A targeted RNAi screen identifies factors affecting diverse stages of receptor-mediated transcytosis

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A targeted RNAi screen identifies factors affecting diverse stages of receptor-mediated transcytosis

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Endosome transport by transcytosis is the primary mechanism by which proteins and other large cargo traverse epithelial barriers in normal tissue. Transcytosis is also essential for establishing and maintaining membrane polarity in epithelia and other polarized cells. To identify novel components of this pathway, we conducted a high-throughput RNA interference screen for factors necessary for the bidirectional transcytosis of IgG by the Fcγ receptor FcγR. This screen identified 23 genes whose suppression resulted in a reproducible decrease in FcγR-mediated transcytosis. Pulse-chase kinetic transport assays on four of the top-ranking genes (EXOC2, EXOC7, PARD6B, and LEPROT) revealed distinct effects on the apical and basolateral recycling and transcytotic pathways, demonstrating that these pathways are genetically separable. We also found a strong dependence on PARD6B for apical, but not basolateral, recycling, implicating this cell polarity gene in assembly or maintenance of the apical endosomal system. This dataset yields insights into how vesicular transport is adapted to the specialized functions of differentiated cell types and opens new research avenues into epithelial trafficking.

Introduction

In many tissues throughout the body, a single layer of polarized epithelial cells functions to separate and affect vastly different environments. Large molecules are transported across these epithelial barriers by transcytosis, a process of intracellular endosome traffic that connects one cell surface with the other. Many roles for transcytosis have been documented (Tuma and Hubbard, 2003). Transcytosis of the immunoglobulins, for example, enables immune surveillance of mucosal surfaces lining the gut, lungs, and urogenital tract (Rojas and Apodaca, 2002; Rath et al., 2015). Transcytosis also contributes to the development of cell polarity by enabling the assembly of distinct apical and basolateral plasma membranes, thus fundamentally shaping cell and tissue function (Apodaca et al., 2012). Microbes, viruses, and microbial toxins exploit transcytosis for invasion of the host (Lencer et al., 1995; Bomsel, 1997; Couesnon et al., 2008), and the pathway holds promise for mucosal delivery of therapeutic peptides and proteins (Spiekermann et al., 2002; Li et al., 2011). Here, we report the results of a high-throughput endoribonuclease-prepared short interfering RNA (esiRNA) screen for components of transcytosis. Several of the newly identified genes are informative of endosome organization in polarized simple epithelia.

The endocytic compartments mediating transcytosis are uniquely adapted to accommodate the polarized cell phenotype (Rodriguez-Boulan et al., 2005; Tzaban et al., 2009; Li et al., 2011). One specialization is the establishment of distinct populations of apical and basolateral early sorting endosomes, which selectively receive cargo from either apical or basolateral surfaces, respectively (Bomsel et al., 1989, 1990; Parton et al., 1989; Sheff et al., 2002). These early sorting endosomes then route internalized cargo to one of several possible destinations, including back to the cell surface where endocytosis originated (the recycling pathway) or to the opposite cell surface via the common/apical recycling endosomes (the transcytotic pathway). The common/apical recycling endosome is another adaptation of the endosomal system of polarized epithelia. It receives cargo from both cell surfaces and serves as a major sorting station for the recycling and transcytotic pathways (Parton et al., 1989; Hughson and Hopkins, 1990; Apodaca et al., 1994; Barroso and Sztul, 1994; Odorizzi et al., 1996; Wang et al., 2000a).

Transcytosis has been best characterized for basolateral-to-apical transport of dimeric IgA (dIgA) by the polymeric immunoglobulin receptor (pIgR; Rojas and Apodaca, 2002), pIgR binds dIgA on the basolateral membrane of mucosal epithelial cells and carries it sequentially into the early basolateral sorting endosome, the common endosome, the apical recycling endosome, and finally to the apical cell surface where the extracellular domain of the receptor is cleaved for release into the...
lumen as a component of secretory IgA. A complex network of genes directly regulate plgR-mediated transcytosis, including several Rab family GTPases (RAB3B, RAB5, RAB11A, RAB17, and RAB25; Hunziker and Peters, 1998; Wang et al., 2000b; van Ijzendoorn et al., 2002), the exocyst complex (Oztan et al., 2007), retromer (Vergés et al., 2004), phosphoinositide-3-kinase (Vergés et al., 2007), MYO5B (Lapierre et al., 2001), RAB11FIP5 (Ducharme et al., 2007; Su et al., 2010), the Rab11 GTPase activating protein TBC1D9B (Gallo et al., 2014), the lipid microdomain-associated protein MAL (de Marco et al., 2002), and signaling cascade mediated through the Src family kinase p62VES (Luton et al., 1999; Su et al., 2010).

Much less is known about apical-to-basolateral transcytosis. One example of transport in this direction is the transcytosis of IgG by the Fcγ receptor FcRn, which physiologically carries its cargo in both directions across epithelial barriers (Roopenian and Akiles, 2007). FcRn trafficking has been shown to depend on some of the same proteins as plgR, namely, MYO5B, RAB25 (Tzaban et al., 2009), calmodulin (Dickinson et al., 2008), and components of the actin cytoskeleton (Zhou et al., 2015). However, there are also clear differences, as FcRn transcytosis does not require RAB11A (Tzaban et al., 2009), a central organizing molecule for plgR transcellular transport. Another model of apical-to-basolateral transcytosis by the Wnt receptor in the Drosophila melanogaster wing imaginal disc, required for transepithelial signaling by Wnt, revealed dependence on the E3 ubiquitin ligase Godzillia (Yamazaki et al., 2016). The involvement of other genes in apical-to-basolateral transcytosis has not been tested, leaving a fragmentary view of this complex multistage and clinically relevant pathway.

