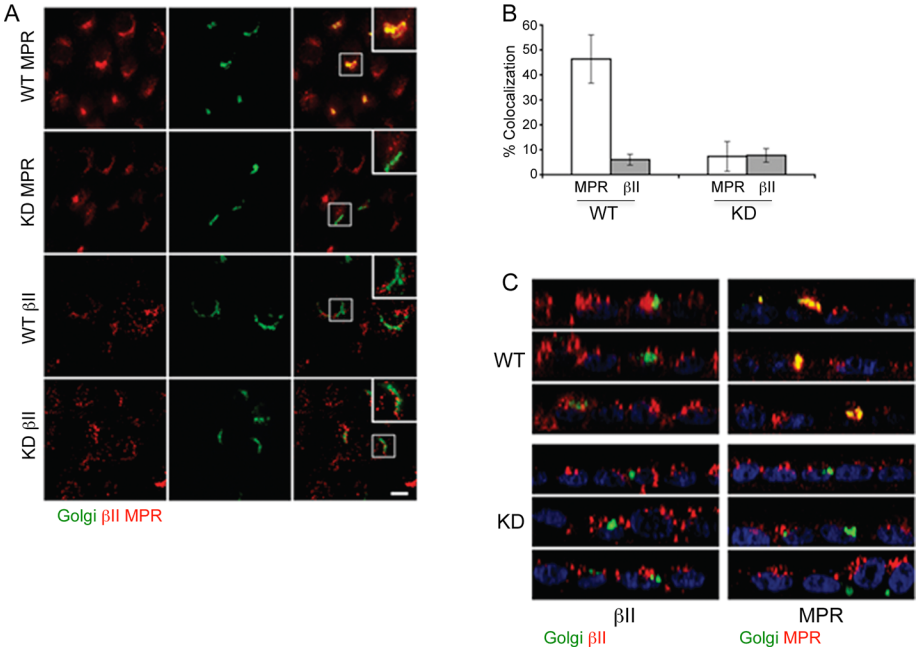

FIGURE 7: Type II TGF-βR trafficking occurs independent of Golgi transit. (A) Nonpolarized MD-1 (WT) and 43-10 (KD) cells transiently transfected with TGN marker galactosyltransferase-GFP (GalT-GFP) were incubated at 37°C in DMEM containing 10 μg/ml monoclonal antibody to CI-MPR (MPR; top two panels) or GM-CSFR-beta (βII; bottom two panels) for 60 min. Cultures were then fixed, permeabilized, and stained with Cy3-conjugated secondary antibodies (red) to detect the internalized CI-MPRs and chimeric type II receptors. Third column depicts merge of fluorescent signals and blow-up of boxed region. (B) Mean ± SD percentage of CI-MPR or chimeric βII colocalization with GalT-GFP for 30 cells in each of three independent experiments from A. (C) Polarized MD-1 (WT) and 43-10 (KD) cells transfected with GalT-GFP and processed as in A.

membrane proteins (Grant and Donaldson, 2009; Hsu *et al.*, 2012). Although more recent studies documented endocytic uptake being regulated via a primarily clathrin-dependent process (Anders *et al.*, 1997; Doré *et al.*, 2001; Yao *et al.*, 2002; Mitchell *et al.*, 2004) and defined roles identified for Rab11 and Dab2 in TβRII recycling (Penheiter *et al.*, 2002, 2010; Di Guglielmo *et al.*, 2003; Mitchell *et al.*, 2004), there is a paucity of information on the operative sorting signals, coat proteins/adaptors, and/or how receptor trafficking is regulated in polarized epithelia. For instance, whereas native and chimeric type I and type II TGF-βRs traffic to, and signal from, the basolateral membrane in polarized epithelial cells (Murphy *et al.*, 2004, 2007), the cellular activities directing and/or maintaining that response are unknown. To directly address that issue, we used a COOH-terminal fragment of the TβRII containing a motif required for appropriate basolateral expression in an attempt to identify interacting proteins important for TβRII trafficking. One such protein, the Vps26 subunit of the mammalian retromer, was identified and selected for further analysis.

