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RESEARCH ARTICLE

Galectin-3 modulates the polarized surface delivery of β1-integrin in epithelial cells

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ABSTRACT

Epithelial cells require a precise intracellular transport and sorting machinery to establish and maintain their polarized architecture. This machinery includes β-galactoside-binding galectins for targeting of glycoprotein to the apical membrane. Galectin-3 sorts cargo destined for the apical plasma membrane into vesicular carriers. After delivery of cargo to the apical milieu, galectin-3 recycles back into sorting organelles. We analysed the role of galectin-3 in the polarized distribution of β1-integrin in MDCK cells. Integrins are located primarily at the basolateral domain of epithelial cells. We demonstrate that a minor pool of β1-integrin interacts with galectin-3 at the apical plasma membrane. Knockdown of galectin-3 decreases apical delivery of β1-integrin. This loss is restored by supplementation with recombinant galectin-3 and galectin-3 overexpression. Our data suggest that galectin-3 targets newly synthesized β1-integrin to the apical membrane and promotes apical delivery of β1-integrin internalized from the basolateral membrane. In parallel, knockout of galectin-3 results in a reduction in cell proliferation and an impairment in proper cyst development. Our results suggest that galectin-3 modulates the surface distribution of β1-integrin and affects the morphogenesis of polarized cells.

KEY WORDS: Galectin-3, β1-integrin, Apical transport, Endocytosis, Cell polarity

INTRODUCTION

Epithelial cells maintain a polarized structure in which the apical membrane domain is separated by a tight junctional complex from the basolateral membrane domain. Layers of epithelial cells thus create a barrier that separates the inside of a body from its outside milieu, which faces apical cell poles. At the basal surface, integrin receptors interact with the basement membrane, a component of the extracellular matrix (ECM). This interaction determines the apico-basal orientation, the mitotic spindle axis and microtubule dynamics (Lee and Streuli, 2014). The apical domain, on the other hand, is enriched in glycosphingolipids and often contains specialized protuberances, such as cilia and microvilli. Protein and lipid components of the two membrane domains are sorted and transported to their final destination by a highly selective machinery that is still not fully understood (Apodaca et al., 2012). N- and O-linked glycans can direct glycoproteins to the apical membrane domain (Fiedler and Simons, 1995; Yeaman et al., 1997; Alfalah et al., 1999). Their recognition involves sugar-binding proteins called galectins, which are exclusively expressed in multicellular organisms. Each member of this family has a specific distribution pattern that ranges from the widely expressed galectin-1 and -3 to the more tissue-specific galectin-2 and -7 (Viguier et al., 2014). Structurally, the chimeric galectin-3 is unique, with a C-terminal carbohydrate recognition domain (CRD) and an N-terminal domain involved in non-classic secretion and oligomerization of the lectin (Menon and Hughes, 1999). Galectin-3 is involved in a variety of cellular processes such as apoptosis, carcinogenesis, cell differentiation, cell migration and protein trafficking. Some of these processes depend on the presence of galectin-3 in body fluids and involve interaction of the lectin with the extracellular matrix or cell surfaces. On epithelial cells, protein bundling into higher-order multimers is induced by association of galectin-3 with the extracellular matrix protein hensin (Hikita et al., 2000). Alternative variants of galectin-3 bundled with glycoproteins into lattice structures can also reduce the mobility of membrane proteins. It has been shown that galectin-3 binds to N-glycans on EGFRs and other surface glycoproteins, thereby slowing down lateral diffusion and preventing endocytosis (Partridge et al., 2004). Furthermore, disruption of the lattice by glycan competition or knockdown of galectin-3 increases the mobility of EGFR-GFP in carcinoma cells about twofold (Lajoie et al., 2007). However, galectin-3 mediates the endocytic uptake of β1-integrins in breast carcinoma cells in a lactose-dependent manner (Furtak et al., 2001). This cellular event includes the functional involvement of galectin-3 in membrane bending and the formation of clathrin-independent carriers, as recently reported (Lakshminarayan et al., 2014).

Here, we analysed the role of galectin-3 in the surface distribution of β1-integrin in epithelial MDCK cells, which sort this transmembrane protein primarily to the basolateral membrane domain. A minor pool of β1-integrin interacts with galectin-3 at the apical plasma membrane and co-internalizes with the lectin. Following CRISPR/Cas-mediated knockout of galectin-3, β1-integrin expression is reduced and the distribution pattern of this integrin is altered, as indicated by its loss at the apical membrane. Apical localization of β1-integrin is restored by the addition of recombinant galectin-3 or by galectin-3 overexpression, which correlate with increased β1-integrin expression. We thus postulate that galectin-3 mediates apical trafficking of endocytosed and newly synthesized β1-integrin in polarized epithelial cells.

RESULTS

Galectin-3 is secreted, bound and endocytosed at the apical membrane domain

In polarized epithelial cells, galectin-3 is synthesized on free ribosomes and secreted via an unconventional mechanism into the

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extracellular milieu (Lindstedt et al., 1993; Sato et al., 1993). To test whether galectin-3 is secreted in a polarized manner, medium was collected from the apical and basolateral chambers of filter-grown MDCK cells and analysed by immunoblotting (Fig. 1A,B). In line with previous data (Lindstedt et al., 1993), the lectin was present predominantly in the apical medium, with only 2.2% of secreted galectin-3 detected in the basolateral medium. The constitutively secreted gp80 (also known as clusterin; encoded by *CLU*) was labelled as a soluble control protein (Urban et al., 1987).