To gain new insight into receptor-mediated transcytosis, we conducted an RNAi screen for genes that operate in the trafficking of IgG by the Fcγ receptor FcRn, which physiologically carries its cargo in both directions across epithelial barriers (Roopenian and Akiles, 2007). FcRn trafficking has been shown to depend on some of the same proteins as plgR, namely, MYO5B, RAB25 (Tzaban et al., 2009), calmodulin (Dickinson et al., 2008), and components of the actin cytoskeleton (Zhou et al., 2015). However, there are also clear differences, as FcRn transcytosis does not require RAB11A (Tzaban et al., 2009), a central organizing molecule for plgR transcellular transport. Another model of apical-to-basolateral transcytosis by the Wnt receptor in the Drosophila melanogaster wing imaginal disc, required for transepithelial signaling by Wnt, revealed dependence on the E3 ubiquitin ligase Godzillia (Yamazaki et al., 2016). The involvement of other genes in apical-to-basolateral transcytosis has not been tested, leaving a fragmentary view of this complex multistage and clinically relevant pathway.

**Results**

**Development of a model for receptor-mediated transcytosis compatible with high-throughput screening**

We have previously established a cell culture model of FcRn-mediated transcytosis built upon the MDCK cell line.

Stably expressing the two subunits of human FcRn, human β2m and the human FcRn heavy chain, which was tagged with the HA epitope at the N terminus and GFP at the C terminus (Tzaban et al., 2009). To measure transcytosis, cells are grown on transwell semipermeable filters, which provide independent experimental access to both the apical and basolateral sides of the monolayer. Then, IgG is added to one side of the monolayer (at pH 6.0 to facilitate FcRn-dependent binding and uptake), and the amount of IgG to pass through the filter after a period of time is quantified. In preparation for an RNAi screen, several modifications were made.

One challenge of the original transcytosis protocol was that the assay used to detect IgG, an ELISA, was not sensitive enough for small-volume (e.g., 96-well) transport studies and furthermore required many binding and washing steps that would be difficult to implement in a high-throughput format. To simplify the assay and increase sensitivity, we constructed a recombinant protein in which the Fc (FcRn-binding) domain of human IgG1 was linked to Gaussia luciferase (Tannous et al., 2005) in place of the variable domains (GLuc-Fc; Fig. 1 A). The Gluc-Fc fusion was produced in CHO cells and purified to >99% homogeneity (Fig. S1 A). The Gluc-Fc fusion can be detected by direct enzymatic assay with approximately five orders of magnitude greater sensitivity and over 2.5 times the dynamic range compared with our original ELISA assay for detecting IgG (Fig. 1 B). To test whether this Gluc-Fc fusion protein crosses MDCK monolayers through an FcRn-mediated pathway, we compared the permeability of Gluc-Fc to that of a similar construct with inactivating mutations in the FcRn-binding domain (Gluc-FcIFHH; Fig. 1 A). The FcIFHH mutation abolishes binding to FcRn, but not to other immune Fc receptors (Spiekermann et al., 2002; Baker et al., 2011), and thus provides a clean measure of FcRn-independent (i.e., nonspecific) permeability. We found that the wild-type Gluc-FcWT protein was transported in both directions across MDCK monolayers >10 times more efficiently than the nonbinding Gluc-FcIFHH mutant (Fig. 1 C), confirming that the luciferase fusion was transported across the monolayer by binding FcRn.

We also took steps to increase the biological signal-to-noise of our transport assays. Several mutations in the FcRn-binding domain of IgG have been identified that bind to FcRn with increased affinity, resulting in enhanced FcRn-dependent trafficking phenotypes in vivo (Zalevsky et al., 2010). We hypothesized that one of these mutations might also lead to more efficient transport in our in vitro cell system. To test this, we introduced two affinity-increasing point mutations into the FcRn-binding region (N434S and M428L) (Tzaban et al., 2009). To measure transcytosis, cells are grown on transwell semipermeable filters, which provide independent experimental access to both the apical and basolateral sides of the monolayer. Then, IgG is added to one side of the monolayer (at pH 6.0 to facilitate FcRn-dependent binding and uptake), and the amount of IgG to pass through the filter after a period of time is quantified. In preparation for an RNAi screen, several modifications were made.

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We next established protocols for consistent and efficient gene knockdown (KD) in MDCK cells. We chose to use esiRNAs because they offer a reproducible approach for gene KD in mammalian cell culture (Kittler et al., 2004) and have been shown to produce fewer off-target effects than single, chemically synthesized siRNAs (Kittler et al., 2007). We prepared esiRNAs enzymatically starting from plasmid DNA or a
custom MDCK cDNA library (Fig. S1, D and E) and optimized transfection protocols for MDCK cells grown on transwell filters. Using esiRNAs directed against FcRn, we observed an 85% reduction in FcRn transcript levels by quantitative RT-PCR and a corresponding 85% reduction in FcRn-mediated transcytosis (Fig. S1, F and G).

We then adapted our cell culture model to a small-volume 96-well format (0.14-cm² filter supports). For the 96-well assays, both esiRNA transfections and transport assays were performed with the aid of robotics, allowing tight control over the conditions and timing of each step. In a pilot screen, we observed significant reductions in GLuc-FcMN transepithelial transport by KD of FcRn, as expected, and by KD of genes involved in clathrin-mediated endocytosis (CLTC, DNM2, and AP2M1; Fig. 1 D and Table S1), a pathway previously implicated in FcRn-dependent trafficking (Rodewald and Kraehenbuhl, 1984; Wu and Simister, 2001). When assessed by microscopy, KD for each gene in the clathrin endocytosis pathway redistributed FcRn from its predominantly endosomal location to the cell surface (Fig. 1 E), a phenotype consistent with blockade of endocytosis. These studies validated our experimental system.