Retromer/cargo binding can occur via Vps complex–dependent as well as –independent mechanisms (McGough and Cullen, 2011). Although TβRII/retromer coimmunoprecipitation can be observed with antibodies to either the Vps26 or Vps35 subunits, no association with native or chimeric type I TGF-βRs was detected (Figure 1 and Supplemental Figure S1). Consistent with that finding are our data showing that retromer knockdown has no detectable effect on TβRI trafficking or Smad phosphorylation (Figure 2, D–F, and Supplemental Figure S2, A and B) yet is critical for appropriate membrane localization of TβRII in polarized epithelial cells (Figures 2–5 and Supplemental Figure S2, C and D). Because steady-state TβRII expression was observed at both the apical and basolateral domains with no diminution in receptor ligand binding in retromer knockdown cells, we initially believed that retromer was providing a targeting function. This was not found to be the case, as when we performed kinetic analysis of TβRII membrane expression, the absence of retromer had no effect on basolateral TβRII delivery/expression during the first 60 min after release from a 20°C Golgi block (Figure 3). Apical TβRII expression did, however, become detectable over the next 30–60 min in the retromer knockdown cells, with steady-state mislocalization levels obtained within the next hour.

The fact that retromer provides "maintenance" rather than direct TβRII "trafficking" function(s) (Figure 3) is analogous to one role of the AP1B complex in basolateral sorting (Fölsch *et al.*, 1999; Gan *et al.*, 2002; Mellman and Nelson, 2008; Gonzalez and Rodriguez-Boulan, 2009; Gravotta *et al.*, 2012). For instance, although initial studies indicated a targeting role of AP1B for a subset of basolaterally sorted proteins, including the low-density-lipoprotein receptor (Fölsch *et al.*, 1999; Gan *et al.*, 2002; Gonzalez and Rodriguez-Boulan, 2009; Gravotta *et al.*, 2012), subsequent analyses recognized that this finding reflected a defect in postendocytic recycling. It should be noted, however, that this is not the case for all cargo, as some traffic directly from the TGN to the recycling endosomes for initial basolateral or apical delivery (Ang *et al.*, 2004; Gravotta *et al.*, 2007). In a similar vein, although we initially expected that retromer/TβRII binding would be through the LTA motif (Murphy *et al.*, 2007), this was not the case (unpublished observations). Given that the LTA motif, however, is necessary for TβRII basolateral targeting, in hindsight it would not be unexpected to find that TβRII basolateral "delivery" and subsequent "maintenance" (via a recycling mechanism; Figures 3–5) might be uniquely controlled. This is analogous to the complex interaction of the CI-MPR with Vps35 not requiring the YSKV sequence involved in CI-MPR internalization (Arighi *et al.*, 2004). Moreover, 1) the LTA sequence (^{529}LTAxxVAxxF^{538}) does not fit the general retromer binding criteria of being highly hydrophobic and rich in aromatic amino acids, as seen for sortilin (FLV), CI-MPR (WLM), DMT1-II (YLL), or sorLA (FANSHY; Fjorback *et al.*, 2012); and 2) most of the aforementioned motifs were identified for their role in endosome-to-Golgi/TGN trafficking. Given that we have now determined that the maintenance of basolaterally expressed TβRII is independent of Golgi transit (Figure 7), it will be of interest to determine how retromer interacts with the comparable region in TβRII.