To determine the binding capacity for galectin-3 to each of the two membrane domains of MDCK cells, recombinant, biotin-labelled human galectin-3 (hGal3-biotin) was added to the apical or the basolateral plasma membrane. The cells were incubated at 4°C to prevent endocytic uptake. After removing unbound lectin from the plasma membrane by extensive washing, cells were lysed and hGal3-biotin was isolated using neutravidin beads (Fig. 1C,D). The externally added recombinant lectin was mainly detected at the apical membrane domain, with only 3% associated with the basolateral domain. This interaction was inhibited in the presence of lactose or at acidic pH (pH 4.5), suggesting binding of a carbohydrate- and pH-dependent galectin to the membrane (Fig. S1A,B). It has previously been shown that

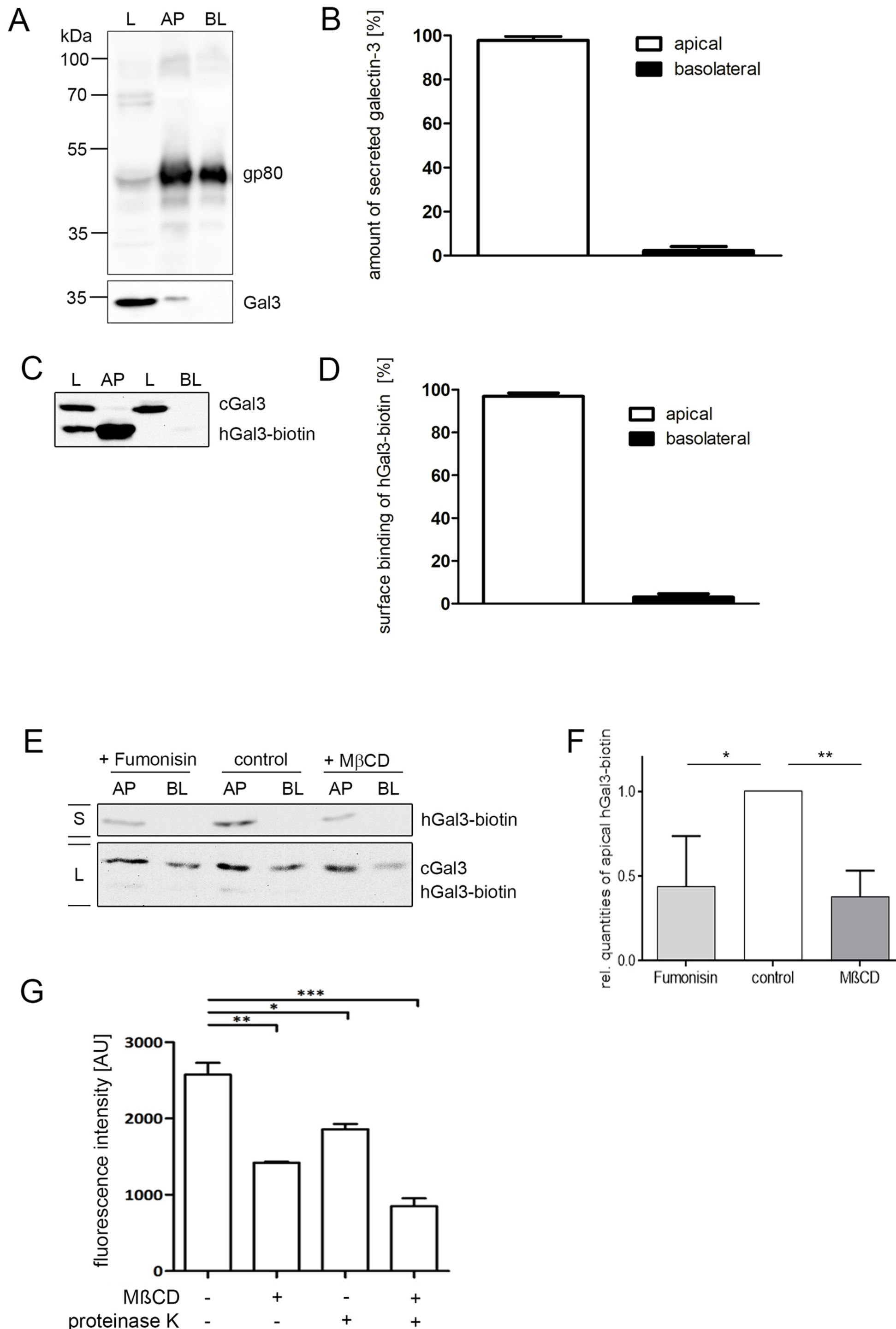

Fig. 1. Apical secretion and binding of galectin-3. (A) Apical and basolateral media from filter-grown MDCK cells were collected after 4 h of secretion at 37°C and analysed by immunoblotting. (B) Quantification of galectin-3 secretion into the apical and basolateral media normalized to total secreted amounts of galectin-3 (100%). Mean±s.d., *n*=3. (C) Apical and basolateral membrane domains were incubated with 1.5 µM recombinant hGal3-biotin at 4°C to allow binding of the lectin to the plasma membrane. Biotinylated hGal3 was precipitated with neutravidin beads and analysed with polyclonal anti-galectin-3 (H-160) by western blotting. (D) Quantification of hGal3 surface binding. Mean±s.d., *n*=5. (E) Apical or basolateral binding to polarized MDCK cells treated or not treated with 10 µM fumonisin or 50 mM MβCD assessed as described above with 500 nM recombinant hGal3-biotin added to the apical or basolateral medium. (F) Quantification of hGal3 binding to the apical membrane. Mean±s.d., *n*=4. (G) GPMVs were treated as indicated with 50 mM MβCD and/or 0.01 mg/ml proteinase K. Binding of Gal3-Alexa647 was quantified by flow cytometry. Fluorescence intensities of Gal3-Alexa647 are shown; 10,000 events were recorded for each condition. Statistical significance was tested with one-way ANOVA and Bonferroni's post-test. *P<0.05, **P<0.01. L, lysate; S, surface; AP, apical; BL, basolateral.