Preparing a targeted esiRNA library
We conducted a targeted rather than whole-genome screen for pragmatic reasons, dictated by the cost and feasibility of polarized epithelial monolayer culture in high-throughput formats. To select candidate genes, we adopted a strategy designed to take advantage of known biology without sacrificing the potential to discover unexpected genes and/or gene families. First, we curated a set of 143 genes known to be involved in endosomal trafficking in epithelia (although many of these genes have not yet been tested for a role in transcytosis; Fig. S1, F and G).

As a second set of candidate genes, we identified genes implicated in epithelial trafficking by one of four indirect lines of evidence: (1) intracellular location, where the gene was localized to the endosomal compartment by proteomics (Foster et al., 2006; Lapierre et al., 2007; Cao et al., 2008) or by GFP tagging (Huh et al., 2003); (2) protein domain, where the gene contains at least one protein domain implicated in membrane trafficking or endosome dynamics (e.g., Rab GTPase, FYVE-finger, and BAR; Table S2); (3) expression in polarized epithelial cells, where the gene was found to be strongly expressed in simple columnar epithelial cells relative to other cell types (Nelms et al., 2016); and
A targeted esiRNA screen yields reproducible modifiers of FcRn-mediated transcytosis

The full esiRNA library was screened over a period of three sessions. The raw data displayed substantial systematic variability caused by batch and edge effects of the multiwell format. Several aspects of our experimental design made it possible to control for this systematic variability and identify reproducible hits. First, we included a large number of control wells distributed across each plate (Fig. S2 A); this layout was designed to fit statistician’s recommendations for high-throughput screens (Zhang, 2008). Second, we performed the screen with substantial replication (Fig. S2 B); every library plate was screened in triplicate in both transport directions, and GLuc-FcMN concentration was measured in duplicate for every well. The raw data were normalized using a linear mixed-effects model (Yu et al., 2011), resulting in a substantial decrease in variability (Fig. S3).

Normalized biological replicates displayed a high degree of reproducibility, with an $R^2$ of 0.907 for basolateral-to-apical transport and 0.966 for apical-to-basolateral transport (Fig. 3 B). Replicate values for each esiRNA were then log-transformed, pooled, and converted into a Z-score, which reflects the number of standard deviations of the phenotype (GLuc-FcMN transport) relative to the negative (mock transfection) controls.

When we compared Z-scores between the two directions of transport, we discovered that the effect of any esiRNA on one direction of transport was always directly proportional to its effect on the other direction (Fig. S4, A and B). This can be explained by the biology of FcRn and the conditions of our transport assays. First, GLuc-FcMN permeability is proportional to the flux of FcRn across the cell; this is caused by several features of FcRn, such as the finding that FcRn trafficking is not dramatically altered by binding to its ligand (Tzaban et al., 2009). Second, because (a) our assays were performed after chronic gene KD and (b) FcRn biosynthesis and degradation are slow relative to recycling and transcytosis, any perturbation in FcRn trafficking will lead to a new steady state where the flux of receptor in both directions across the cell is essentially equal. For example, if an esiRNA KD specifically inhibits basolateral endocytosis, FcRn will accumulate at the basolateral surface until the overall rate of FcRn flux in both directions across the cell is again balanced. Therefore, any perturbation on FcRn trafficking will manifest as an overall increase or decrease in FcRn-mediated transport, regardless of the molecular step affected or direction of Gluc-FcMn transcytosis. We thus pooled together the Z-scores in both directions, resulting in a single pooled Z-score that reflects the chance that an esiRNA affects transcellular FcRn flux.

An ordered plot of pooled Z-scores is shown in Fig. 3 C. The 112 negative control wells closely fit a standard normal distribution (Fig. 3 C, blue crosses and histogram on the right; $P = 0.498$, Shapiro–Wilk normality test). In contrast, many esiRNAs produced a phenotype far more extreme than the negative controls. For instance, esiRNAs targeting 7 out of 9 genes encoding subunits of the exocyst complex (red circles in Fig. 3 C) resulted in a decrease in transport larger than all negative controls. The exocyst is a large, multimeric protein complex that tethers vesicles to the membrane; it has previously been linked to related membrane trafficking pathways in epithelia (Heider and Munson, 2012), including in basolateral-to-apical transcytosis of pIgR (Oztan et
al., 2007), and the identification of the majority of subunits of this complex supports the validity of our results. Furthermore, our top hit, ACTR3B, is a component of the ARP2/3 complex recently implicated in IgG trafficking across the intestine in mice (Zhou et al., 2015). All esiRNAs that resulted in a Z-score less than $-3$ (23 “hits”) are shown in Table 1 and the entire list is shown in Table S4. This Z-score cutoff corresponds to a false discovery rate (FDR) of 0.03, signifying that only approximately one false positive is expected among these 23 genes by chance.

Eight of the top-scoring genes that caused a decrease in GLuc-FcMN transport (Table 1) were selected for validation in transport assays using monolayers grown on larger 0.33-cm²
filter supports. We repeated all validation assays on multiple independent days (to assess day-to-day variability), and with independent esiRNAs that target a distinct region of each gene’s transcript (to rule out potential off-target effects). Treatment with all eight original esiRNAs led to a significant reduction in GLuc-FcMN permeability that was reproducible between days (Fig. 4), confirming the phenotype from our screen. In addition, the phenotype was validated for seven out of eight genes using independent esiRNAs, suggesting that these phenotypes are unlikely to be caused by off-target effects. The validated genes include both genes with an established role in epithelial membrane trafficking (e.g., EXOC2 and ACTR3B) and genes that have not been linked to epithelial trafficking before (e.g., LEPROT and ARMT1). Thus, this screen identified several genes previously unknown to be necessary for receptor-mediated transcytosis across polarized epithelia.