Although a related role for retromer in β2AR endosome-to-plasma membrane trafficking has been reported (Temkin *et al.*, 2011), in contrast to β2ARs, internalized type II TGF-βRs undergo apical missorting with no significant change in steady-state receptor levels (i.e., misrouting to a degradative compartment; Figure 2B). Furthermore, because TGF-βRs use a Rab11-regulated pathway and there are no recognizable PDZ domains in the TβRII (distinct from the Rab4 association and PDZ requirement for β2AR recycling;

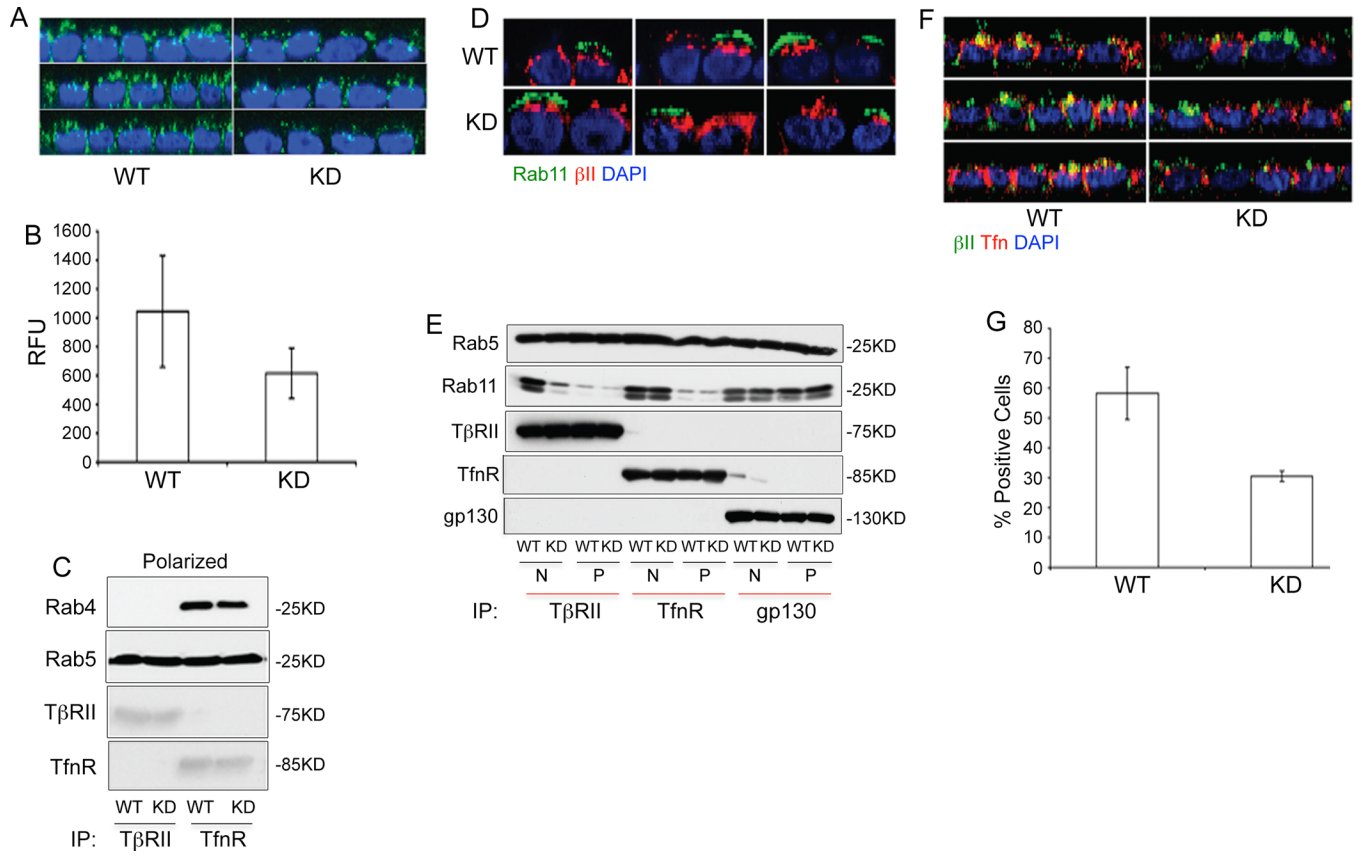

FIGURE 8: Apical type II TGF-βR mislocalization in polarized retromer knockdown cells is independent of the Rab11-positive apical recycling endosome. (A) Recycling of the chimeric βII receptor in polarized MD-1 (WT) and 43-10 (KD) cells was determined as in *Materials and Methods*. (B) Data from A represented as mean arbitrary units of fluorescence ± SEM of 40 cells from three independent experiments. (C) Microsomes were purified from polarized MD-1 (WT) or 43-10 (KD) cells and processed as described in Figure 6E and *Materials and Methods*. Cofractionation of Rab4 or Rab5 with TβRII (lanes 1 and 2) or TfnR (lanes 3 and 4) was assessed by Western blotting. (D) Polarized MD-1 (WT) and 43-10 (KD) cells were transiently transfected with GFP-Rab11 for 24 h. After fixation and permeabilization, the cultures were stained with GM-CSFR-β antibody (βII) and Cy3-conjugated secondary antibody (Red) to detect any colocalization. (E) Microsomes were prepared from nonpolarized (N) or Transwell polarized (P) MD-1 (WT) or 43-10 (KD) cells as described. The cofractionation of Rab5 (top panel) or Rab11 (second panel) with immunoprecipitated TβRII (lanes 1–4), TfnR (lanes 5–8), or the apical-directed protein gp130 (lanes 9–12) was then assessed by Western blotting. Bottom three panels depict cofractionation of the indicated protein with the immunoprecipitated target. (F) Subsequent to Transwell polarization, MD-1 (WT) and retromer depleted 43-10 (KD) cells were incubated with phenol red–free DMEM at 37°C for 2 h, washed with cold DMEM (3×), and then treated with 25 μg/ml Alexa Fluor 555–conjugated Tfn (red) diluted in DMEM at 10°C for 15 min, followed by incubation at 