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galectin-3 associates with detergent-resistant membrane microdomains (DRMs) at the apical plasma membrane (Straube et al., 2013) and the lectin triggered a glycosphingolipid-dependent endocytic process in non-polarized cells (Lakshminarayan et al., 2014). We therefore assessed whether apical binding of galectin-3 depends on the integrity of DRMs. DRMs of polarized MDCK cells were disrupted by treatment with 10 µM fumonisin, an inhibitor of sphingolipid synthesis (Wang et al., 1991), or 50 mM methyl-β-cyclodextrin (MβCD), which specifically removes plasma membrane cholesterol (Neufeld et al., 1996). Fig. 1E,F indicates that MβCD treatment significantly reduced the binding capacity of galectin-3 to the apical plasma membrane. The effects of fumonisin on galactin-3 binding capacity were less than that of MβCD, but still significant. The inhibitory impact of MβCD on galectin-3 binding was corroborated when the interaction of fluorescently tagged galectin-3 with giant vesicles from the apical plasma membrane (GPMVs) was studied by cytometry (Fig. 1G). DRM disruption by MβCD, as well as proteinase K treatment to remove exposed membrane-attached glycoproteins, significantly reduced the interaction of fluorescently labelled galectin-3 (Gal3-A647) with GPMVs. The greatest reduction in the binding capacity of galectin-3 was observed when GPMVs were depleted of both DRMs and exposed glycoproteins at the same time. Altogether, these experiments indicate that the integrity of DRMs and the presence of glycosphingolipids and glycoproteins facilitate binding of galectin-3 to the apical membrane.

Galectin-3 interacts with integrins at the apical membrane domain

Since extracellular galectin-3 binds predominantly to the apical plasma membrane, we wanted to identify putative interaction partners of the lectin at this membrane domain. To this end, recombinant hGal3-biotin was added to the apical medium of polarized MDCK cells and isolated using neutravidin beads. Analysis of co-precipitating proteins by mass spectrometry revealed α2-integrin as a putative interaction partner for galectin-3 (Fig. 2A, Table S1). We also detected endogenous canine galectin-3 in co-precipitates, corroborating the idea that the recombinant lectin oligomerizes with the endogenously expressed orthologue into larger protein complexes or lattices (Nabi et al., 2015). The interaction of endogenous galectin-3 and α2-integrin, as well as another member of the integrin family, β1-integrin, was further confirmed by co-immunoprecipitation experiments (Fig. 2B). Moreover, addition of recombinant hGal3-biotin to apical and basolateral MDCK plasma membrane domains and subsequent isolation with neutravidin beads showed that both, α2- and β1-integrin, are bound by galectin-3 predominantly at the apical membrane domain (Fig. 2C). When the integrity of DRMs was disrupted in the presence of 10 µM fumonisin or 50 mM MβCD, the β1-integrin–galectin-3 interaction was significantly perturbed (Fig. S1C,D), thus confirming that this interaction requires a particular membrane context. This is in line with the observation that only minor amounts of recombinant galectin-3 were bound to the basolateral membrane (Fig. 2C), which comprises lower amounts of the DRM-forming glycosphingolipids and sphingomyelin compared with levels in the apical domain (van Meer et al., 1987).

Next, we assessed the surface distribution of α2- and β1-integrin by biotinylation of the apical or basolateral membrane domain (Fig. 2D). Both, α2- and β1-integrin are located primarily at the basolateral membrane and only about 30% is apically located. Galectin-3 preferentially associates with this minor apical pool of integrins (Fig. 2C). We confirmed the prevailing basolateral localization of β1-integrin using confocal fluorescence microscopy (Fig. 2E,F). The focal plane was adjusted to image the apical/subapical region of the cells. β1-integrin was detected on the subapical area of lateral plasma membranes. Note that at the apical membrane β1-integrin appeared in a punctate pattern, which is typically observed for apical membrane proteins in epithelial cells as a result of microvilli folding (Fuller et al., 1985; Schoeneberger et al., 1994; Straube et al., 2013). Endogenous canine galectin-3 was found in punctate structures of vesicular appearance, some of which colocalized with β1-integrin-positive structures in the apical and subapical region (Fig. 2E). To analyse whether this colocalization occurs at the apical surface rather than intracellularly, we applied Alexa Fluor 555-conjugated recombinant human galectin-3 (hGal3-Alexa555) to the apical plasma domain of polarized MDCK cells. To exclude internalization, binding of the lectin was performed at 4°C. Thereafter, cells were fixed and stained for β1-integrin (Fig. 2F). Interestingly, colocalization with exogenously added hGal3-Alexa555 was also observed in the apical region and close to the cell borders. Estimation of the colocalization efficiency revealed a higher Manders' coefficient if exogenously added galectin-3 was co-stained with β1-integrin, which can be explained by the interaction of galectin-3 and β1-integrin at the cell surface.