**KD of EXOC2/7, LEPROT, and PAR6 affect diverse steps of the transcytotic and recycling pathways.**

Trafficking pathways in polarized epithelia involve separate apical and basolateral early endosomal pathways and endosome routes connecting both cell surfaces with each other and with other intracellular organelles. A defect in any of these routes could result in disruption of FcRn-mediated transcytosis. To determine which trafficking steps were disrupted during our screen, we selected four of the top-ranked genes for further characterization. These include two subunits of the exocyst complex (EXOC2 and EXOC7), the polarity regulator PAR6B, and the tetra-spanning membrane protein LEPROT.

First, we looked for possible indirect effects on membrane polarity or monolayer integrity after knocking down these genes. In each of the four cases tested, the cells assembled into tight epithelial monolayers as assessed by transepithelial electrical resistance (TEER), with PAR6B KD producing a slight increase in TEER and all other genes exhibiting no change (Fig. 5 A). There were no visible defects in monolayer integrity or cell morphology when the cells were imaged by confocal microscopy (Fig. 5 B and Fig. S5), and the membrane polarity markers podocalyxin/GP135 (apical membrane) and E-cadherin (lateral membrane) were normally localized to the appropriate plasma membrane domain (Figs. 5 B and S5 A). We suspect that none of these four genes had a defect in monolayer integrity because they were selected from genes whose KD caused a decrease in GLuc-FcMN permeability. Most gene KDs that disrupt the monolayer in any way are likely to result in an increase in permeability for GLuc-FcMN, and so the genes whose KD led to an increase in permeability during our screen may contain a much higher proportion of genes that affect tight junction integrity.

We next assessed changes in FcRn expression or localization after gene suppression. Genes that affect the biosynthetic delivery of FcRn could indirectly affect transcytosis, but such phenotypes should be readily distinguished by a reduction in FcRn expression, relocalization to a biosynthetic organelle such as the ER, or both. The total cellular content of FcRn was unaltered by any of the four esiRNAs as determined by Western blot against HA-FcRn-EGFP (Fig. 5 C) and direct epifluorescence microscopy (Figs. 5 D and S5 B). This finding rules out gross biosynthetic defects or mis-sorting of FcRn to the lysosome as causes of the transcytosis phenotype. We also found no change in FcRn localization away from the endosomal compartment or alteration of FcRn-containing endosome morphology (Figs. 5 D and S5 B). This is in contrast to the dramatic shift in localization of FcRn from endosome to plasma membrane after blockade of clathrin-mediated endocytosis (Fig. 1 E), suggesting that EXOC2, EXOC7, LEPROT, and PAR6B all act primarily in steps distinct from endocytosis.

**Table 1. Gene KD resulting in decreased transport phenotype**

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<sup>*Genes selected for secondary validation.</sup>
Having found no overt effects on cell monolayer health or FcRn localization, we sought to determine which trafficking pathways were affected by KD of each gene. To this end, we turned to pulse-chase transport assays, where it is possible to measure not only the amount of transepithelial transport (transcytosis), but also the amount of recycling back to the same surface and the amount of cargo internalization. Cell monolayers were loaded with GLuc-FcMN from either the apical or basolateral side for 60 min, washed thoroughly at 4°C, and then either returned to 37°C to measure the time course of apical or basolateral GLuc-FcMN output (recycling or transcytosis) or lysed in detergent to measure GLuc-FcMN internalization.

In the pulse-chase assays, we observed less transcytosis in both directions for all four gene KDs (Fig. 6, A and B), similar to what was observed in the steady-state transport experiments during the screen and subsequent validation (Table 1 and Fig. 4). Unlike transcytosis, however, recycling and endocytic uptake were affected differently by knocking down each gene, revealing three distinct trafficking phenotypes (Fig. 6, C–E).

In the first phenotype, shared by both subunits of the exocyst complex (EXOC2 and EXOC7), there was very little change in apical or basolateral recycling or in the amount of GLuc-FcMN internalization from either surface. Thus, exocyst KD appears to specifically affect trafficking into the transcytotic pathway while being dispensable for recycling and internalization. A different phenotype was observed after LEP ROT KD, in which recycling and internalization were both inhibited from either surface. This is unlikely to be explained by a biosynthetic or endocytic defect, because FcRn is normally expressed and does not accumulate at the cell surface (Fig. 5 and Fig. S5). Our interpretation is that LEP ROT is necessary for a key postendocytic trafficking step (or steps), consistent with the reported effects of LEP ROT on leptin receptor trafficking in nonpolarized cells (Séron et al., 2011). Finally, KD of PARD6B had opposing effects on recycling and internalization from each cell surface: whereas apical recycling and cargo uptake were strongly inhibited, basolateral recycling was enhanced and basolateral uptake was unaffected (Fig. 6), suggesting that PARD6B impinges on trafficking pathways particularly relevant to the apical cell surface.

Discussion

One major challenge in the field of endosome trafficking is to understand how the general rules of vesicular transport learned from yeast and cultured nonpolarized cells can be adapted to the specialized functions of differentiated cell types. Here, we describe the most comprehensive assessment to date of genes associated with a specialized endosomal transport pathway in polarized epithelial cells. Screens for genes operating in trafficking across polarized mammalian epithelial cells have not been reported before, and several challenges discussed in the Results section were uniquely overcome to make this study successful. Our results identified numerous cases in which the KD of specific genes affects bidirectional transcytosis of IgG by the Fcγ receptor FcRn. We further observed several distinct phenotypic classes, indicating that these genes are likely to operate in diverse stages of the transcytotic pathway and that the apical and basolateral recycling and transcytosis pathways are genetically separable. These results may apply to other constitutively polarized cell types, such as neurons or endothelial cells, which share some molecular mechanisms (Bonifacino, 2014).