37°C for 5 min. After DMEM wash (3×) and incubation at 37°C for 20 min, cells were acid stripped, fixed, permeabilized, and incubated with Alexa Fluor 488–conjugated secondary antibody (green) to detect the chimeric type II receptor (βII). (G) Percentage of cells that showed colocalization of chimeric βII with pulse-chased Tfn from F represented as the mean ± SD for 384 cells from three independent experiments.

Mitchell *et al.*, 2004; Penheiter *et al.*, 2010), retromer differentially regulates TβRII and β2AR recycling. These differences, however, are not surprising, considering 1) the various roles these receptor families have, 2) the differing experimental models used, and, most important, 3) the myriad retromer functions that are just now being identified (Verges, 2008; McGough and Cullen, 2011; Cullen and Korswagen, 2012; Seaman, 2012).

It is of interest that we do not see any detectable colocalization of the type II TGF-βR and Rab4 (Figure 6, C and D), as Rab4 has been shown to colocalize with the early endosome in conjunction with other cargo, such as β2AR (Cao *et al.*, 1999; Seachrist *et al.*, 2000) or transferrin receptor (Sheff *et al.*, 1999; Sonnichsen *et al.*, 2000). In the majority of those studies, however, Rab4 is shown to functionally affect the trafficking of the cargo being studied. This is contrasted by our previous determination that Rab4 had no identifiable role in TβRII recycling (Mitchell *et al.*, 2004). As such, since we do not see colocalization (Figure 6, C and D) or cofractionation (Figures 6E and 8C) of chimeric or native type II TGF-βRs with Rab4, yet have documented both colocalization (Supplemental Figure S4A) and cofractionation (Figures 6E and 8C) of the transferrin receptor (i.e., positive control) with Rab4, this suggests 1) TβRIIs traffic via distinct population(s) of early endosomes devoid of Rab4, and/or 2) TβRIIs sufficiently segregate from Rab4 on the early endosome membrane such that they do not appear to colocalize or precipitate within the same microsome fragments. This latter point is consistent with the data of Sonnichsen *et al.* (2000), who proposed that endosomes are a mosaic of distinct domains defined by their composition of Rab proteins.