In the orthogonal views of Fig. 2E,F we found β1-integrin staining primarily at the lateral and rarely at the basal membrane. To better visualize the apical and basal distribution pattern of β1-integrin in tissues, we studied human and mouse renal epithelial cells. Renal tissue was sectioned and immunostained with antibodies directed against β1-integrin and apical villin (mouse) or β1-integrin and galectin-3 (human) (Fig. S2). The majority of integrin labelling was found on the basal membrane of kidney epithelial cells. Apical membranes were faintly stained by anti-integrin antibodies and this staining clearly overlapped with the galectin-3 localization, which corroborates the MDCK cell data and suggests that minor fractions of β1-integrin are delivered to the apical membrane in renal tubule cells. Together, these data point towards an interaction of galectin-3 with α2- and β1-integrin at or in close proximity to the apical membrane domain.

The surface distribution of β1-integrin is modulated by galectin-3

Next, the relationship between galectin-3 and β1-integrin was analysed in MDCK cells. First, we compared β1-integrin expression levels in fully polarized MDCK cells, galectin-3-knockout cells ($MDCK_{\Delta Gal3}$) and galectin-3-overexpressing cells ($MDCK_{Gal3\text{-}YFP}$). $MDCK_{\Delta Gal3}$ cells exhibited decreased β1-integrin expression, whereas remarkably higher protein levels of β1-integrin were found in $MDCK_{Gal3\text{-}YFP}$ cells (Fig. 3A-C). This suggests that alterations in the cellular level of galectin-3 directly or indirectly affect total quantities of β1-integrin. To assess if this is based on altered β1-integrin gene transcription or protein stability, we first determined β1-integrin mRNA levels in MDCK, $MDCK_{\Delta Gal3}$ and $MDCK_{Gal3\text{-}YFP}$ cells using quantitative RT-PCR (Fig. S3A). β1-integrin transcript levels did not vary significantly in the three cell lines. On the other hand, at the protein level, β1-integrin increased following neutralization of lysosomal pH in MDCK cells (Fig. S3B,C). This effect was even more prominent in $MDCK_{\Delta Gal3}$ cells, whereas negligible alterations were observed in $MDCK_{Gal3\text{-}YFP}$ cells treated with chloroquine. A strong chloroquine dependency of the β1-integrin stability in $MDCK_{\Delta Gal3}$ cells suggests that, in the absence of galectin-3, significant amounts of β1-integrin are degraded within lysosomes. This is corroborated by a steeper decline of

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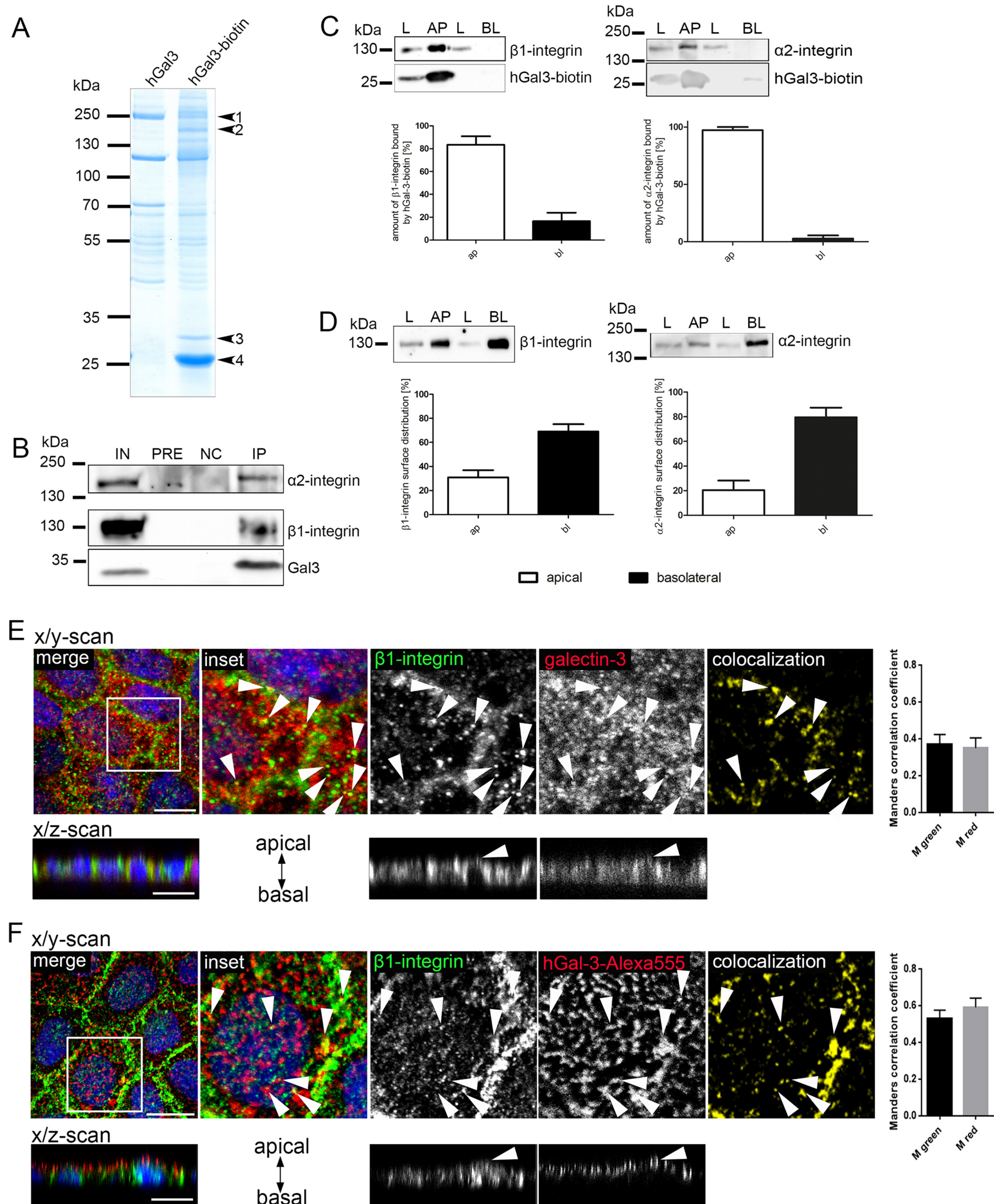