An immediate finding from our screen was a clear requirement for the exocyst complex in FcRn-mediated transcytosis. The exocyst is a large, octomeric protein complex that mediates exocytosis of some Golgi- and endosome-derived vesicles at the plasma membrane. This finding was unexpected, as the exocyst has previously been implicated in endocytosis and exocytosis at the plasma membrane (Bonifacino, 2014). However, our results suggest that the exocyst plays a role in the postendocytic trafficking of FcRn, specifically in the transcytotic pathway.

Our results also highlight the importance of LEP ROT, a gene that affects recycling and internalization from both surfaces. This suggests that LEP ROT may be involved in a key postendocytic step that is common to both recycling and internalization pathways. Further studies are needed to clarify the mechanism by which LEP ROT affects trafficking.

Finally, our results suggest that PARD6B plays a role in the apical recycling and internalization pathways. This is consistent with previous reports that PARD6B is involved in endocytosis and recycling at the plasma membrane (Bonifacino, 2014). Our findings suggest that PARD6B may be involved in the apical recycling pathway, which is consistent with the observed effects of KD on apical recycling and internalization.

In conclusion, our study identifies numerous genes that affect bidirectional transcytosis of IgG by FcRn in polarized epithelial cells. These results provide new insights into the molecular mechanisms underlying endosome trafficking and may have implications for the development of therapies for diseases associated with defects in transcytosis.
specified regions of the plasma membrane (Heider and Munson, 2012). The complex can act at both apical and basolateral cell surfaces of polarized epithelia and is necessary for apically directed transcytosis of dimeric IgA by pIgR (Oztan et al., 2007). In our pulse-chase studies on EXOC2 and EXOC7, we found that both subunits led to a decrease in bidirectional GLuc-FcMN transcytosis without any significant effect on recycling or uptake from either surface. It is striking that there was very little effect on FcRn recycling after exocyst KD, as many other receptors require the exocyst for this process, including the transferrin receptor (TfR; Prigent et al., 2003) and pIgR (Oztan et al., 2007). One explanation for this difference may be that FcRn follows a different intracellular itinerary. For instance, though there is very strong intracellular colocalization between TfR and FcRn (Ober et al., 2004; Goebl et al., 2008; Tzaban et al., 2009; Jerdeva et al., 2010), recycling by both receptors have a distinct dependence on several genes: TfR recycling in polarized epithelia requires exocysts (Prigent et al., 2003), but not RAB11A (Wang et al., 2000b; Tzaban et al., 2009) and not LEPROT in HeLa cells (Séron et al., 2011). FcRn recycling, on the other hand, does not require exocysts (this study) but requires RAB11A (Ward et al., 2005; Tzaban et al., 2009) and LEPROT (this study). This result emphasizes that the apparent effect of any perturbation of trafficking machinery can be dependent on the specific receptor being studied and provides further evidence that trafficking pathways in epithelial cells are heterogeneous with multiple possible routes back to the cell surface (Goldenring, 2015).

We observed a polarized phenotype for PARD6B KD, with a strong defect on apical uptake and recycling but little effect on, or even an enhancement of, basolateral recycling. PARD6B encodes for a member of a protein family required for the establishment and maintenance of epithelial cell polarity (Chen and Zhang, 2013); it acts as a scaffolding protein in complex with Par3 and atypical protein kinase C to specify the apical membrane domain (Martin-Belmonte and Mostov, 2008). Although the phenotype we observed for PARD6B KD could in principle be an indirect effect caused by a defect in cell polarity, we observed no change in the polarity of two canonical apical and basolateral membrane markers, cell morphology, or tight junction formation. There is mounting evidence that Par6 family members can regulate membrane trafficking independent of their role in cell polarity (Balklava et al., 2007; Shivas et al., 2010), raising the possibility that the PARD6B KD phenotype could be a result of a direct role in endosome function. For instance, expressing a dominant negative Par6b in HeLa cells inhibited TfR internalization and major histocompatibility complex (MHC) class I recycling (Balklava et al., 2007), even though this cell line exhibits no constitutive polarity. Furthermore, Par6c has been shown to directly interact with and regulate trafficking genes, including the exocyst complex (Das et al., 2014).
In mammals, there are three isoforms of Par6 (PARD6A, PARD6B, and PARD6G), and it is unclear to what extent these operate differently in establishing cell polarity or regulating membrane trafficking. PARD6B was the only isoform included in our screen, and it was included because it was predicted to be strongly expressed in polarized epithelia (Nelms et al., 2016). Indeed, antibody staining from the human protein atlas (http://www.proteinatlas.org/; Uhlén et al., 2015) showed that PARD6B was strongly enriched in simple epithelia, whereas the other two Par6 isoforms were not. Moving forward, it will be important to assess any differences in the function of these three isoforms. The epithelial-enriched expression pattern of PARD6B suggests that it may be vital for a function unique to polarized epithelial cells.
FcRn trafficking, we speculate that PAR6B may contribute to endosome specialization in polarized epithelia, perhaps by helping to establish or regulate an apical membrane-specific endosome compartment.