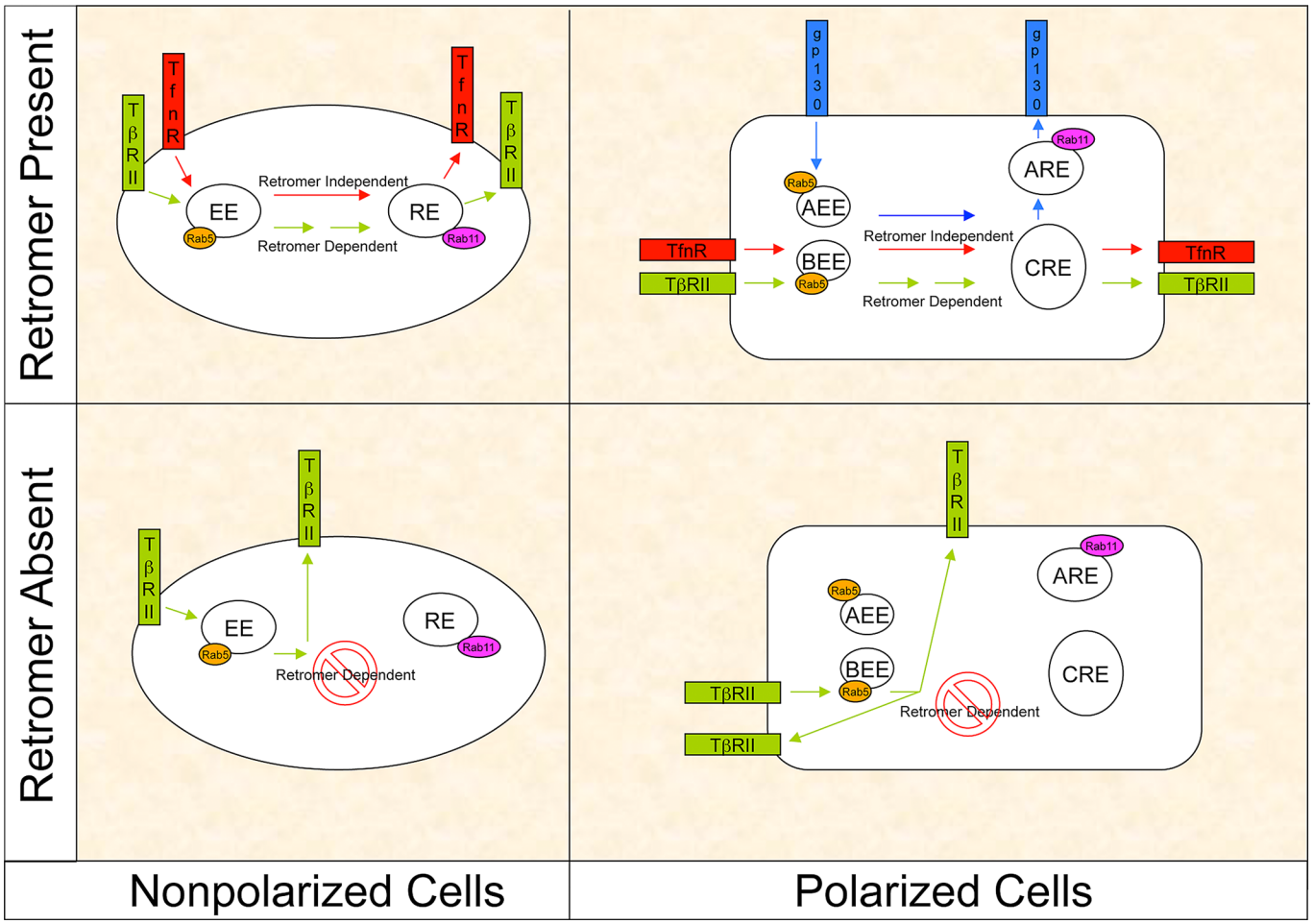

FIGURE 9: Model depicting role of retromer in maintaining steady-state basolateral expression of the type II TGF-βR. Nonpolarized cells: TβRII and TfnR undergo constitutive clathrin-dependent internalization to the Rab5-positive early endosome (EE) and recycling via Rab11 (rapid TfnR recycling through Rab4 is not depicted). In the absence of retromer, whereas TfnR recycling and steady-state TβRII membrane levels are unaffected, TβRIIs are unable to enter the Rab11-positive compartment and show diminished recycling. Polarized cells: TβRII and TfnR are similarly internalized to the basolateral early endosome (BEE) and recycled through the common recycling endosome (CRE) to the basolateral plasma membrane. Entry of TβRII, but not TfnR, to the CRE depends on retromer such that retromer depletion results in TβRII mislocalization to both apical and basolateral membranes. In contrast to the apical-directed gp130 protein, apical TβRII trafficking occurs independent of the Rab11-positive apical recycling endosome (ARE). The ARE and CRE are depicted as distinct compartments for ease of presentation. The recycling endosome, however, may consist of a single endosome with multiple subdomains (Ang and Folsch, 2012). Given that retromer functions downstream of Rab5 and it is unknown whether apical and/or basolateral TβRII delivery in retromer knockdown cells occurs via distinct compartments/markers, TβRII membrane return is shown not originating from a defined locale.

Our findings showing that retromer knockdown 1) has no effect on initial basolateral TβRII delivery (Figure 3, A and B), 2) inhibits recycling downstream of clathrin-dependent internalization (Figures 5, 6, and 8, A–C), and 3) results in the mislocalization of TβRII to the apical plasma membrane (Figures 2–5 and Supplemental Figure S2, C and D) support a unique role for the mammalian retromer complex in regulating the homeostatic expression of type II TGF-βRs in polarized epithelia. Specifically, after basolateral cell surface delivery, TβRIIs undergo constitutive clathrin-dependent internalization and transit to the Rab5-positive BEE. Retromer and potentially other associated proteins such as Dab2 or Rab7 (Rojas *et al.