Fig. 2. See next page for legend.

surface-biotinylated β1-integrin in $\mathrm{MDCK}_{\Delta\mathrm{Gal3}}$ cells (Fig. S3D,E). By comparison, elevated quantities of β1-integrin in galectin-3-overexpressing cells can then be explained by a reduction in the recruitment of lysosomal β1-integrin. Galectin-3 thus seems to modulate the cellular β1-integrin pool by protecting it from lysosomal degradation.

A substantial decrease in the level of surface-exposed β1-integrin has already been described for iron-exposed renal tubular LLC-PK1 cells and this was accompanied by a reduction in cell proliferation (Sponsel et al., 1996). Interestingly, we also observed a significant decrease in the proliferation of $\mathrm{MDCK}_{\Delta\mathrm{Gal3}}$ cells compared with $\mathrm{MDCK}_{\mathrm{wt}}$ cells (Fig. S3F). The growth of $\mathrm{MDCK}_{\mathrm{Gal3\text{-}GFP}}$ cells, on

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Fig. 2. Galectin-3 interacts with integrins at the apical membrane domain. (A) Apical domains of polarized MDCK cells were incubated with 1.5 µM hGal3-biotin at 4°C. Biotinylated hGal3 was precipitated with neutravidin beads and analysed by mass spectrometry. Scored hits for bands indicated by arrowheads are: (1) thrombospondin-1, (2) α2-integrin, (3) galectin-3, (4) human galectin-3. (B) Endogenous galectin-3 was precipitated from polarized MDCK cells and co-precipitating proteins analysed by immunoblotting. IN, input; PRE, preclearing; NC, negative control; IP, immunoprecipitation. (C) Relative amounts of surface β1-integrin and α2-integrin bound by apically or basolaterally added hGal3-biotin. Amounts of galectin-3-bound integrins at the apical and basolateral membrane were normalized to integrin levels in the corresponding lysates. Mean±s.d., *n*=3. (D) Surface distribution of β1-integrin and α2-integrin at the apical and basolateral plasma domains of polarized MDCK cells. Amounts of integrins at the apical and basolateral membranes were normalized to integrin levels in the corresponding lysates. Mean±s.d., *n*=3. (E,F) Localization of β1-integrin (green) and endogenous galectin-3 (E, red) or exogenously added hGal3 (F, red) at the apical membrane of polarized MDCK cells analysed by confocal microscopy. Nuclei are stained blue with Hoechst 33342. Colocalization channels show positive product of differences from the mean (PDM). Arrowheads indicate colocalizing structures. Scale bars: 10 µm. Manders' correlation coefficient was used for quantification of experiments (shown on the right). Mean±s.e.m. of 25 images from three independent experiments (E) and 18 images from three experiments (F) were analysed. *x-z* scans are shown below.

the other hand, was impaired at the beginning of the time course (day 3); however, we did not detect significant alterations in the growth rates of these cells after 10 days in culture.

Owing to the fact that galectin-3 also alters polarized trafficking of glycoproteins (Hoenig et al., 2015), a correlation between the polarized distribution and the subcellular concentration of β1-integrin seems plausible. To test this directly, we analysed the surface distribution of β1-integrin in galectin-3-overexpressing and galectin-3-knockout cells by surface biotinylation. Compared with wild-type cells, $MDCK_{Gal3\text{-}YFP}$ cells showed significantly enhanced levels of β1-integrin at the apical surface and reduced basolateral β1-integrin (Fig. 3D,E). Conversely, $MDCK_{\Delta Gal3}$ cells displayed reduced amounts of apical β1-integrin (Fig. 3F,G). Moreover, supplementation of $MDCK_{\Delta Gal3}$ cells with hGal3 for 8 h was sufficient to rescue and even increase levels of apical β1-integrin. The effect of hGal3 administration to $MDCK_{\Delta Gal3}$ cells was also analysed by confocal microscopy (Fig. 3H). Here, the overall intensity of β1-integrin-positive signals in the subapical area of the cells was elevated after 8 h (Fig. 3I). Quantification of the number of β1-integrin-positive puncta at or just below the apical membrane, excluding highly stained cell borders, also revealed a significant increase in these structures after up to 8 h of hGal3 administration. No significant increase in the apical cell area was observed. Instead, the density and intensity of puncta per µm^2 was enhanced in the presence of galectin-3. To further characterize these β1-integrin-positive puncta in the apical cell area we used Alexa Fluor 488-conjugated phalloidin to stain actin-based microvilli as previously described (Poole et al., 2004). This microvilli staining overlaps with β1-integrin (Fig. S4). Manders' correlation coefficients were even higher following 8 h of hGal3 administration, which suggests that exogenously added galectin-3 elevates β1-integrin levels on apical microvilli.