Other genes identified in this screen have not been studied in the context of polarized epithelial cells before. LEPROT encodes a four-pass transmembrane protein that regulates a postendocytic step of LDL and growth hormone receptor trafficking (Séron et al., 2011). In nonpolarized cells, the requirement for LEPROT is receptor specific, as epidermal growth factor receptor (EGFR) and TIR are unaffected by LEPROT KD (Séron et al., 2011). LEPROT KD resulted in a general slowdown of all FcRn trafficking pathways, suggesting a defect in a rate-limiting step of FcRn transport. Another gene, ARMT1, was recently shown to function in the DNA damage response by negatively regulating PCNA through enzymatic methyltransferase activity (Perry et al., 2015). It is plausible that ARMT1 KD affected FcRn-mediated transcytosis indirectly by altering gene expression, but very little is known about this protein, including its intracellular localization and potential binding partners, and so it may have a direct function in endosomal trafficking distinct from its role in DNA damage.

Our screen implicates many other genes in the process of transcytosis, all with FDRs less than 0.01. Of the validated genes, a few are worthy of further note. ARL14 (Z-score = −5.33) is a small GTPase that recruits MYO1E to MHC class II–containing vesicles to affect movement along the actin cytoskeleton in dendritic cells (Paul et al., 2011). The guanine nucleotide exchange factor for ARL14 is PSD4. PSD4 was not identified in our screen but the PI kinase that regulates its membrane association, PIP5K1A, scored highly (Z-score = −2.52, FDR < 0.1). The screen also identified the actin-related protein ACTR3B, a component of the ARP2/3 complex (Z-score = −5.41). Both results are consistent with the dependency of endosome trafficking on actin dynamics (Sheff et al., 2002; Guerriero et al., 2006; Salvarezza et al., 2009), and it will be interesting to identify where the transcytotic pathway ARL14 and ACTR3B operate. Three other genes identified (VPS13C, STAM, and CCZ1) point to sorting steps associated with the transition from early to late endosomes. VPS13C (Z-score = −4.47) encodes a large protein expressed in multiple cell types and of essentially unknown function (Velayos-Baeza et al., 2004). CCZ1 (Z-score = −4.15) assembles with the protein encoded by Mon1 to act as a Rab7 guanine nucleotide exchange factor (Nordmann et al., 2010; Gerondopoulos et al., 2012) and with RABX5/Rab5 to effect the early-to-late endosome transition (Poteryaev et al., 2010). STAM1 (Z-score = −3.75) is a subunit of the ESCRT-0 complex, which also acts at early steps in the early-to-late endosome transition to effect protein sorting (Bache et al., 2003; Ren and Hurley, 2010). One aspect of this transition involves the sorting of selected cargo away from the lysosome, a characteristic feature of trafficking for FcRn (Roopenian and Akilesh, 2007).

We present several resources that will enable research into epithelial trafficking: we identified a large number of genes whose KD affects FcRn-mediated transcytosis and also established more sensitive assays to measure both continuous and pulse-chase FcRn-mediated transport. It should be emphasized that this screen does not provide a complete picture of the genes involved in transcytosis; several may have been missed because of insufficient KD or redundancy with other cellular proteins (e.g., many Rab proteins have isoforms with partially redundant functions). Genes that were not targeted may also play interesting roles in the pathway. In addition, because our cell model was built using the human FcRn in a canine cell line, it is possible that this species difference affected our results; the available data, however, are consistent with the receptor following a conserved intracellular itinerary among epithelial cells from different species where FcRn actively transports cargo.

Of the newly implicated genes we have characterized so far, we find distinct phenotypes on endosomal trafficking, suggesting that these genes operate in diverse stages of the transcytotic pathway. The next steps will be to determine which trafficking events are affected by each gene KD, whether the effects are direct or indirect, and how these genes operate together to maintain the specialized endosomal system of epithelial cells. These results serve as the foundation for future work on the genetic and molecular basis of transcytosis, and advance our understanding of how epithelial cells traffic IgG, sort membrane proteins, and maintain polarity to function at the interface between host and environment.

Materials and methods

Cell culture
An MDCKII cell line stably expressing human β2M and HA-FcRn-EGFP has been previously described (Tzaban et al., 2009). In brief, monomeric EGFP was fused to the FcRn C terminus with a GSSGSSS linker between FcRn and EGFP (pcDNA3.1; Invitrogen). The FcRn-EGFP fusion was transfected into MDCKII cells stably expressing human β2M (Claypool et al., 2002). This cell line was sorted by FACS to isolate cells with uniformly high GFP fluorescence, gating for cells that express detectable but low levels of GFP. The cell line was frozen down after sorting and used within 3 to 15 passages after thawing for all experiments. Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and penicillin and streptomycin, all obtained from Thermo Fisher Scientific. Cells were routinely confirmed by PCR to be negative for mycoplasma using a PCR kit from MB Biosciences.

Gluc-FcMN production and purification
Humanized Gaussia luciferase (Tannous et al., 2005) was cloned into the pcDNA3.1 backbone vector with G418/neomycin resistance (Thermo Fisher Scientific), with an AGGV linker and the Fc domain of human IgG1 on the N terminus and the signal sequence of mouse MHC class I H2-K b (Platzer and Fiebig, 2010) on the C terminus. All fusion-protein plasmids were verified by Sanger sequencing. Each fusion protein construct was transfected into CHO cells. These cell lines were placed under selection using G418 and then subcloned to obtain stable cell lines with strong expression of each Gluc-Fc variant. The stable cell lines were grown on Cytodex3 microcarrier beads (GE Healthcare) in Techne spinner flasks for up to 9 d, and media was collected every day for protein purification. Media for protein production was OptiMEM GlutMAX (Thermo Fisher Scientific) supplemented with 2% ultra-low IgG FBS (Thermo Fisher Scientific), 1 mM CaCl 2 (Sigma-Aldrich), nonessential amino acids (Thermo Fisher Scientific), and penicillin and streptomycin (Thermo Fisher Scientific).