*, 2008; Balderhaar *et al.*, 2010; Penheiter *et al.*, 2010) function to promote exit and subsequent trafficking from the CRE back to the appropriate plasma membrane domain. In nonpolarized cells this occurs via a Rab11-dependent process (Figure 6; Mitchell *et al.*, 2004). In the absence of retromer, however, TβRIIs aberrantly sort in polarized cultures independent of Golgi transit and the Rab11-positive ARE (Figures 7, A–C, and 8, D and E) such that both basolateral and apical expression is observed. A model depicting these findings is presented in Figure 9. Ongoing studies focus on identifying and characterizing the retromer interacting motif(s), sorting nexin requirement(s) (Harterink *et al.*, 2011), and accessory proteins to better understand and integrate retromer in maintaining TβRII polarity with its disparate effects on cargo such as basolateral-to-apical transcytosis of polymeric immunoglobulin A (Verges *et al.*, 2004) and apical localization of Crumbs in *Drosophila* larvae (Pocha *et al.*, 2011).

MATERIALS AND METHODS

Cell culture

MDCK cells were maintained in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) at 37°C and 5% CO_2. For Transwell culture (12-mm Costar polycarbonate membranes), cells were plated at a density of 5×10^4 cells/ml in 0.5 ml of culture medium. Fully polarized monolayers were achieved after 3 d (Murphy *et al.*, 2004, 2007).

MD-1 cells represent a MDCK clone stably expressing chimeric type I and type II TGF-βRs (Murphy *et al.*, 2004, 2007). The designations αI and βII refer to chimeric receptors expressing the extracellular ligand-binding domain of the GM-CSF α or β receptor coupled to the transmembrane and cytoplasmic domain of the TGF-β type I and type II receptors, respectively (Anders and Leof, 1996). Previous work documented that chimeric and native TGF-βRs have analogous signaling and trafficking activity regardless of the culture conditions or cell type tested (Anders and Leof, 1996; Anders *et al.*, 1997; Yao *et al.*, 2002; Mitchell *et al.*, 2004; Murphy *et al.*, 2004).