Next, we tested whether the hGal3-dependent increase of apical β1-integrin is reversible. $MDCK_{\Delta Gal3}$ cells were supplemented with recombinant hGal3 for up to 4 h. Afterwards, cells were incubated in fresh medium for different periods of time in the absence of recombinant hGal3 (Fig. S5A,B). Indeed, we found that β1-integrin at the apical membrane peaked in conjunction with the application of hGal3 and declined thereafter. A similar effect was observed when MDCK cells were supplemented with hGal3 for longer periods of 8 h (Fig. S5C,D). Otherwise, addition of an N-terminally truncated variant of galectin-3, hGal3C-TRX (Saraboji et al., 2012), did not enhance apical delivery of β1-integrin (Fig. S5E,F). This suggests, that the N-terminus of galectin-3, which is involved in oligomerization of the lectin (Menon and Hughes, 1999), is crucial for redirection of β1-integrin to the apical cell surface.

Galectin-3-dependent surface distribution of β1-integrin was further analysed in MDCK cells grown in a three-dimensional Matrigel matrix. Fig. 4 depicts MDCK cell cysts in which apical gp135 (podocalyxin) and lateral β-catenin were labelled to visualize cell polarity. $MDCK_{wt}$ cells developed uniform cysts consisting of epithelial monolayers with the apical surfaces facing the central lumen (Montesano et al., 1991). Antibodies directed against β1-integrin faintly stained the gp135-positive apical cell surface. Cysts formed by $MDCK_{\Delta Gal3}$ cells showed defects in cyst organization and developed multiple small lumens or tubes within the cysts, which is in line with previous data on the influence of galectin-3 on epithelial morphogenesis (Koch et al., 2010). Membranes positive for gp135 were only occasionally co-stained by β1-integrin, which suggest that a regular minor pool of apical β1-integrin is linked to correct cyst development. On the other hand, cysts of galectin-3-overexpressing $MDCK_{Gal3\text{-}GFP}$ cells had a single, regular central lumen and showed distinct β1-integrin immunofluorescence staining at the apical membrane. Together, these results demonstrate that epithelial cyst formation and the surface distribution of β1-integrin are affected in the absence of galectin-3.

Galectin-3 enhances β1-integrin endocytosis at the apical membrane

We next addressed the question of how galectin-3 enhances the apical pool of β1-integrin. As galectin-3 binds predominantly to the apical membrane (Fig. 1C,D), we first studied internalization of this lectin from the apical or the basolateral membrane domain. Gal3-A647 was added to the apical and basolateral domains of filter-grown MDCK cells and incubated for 30 min at 37°C to allow internalization. Residual galectin-3 at the cell surface was removed and basolateral uptake of fluorescent canine transferrin (cTf-Alexa555) was used in control experiments. Fig. S6A depicts predominantly apical endocytosis of Gal3-A647. In a similar approach internalization of recombinant hGal3 from the apical or basolateral membrane domain of polarized MDCK cells was monitored by immunoblotting (Fig. S6B). Within 60 min of uptake, galectin-3 was exclusively detected at the apical membrane domain. We then focused on the internalization of β1-integrin from this membrane domain. We therefore labelled β1-integrin with a reducible biotin conjugate and endocytosis was allowed for 0 or 30 min at 37°C. Thereafter, reduction with glutathione removed the biotin label from polypeptides at the cell surface. Fig. 5A (left panel, control) shows that non-reduced internalized β1-integrin and endogenous galectin-3 were significantly increased after 30 min of internalization. Since integrin is rapidly endocytosed and recycles back to the cell surface (Arjonen et al., 2012), we studied the influence of membrane recycling in these experiments by using the lysosomotropic amine, primaquine, which blocks endosomal recycling (Somasundaram et al., 1995). We observed a dose-dependent reduction of non-reduced, internalized β1-integrin in the presence of 0.3 or 0.6 mM primaquine (Fig. 6C,D). This indicates that internalized β1-integrin is degraded if endosomal recycling is blocked, most likely by lysosomal degradation. Further analysis of the efficiency of β1-integrin internalization and recycling demonstrates that it is reduced in the presence of lactose, a galectin-3 ligand, and is drastically reduced by