Collected media was filtered through a 0.2-µm filter to remove cell debris and incubated with protein A or protein G Sepharose beads (GE Healthcare) in Techne spinner flasks for up to 9 d, and media was collected every day for protein purification. Media for protein production was OptiMEM GlutMAX (Thermo Fisher Scientific) supplemented with 2% ultra-low IgG FBS (Thermo Fisher Scientific), 1 mM CaCl 2 (Sigma-Aldrich), nonessential amino acids (Thermo Fisher Scientific), and penicillin and streptomycin (Thermo Fisher Scientific).
Steady-state transport assays

Cells were transfected at the same time of plating (reverse transfection) using Lipofectamine RNAiMAX (Thermo Fisher Scientific). For the transfections, lipid esiRNA complexes were prepared by incubating 2 ng/µl esiRNA in OptiMEM (Thermo Fisher Scientific) with 1.5% by volume RNAiMAX for 15 to 30 min. During the transfection period, 34.6 µl lipid esiRNA complex was added to the apical reservoir of a 6.5 mm polycarbonate transwell plate with 0.4 µm pore size (Corning). Meanwhile, MDCK-FcRn cells were trypsinized and resuspended to a density of 1.2 × 10^5 cells/ml in DMEM with 10% FBS and without penicillin and streptomycin, and 138.5 µl of this cell solution was added to the apical reservoirs of the transwell plate with lipid esiRNA complexes. It was important to ensure that the OptiMEM was prewarmed to room temperature and the DMEM was prewarmed to 37°C for a successful transfection. The cells with complexes were then incubated for 16 to 20 h at 37°C without media in the basolateral reservoir. For the follow up experiments (Figs. 4, 5, and 6), a second transfection was subsequently performed to ensure more robust gene KD; in this second transfection, lipid esiRNA complexes were prepared as for the first transfection and then diluted with DMEM with 10% FBS and added to both the apical and basolateral chambers for 4 to 8 h. After transfection, the media was exchanged for prewarmed DMEM with 10% FBS and penicillin and streptomycin. Media was changed again 3 d after plating, and transport assays were performed the next day.

For the transport assays, media was exchanged for DMEM with 10% ultra-low IgG FBS (Thermo Fisher Scientific), 1 µg/ml GLuc-Fc, and 20 mM MES pH 6.0, on the input side and DMEM with 10% ultra-low IgG FBS, pH 7.4, on the output side. The filters were incubated for 90 min at 37°C, and then contents on the output side of the filter were gently mixed and 20-µl aliquots withdrawn for protein concentration determination. To measure the concentration of GLuc-Fc, 80 µl of PBS with 12.5 µM coelenterazine (NanoLight Technology) was added to 2 x 10^5 total cDNA molecules as estimated by dilution PCR and 15 out of 20 sequenced clones representing full-length transcripts. Finally, the library was amplified by emulsion PCR using the Miscellula emulsion PCR kit (CHI MERx) and the PCR primer 5′-AACGAGTTGTATCAACCGCAGA GTAC(T)30VN. Then, to facilitate the addition of a predetermined sequence at the 3′ end of the cDNA (5′ end of the template RNA) by template switching, MnCl2 was added to a final concentration of 2 mM along with Superscript II reverse transcription and the primer 5′-AACGAGTTGTATCAACCGCAGATTACGGRG-3′ (phosphate), and the reaction was incubated at 42°C for 90 min. This protocol leaves an identical priming site on both ends of the cDNA (5′-AACGAGTTGTATCAACCGCAGAT-3′) that can be used to amplify the library by PCR. The cDNA synthesis resulted in substantial library diversity, with ~2 × 10^6 total cDNA molecules as estimated by dilution PCR and 15 out of 20 sequenced clones representing full-length transcripts. Finally, the library was amplified by emulsion PCR using the Miscellula emulsion PCR kit (CHIMERx) and the PCR primer 5′-AACGAGTTGTATCAACCGCAGAGT-3′.

Normalization and hit selection for RNAi screen

To analyze the data from the screen, a strategy was developed to assess esiRNA activity at the same time as normalization, estimating the consistent effect among all esiRNA replicates that cannot be explained by known systematic sources of variation. The method was adapted from (Yu et al., 2011). Let \( X_{\text{ipr}} \) be the log-transformed luciferase intensity of well \( i \), treated with esiRNA \( e \), and measured at location \( p \) (\( \phi = (b,p,r,c) \) for batch \( b \), plate \( p \), row \( r \), and column \( c \)). We fit the measured intensities \( X_{\text{ipr}} \) to a mixed-effects linear model with fixed esiRNA effects \( \mu_e \), random systematic effects \( S_p \), and residual error \( \epsilon \): \( X_{\text{ipr}} = \mu_e + S_p + \epsilon \), where systematic effects are the sum of batch effects \( B_p \), plate effects \( P_p \), row effects \( R_p \), and column effects \( C_p \). This model was fit separately for each direction of transport using the lme4 R package (Bates et al., 2015). Normalized signal intensities, which have been corrected for systematic errors (\( X_{\text{ipr}} - S_p \)), are plotted in Fig. S3.
To assess statistical significance of each esiRNA effect $\mu$, a permutation test was used. A single mock transfection well on the library plate was labeled as a gene named MOCK, and then the mixed linear model fit was repeated, obtaining an estimate of the “esiRNA” effect for a known negative control, $\hmu_{\text{MOCK}}$. This was then repeated for the remaining mock transfection wells (112 in total), providing an empirical null distribution under the mock transfection condition. The null distribution, $\hmu_{\text{MOCK}}$, fits a normal distribution for both directions of transport ($P = 0.82, 0.40$ for basolateral-to-apical and apical-to-basolateral transport, Shapiro–Wilk test).