Plasmids (pLKO.1-puro) encoding shRNAs targeting human Vps35 were purchased from the Mayo Clinic RNA Interference Technology Resource (Rochester, MN). The production of lentivirus and the transduction of MD-1 cells were as described previously (Rahimi *et al.*, 2009). The human Vps35 shRNA sequences CAGTGAAGAAACAGAGCAGAT (#3; base pairs 2237–2257) and GCAGATCTCTACGAACTTGTA (#4; base pairs 234–254) were used to generate clones 33-1/33-18 and 43-10, respectively. The 33 clones required two transfections of shRNA #3, and the 43 clone was sequentially transfected with #4 and then with #3 shRNA. Stable cell lines were maintained in the presence of 1.5 μg/ml puromycin.

Inulin flux was measured by plating 5×10^4 cells/12-mm Transwell dish in 10% FBS/DMEM and allowing them to polarize over 3 d. Medium was removed and the apical and basal chambers washed 2× with serum-free DMEM containing 0.5 mM inulin (Sigma-Aldrich St. Louis, MO). After addition of fresh DMEM/inulin (to both chambers) and a 10-min incubation at 37°C, the apical medium is replaced with fresh DMEM supplemented with 0.2 μCi/ml [^{14}C]inulin (PerkinElmer, Waltham, MA). Transwells are returned to 37°C and at the indicated times aliquots removed from the basal chamber and processed for liquid scintillation counting.

Immunostaining and microscopy

Transwell cultures were rinsed with filter sterilized HMEM+G (13.8 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 13.7 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 2.0 mM glutamine, 0.44 mM KH_2PO_4, 0.18 mM Na_2HPO_4, 1× MEM vitamins, 1× MEM amino acids, pH 7.4; Singh *et al.*, 2007) and incubated with primary antibody diluted in ice-cold HMEM+G at 10°C for 45 min. After washing with ice-cold PBS, the cultures were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. The cultures were then quenched with 50 mM NH_4Cl/PBS before incubating (room temperature, 45 min) with secondary antibodies (Cy3-labeled donkey anti-mouse immunoglobulin G [red; 715-165-150, Jackson ImmunoResearch Laboratories, West Grove, PA] or Alexa Fluor 488–labeled goat anti-rabbit IgG [green; A11008, Invitrogen, Carlsbad, CA]) and 4′,6-diamidino-2-phenylindole (DAPI) diluted in 5% normal donkey serum (NDS)/1% BSA/PBS (blocking buffer). For costaining, cells were treated with blocking buffer at room temperature for 2 h and incubated with antibody diluted in blocking buffer at room temperature for 1 h. After PBS wash (5 × 5 min), cultures were incubated with secondary antibodies and DAPI diluted in blocking buffer. Slides were mounted using Vectashield (Vector Laboratories, Burlington, CA) and fluorescence internalization performed using an AX-70 Olympus microscope (Olympus, Tokyo, Japan) equipped with 60×/1.4 numerical aperture (NA) or 100×/1.35 NA oil immersion objectives. Images were acquired at room temperature using a C4742-95-12NR (Hamamatsu, Hamamatsu, Japan) camera. Confocal images were acquired with a 100× objective (1.3 NA oil lens) on a Zeiss LSM 510 confocal system (Carl Zeiss, Jena, Germany). Individual figures were prepared using Photoshop CS (Adobe, San Jose, CA). Two-dimensional deconvolution of nearest neighbors was used for Figure 6, C and F, and Supplemental Figure S4. No three-dimensional reconstructions, surface or volume rendering, or gamma adjustments were performed. MetaMorph, version 7.3.2 (Molecular Devices, Sunnyvale, CA), was used for all quantitation.