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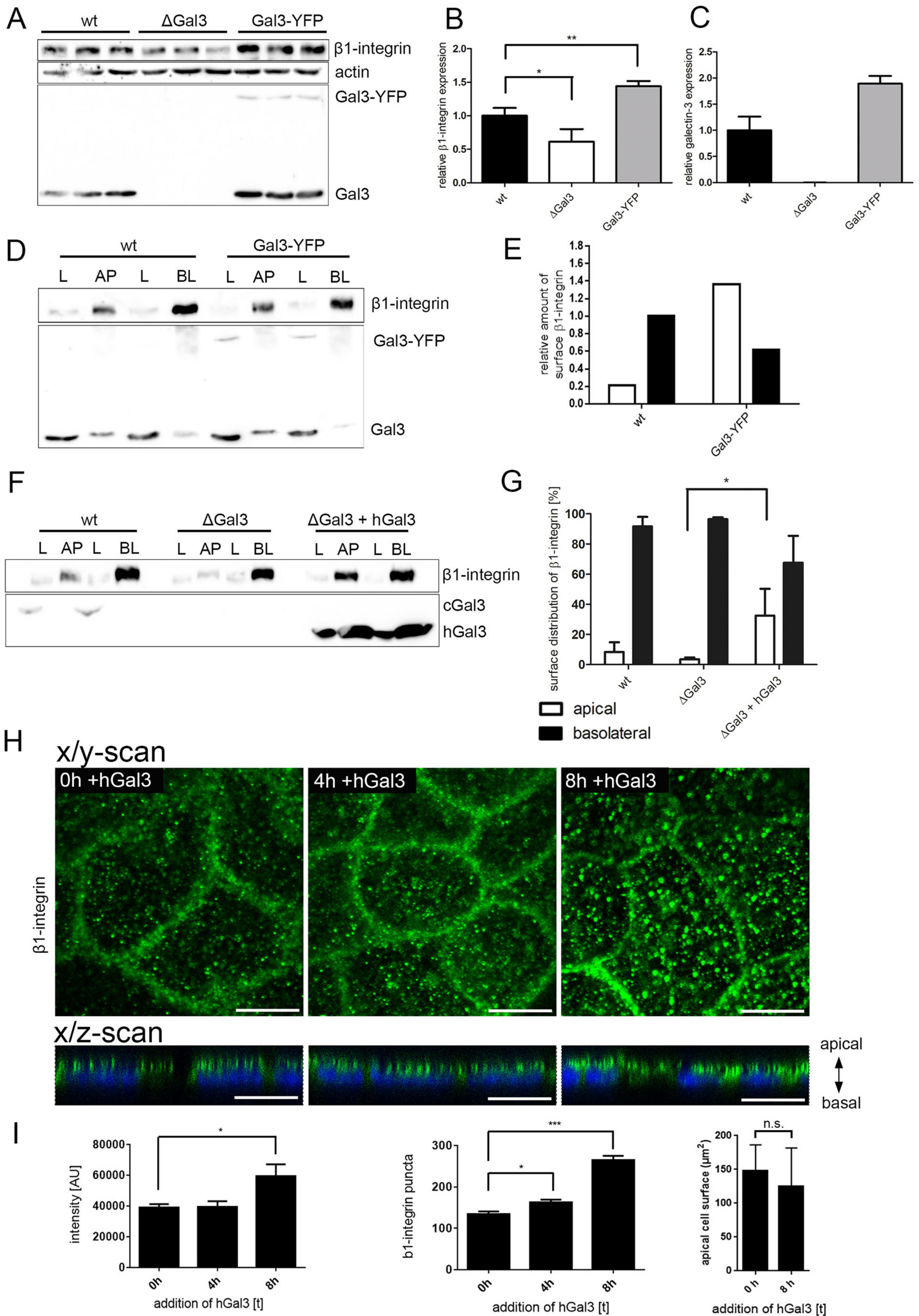

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Fig. 3. Surface distribution of β1-integrin is modulated by galectin-3. (A) Expression levels of β1-integrin analysed in polarized $MDCK_{wt}$, $MDCK_{\Delta Gal3}$ and $MDCK_{Gal3\text{-}YFP}$ cells by immunoblotting. Protein concentrations of the lysates were measured and 20 µg loaded on each lane of the SDS-PAGE. (B) Relative β1-integrin protein expression compared with $MDCK_{wt}$. Mean±s.d., $n=3$. Statistical significance was tested with Student's *t*-test. *$P<0.05$, **$P<0.01$. (C) Relative galectin-3 protein expression compared with $MDCK_{wt}$. Mean±s.d., $n=3$. (D,E) Surface distribution (D) and quantification (E) of β1-integrin in polarized $MDCK_{wt}$ and $MDCK_{Gal3\text{-}YFP}$ cells. Relative amounts of β1-integrin at the apical and basolateral membrane domains were normalized to actin levels in lysates. (F,G) Surface distribution (F) and quantification (G) of β1-integrin in polarized $MDCK_{wt}$ and $MDCK_{\Delta Gal3}$. $MDCK_{\Delta Gal3}$ cells were treated with 1.5 µM hGal3 every hour 8 h prior to experiment. Amounts of β1-integrin at the apical and basolateral membrane domains were normalized to tubulin levels in lysates and are plotted as a fraction of total (apical and basolateral) β1-integrin surface levels. Mean±s.d., $n=3$. Statistical significance was tested with Student's *t*-test. *$P<0.05$. (H) Polarized $MDCK_{\Delta Gal3}$ cells were incubated for 0, 4 or 8 h with recombinant hGal3, fixed, immunolabelled for β1-integrin with Alexa488 (green) and analysed by confocal microscopy. *x-y* and *x-z* scans with nuclei depicted in blue are shown. Scale bars: 10 µm. (I) Quantification of H, total fluorescence intensity and amount of β1-integrin vesicles per cell. Mean±s.e.m., $n=3$. Statistical significance was tested with one-way ANOVA and Bonferroni's post-test. *$P<0.05$, ***$P<0.0001$. No significant difference in the apical cell area from 68 cells before or after 8 h galectin-3 administration was detected.