A $Z$-score was then calculated for each gene and direction of transport by dividing the estimated esiRNA effect, $\mu$, by the standard deviation of the empirical null distribution (i.e., the standard deviation of $\hmu_{\text{MOCK}}$). $Z$-scores for each direction were then pooled using Stouffer’s method:

$$Z_{\text{pooled}} = \frac{Z_{A} + Z_{B}}{\sqrt{2}},$$

resulting in a single overall $Z$-score for each esiRNA in the screen.

### General cell health characterization

For the general cell health characterization (Fig. 5), MDCK-FcRn cells were subjected to the double-transfection protocol described in the Steady-state transport assays section and then grown for 4 d on transwell filters. For microscopy, cells were fixed using 4% paraformaldehyde (Electron Microscopy Sciences) and then stained and imaged as described previously (Tzaban et al., 2009) using a mouse mAb against GP135 (Ojakian and Schwimmer, 1988) and a rat mAb against E-cadherin (U3254; Sigma-Aldrich). Western blot for HA-FcRn-GFP was performed using a high-affinity rat anti-HA mAb (clone 3F10; Roche) and for β-actin using a mouse mAb (A5316; Sigma-Aldrich). HRP-conjugated secondary antibodies were obtained from Sigma-Aldrich. TEER was measured using an EVOM2 (World Precision Instruments).

#### Pulse chase assays

For the pulse chase transport assays, MDCK-FcRn cells were subjected to the double-transfection protocol described in the Steady-state transport assay section and grown for 4 d on transwell filters. To load the cells with GLuc-FcMN, media was exchanged for DMEM with 10% FBS, 1% FBS, and Complete protease inhibitor cocktail (Thermo Fisher Scientific) for wide-field illumination. Solid-state lasers (480, 568, 40, 1% FBS, and Complete protease inhibitor cocktail (Thermo Fisher Scientific) for uptake assays. Luciferase activity was measured as described in the Steady-state transport assay section and converted to protein concentration using a linear least squares fit to a standard curve.

#### Microscope and image acquisition

Confocal images were acquired at 37°C using a spinning disk confocal head (CSU-X1; PerkinElmer) coupled to a highly motorized inverted Axiovert 200-M microscope (ZEISS) equipped with Plan Apochromat 63x/1.4 oil DIC objective and with a X-Cite 120LED light source (Excito) for wide-field illumination. Solid-state lasers (480, 568, and 660 nm) coupled to the spinning head through a fiber optic were used as a light source. An acoustic-optical tunable filter was used to switch between different wavelengths. The imaging system operates under control of SlideBook 6 (Intelligent Imaging Innovations, Inc.) and includes a computer-controlled spherical aberration correction device (SAC; Intelligent Imaging Innovations, Inc.) installed between the objective lens and the charge-coupled device camera (QuantEM: 512SC; Photometrics). Postacquisition images were scaled linearly using ImageJ. For immunofluorescence, MDCK monolayers were fixed in 4% paraformaldehyde, washed, and stained for HA-FcRn-EGFP, E-cadherin, or GP135 as described in General cell health characterization. Secondary anti–mouse Fab2 labeled with Alexa Fluor 647 and anti–rat Fab2 labeled with Alexa Fluor 568 antibodies from Thermo Fisher Scientific were used.

### Statistics and analysis

For Figs. 1 and 5, results were summarized as the mean ± SD of a representative experiment. Statistical significance was estimated using a Student’s $t$ test adjusted for multiple hypothesis testing with Holm’s method. For Figs. 4 and 6, data were pooled from multiple independent experiments. Raw luminescence values from each experiment were normalized by dividing by the mean value of the nontargeting control wells from that experiment (the 90-min time point of the nontargeting control wells for the pulse-chase assay). Results were then summarized as the weighted mean ± the weighted standard deviation between experiments, with weights given by 1 over the variance of the nontargeting control wells in each experiment. The reason for including weights is that a few experiments resulted in a large standard deviation for the normalizing negative control condition, and the weighted mean allowed for a smooth decrease in the value of these noisy experiments without having to assess or remove outliers. No experiments or data points were excluded from the analysis. Statistical significance was estimated using a Wilcoxon signed-rank test and adjusted for multiple-hypothesis testing with Holm’s method.

### Online supplemental material

Fig. S1 shows SDS-PAGE of purified recombinant Gluc-Fc fusion proteins, dose dependency of Gluc-FcMN transport, schematic for preparation of esiRNAs and validation of final products, and validation of esiRNA KD for FcRn. Fig. S2 shows the plate layout and replication schema for the high-throughput screen. Fig. S3 shows plots of normalized and unnormalized luciferase measurements from the screen. Fig. S4 shows the comparison of apical to basolateral and basolateral to apical transport. Fig. S5 shows the immunolocalization of apical (GP135) and basolateral (E-cadherin) membrane markers and the localization of HA-tagged FcRn-EGFP after esiRNA depletion of EXOC2, EXOC7, and LEPROT. Table S1 shows the relative permeabilities for all genes used in the pilot screen. Table S2 shows the final list of genes curated for the screen, the list of genes curated in the high-throughput and literature gene sets, and the list of genes curated based on their containing a domain linked to trafficking. Table S3 shows the list of genes and sequences used to prepare esiRNA for the full and pilot screens and the second esiRNAs prepared for the validation studies. Table S4 shows the entire list of ranked $Z$-scores for each gene studied.

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Author contributions: B. Nelms and W. Lencer conceived and designed the study. B. Nelms and N.F. Dalomba acquired data. B. Nelms and W. Lencer analyzed and interpreted data. B. Nelms and W. Lencer drafted and revised the manuscript, with input from N.F. Dalomba.

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