Direct recycling assay

Recycling in nonpolarized and Transwell polarized cultures was based on a protocol by Fraile-Ramos *et al.* (2001). This was previously described in detail, in which an antibody recognizing the extracellular receptor domain is visualized through 1.5 cycles of recycling (Mitchell *et al.*, 2004). Because the fluorescent secondary antibody binds only those receptors that return to the cell surface with attached primary antibody, intracellular fluorescence is observed only after an additional internalization event. For polarized cells, primary and secondary antibody was added to both apical and basal chambers.

Immunoblotting and immunoprecipitation

Cells were lysed in NP40 lysis buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.2% NP-40) including protease inhibitor cocktail (Roche, Indianapolis, IN) on ice for 1 h. The lysate was clarified at 21,000 × *g* for 15 min. Equal protein was incubated with primary antibody at 4°C overnight with agitation and then with protein A– or G–agarose beads for 2 h. The beads were washed three times with lysis buffer and bound proteins recovered in 2× Laemmli sample buffer. Clarified lysate or immunopurified protein was resolved on SDS–PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat milk in 10 mM Tris (pH 7.4)/0.1% Tween 20 (TBST). The membranes were incubated with antibodies diluted in blocking solution overnight at 4°C, washed with TBST, and incubated with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. After washing with TBST, membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and bands detected with an X-OMAT 2000A. GST pull down was performed as previously described (Yao *et al.*, 2002).

Microsome cofractionation

A modification of the Qproteome Plasma Membrane Kit (37601; Qiagen, Valencia, CA) was developed that allows isolation of not only plasma membrane and plasma membrane–derived vesicles, but also of associated/cofractionated constituents such as Rab proteins. Briefly, cell pellets were collected (4 × 100 mm plates or 6 × 24 mm Transwells per condition) and lysed by mechanical disruption through a 27 gauge needle (15×) in lysis buffer (125 mM HEPES, pH 7.5, 2% NP40, 750 mM NaCl, 50 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, Roche protease inhibitor cocktail). Subsequent to centrifugation (20 min, 12,000 × *g*) to remove nuclei, large organelles, and cell debris, the resulting supernatant (consisting of 20- to 200-nm microsomes derived from the endoplasmic reticulum, Golgi, and plasma membrane) was then incubated (gentle rocking) for

60 min at 4°C with a Kit-supplied (Qiagen, Valencia, CA) lectin ligand (in HEPES lysis buffer) specific for plasma membrane molecules. After addition of *Strep*-Tactin magnetic beads (Qiagen) for an additional 60 min (4°C) and gentle washing (3×, HEPES lysis buffer), intact vesicles were eluted from the beads/lectin ligand in Kit (Qiagen) elution buffer. Cofractionated proteins were assessed by immunoprecipitation and Western blotting as described.

Biotinylation of cell surface receptors

To detect biotinylated cell surface receptors, 4.5×10^5 MD-1 or retromer knockdown 43-10 cells were plated in 24 mm Transwell or six-well plates for 72 h with a medium (10% FBS/DMEM) change after 48 h. After three washes with ice-cold Hank's balanced salt solution (HBSS; Mediatech, Manassas, VA), freshly made sulfo-NHS-SS biotin/HBSS (1 mg/ml; Thermo Scientific, Waltham, MA) was added to the six-well plate (1 ml) to assess total (T) labeling. Domain-specific receptor expression was determined by sulfo-NHS-SS biotin/HBSS addition to the apical (0.5 ml) or basolateral (1 ml) surfaces of polarized Transwell cultures. Plates were rocked for 1 h at 4°C before washing with ice-cold 5 mM Tris/HBSS and lysis in 0.2 ml of modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 10 mM NaF) with protease inhibitors on ice for 1 h. Streptavidin–agarose (100 µl; 21331; Thermo Scientific) was mixed with 0.5–1 of mg protein in 1 ml of total volume for 2 h at 4°C. The agarose was washed four times with lysis buffer (1 ml) and the biotin-bound proteins eluted by boiling for 10 min in 2× Laemmli buffer. Samples were resolved on 10% SDS–PAGE and the target protein detected by Western blotting.

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