N-acetyl-D-lactosamine (LacNAc) (Fig. 5A). These data suggest that galectin-3 positively affects apical β1-integrin internalization and recycling, which was corroborated by enhanced endocytic uptake of β1-integrin in the presence of 1.5 µM recombinant human galectin-3 (Fig. 5C,D). In addition, low levels of β1-integrin internalization and recycling in $MDCK_{\Delta Gal3}$ cells were elevated by apical supplementation of the cells with 100 or 500 nM hGal3 (Fig. 5E,F). Therefore, we conclude that galectin-3 promotes apical β1-integrin internalization and recycling. Moreover, Fig. S6E,F indicates that the two polypeptides are co-internalized from the apical membrane of MDCK cells within 10 min, which is consistent with a previous study showing that both proteins are co-internalized from the plasma membrane of breast carcinoma cells (Furtak et al., 2001).

Once internalized for 10 min at 37°C from the apical membrane of MDCK cells, the interaction between β1-integrin and hGal3-biotin remains at constant levels. However, total amounts of galectin-3 and the co-precipitated β1-integrin start to decline about 30 min after the onset of internalization (Fig. S6E,F). This decline can be explained by recycling of galectin-3 to the apical membrane and release into the medium, as previously shown (Straube et al., 2013). Nevertheless, such endo/exocytic shuttling of β1-integrin would, at the most, lead to an equilibrium of apical β1-integrin and does not explain why the addition of hGal3 increases levels of β1-integrin at the apical membrane (Fig. 3). To further address this question we analysed trafficking pathways that deliver β1-integrin to the apical membrane of MDCK cells.

Newly synthesized β1-integrin is transported to the apical and basolateral membrane domains

Initially, we checked whether galectin-3 directly guides newly synthesized β1-integrin to the apical domain, which would increase the apical pool of β1-integrin. Transport kinetics of β1-integrin to the apical or basolateral membrane was recorded by biosynthetic labelling of MDCK cells with $[^{35}S]$methionine followed by a chase for different periods of time. Apically or basolaterally delivered proteins were biotinylated and isolated followed by immunoprecipitation of β1-integrin (Fig. 6A,B). By 1 h of chase, newly synthesized β1-integrin was detected at the apical and basolateral plasma membranes and levels of integrin delivered to each membrane increased over the following 5 h. Owing to low signal intensities in experiments with chase periods of less than 1 h, we cannot exclude the possibility that β1-integrin is indirectly transcytosed from the basolateral to the apical domain within this early time interval. Decreased basolateral β1-integrin levels after the overnight chase period compared with the rising levels of apical β1-integrin suggest that a portion of basolateral integrin is transcytosed to the apical domain after longer chase periods. However, the gradual increase of β1-integrin from 1 to 6 h of chase at each membrane domain shows a direct delivery of the polypeptide to the apical and to the basolateral cell pole. This does not rule out the passage through intermediate compartments between the TGN and the plasma membrane. Galectin-3 was shown to assist in the sorting of apical glycoproteins in a post-Golgi compartment (Delacour et al., 2006) and thus most likely sorts β1-integrin into carriers directed towards the apical plasma membrane.

To test this hypothesis, we compared polarized membrane targeting of biosynthetically labelled β1-integrin in MDCK, $MDCK_{\Delta Gal3}$ and $MDCK_{\Delta Gal3}$ cells supplemented with hGal3 during the chase period. In contrast to MDCK cells, $MDCK_{\Delta Gal3}$ cells did not transport substantial quantities of newly synthesized β1-integrin to the apical membrane domain (Fig. 6C,D). However, addition of hGal3 increased apical β1-integrin delivery after 6 h of chase. This suggests that exogenously added galectin-3 enhances the sorting of newly synthesized β1-integrin to the apical membrane and confirms that galectin-3 is involved in apical trafficking of this integrin.

Previous data showed that galectin-3 mediates protein sorting by forming high molecular weight clusters (HMWCs) that recruit apical cargo into transport vesicles (Delacour et al., 2007). As such, we predict that galectin-3 will stabilize crosslinked complexes of β1-integrin and glycoprotein. To test this hypothesis, we analysed the distribution of β1-integrin in velocity sedimentation experiments. In linear gradients from 2.5 to 25% Nycodenz, β1-integrin was predominantly detected in high molecular weight fractions 10-12 (Fig. S7). The appearance of β1-integrin and galectin-3 in the high molecular weight fractions indicates that a considerable pool of receptor and lectin associates with HMWCs of about 450-600 kDa. By contrast, in $MDCK_{\Delta Gal3}$ cells, substantial quantities of β1-integrin were additionally found in the lighter fractions 7 and 8, corresponding to a molecular weight of ∼250 kDa. This indicates that β1-integrin HMWCs are destabilized in the absence of galectin-3 and partially dissociate into smaller complexes. Exclusive sorting of β1-integrin into high molecular weight fractions 10-12 was then reconstituted in $MDCK_{\Delta Gal3}$ cells by supplementation with hGal3. Together, these data suggest that galectin-3 stabilizes β1-integrin in HMWCs, most likely by direct crosslinking, and that HMWCs may also play a role in apical sorting of newly synthesized β1-integrin by galectin-3.

Galectin-3 promotes redistribution of β1-integrin from the basolateral towards the apical domain

In addition to the transport of newly synthesized β1-integrin to the apical membrane, an increase in apical β1-integrin based on basolateral to apical transcytosis is also conceivable. To assess this possibility, basolateral membrane proteins of polarized and filter-grown $MDCK_{\Delta Gal3}$ cells were labelled with a reducible biotin conjugate. Internalization of biotin-labelled membrane proteins was allowed for 15 min at 37°C. Subsequently, biotin was removed from non-internalized biotinylated proteins by addition of glutathione. Endocytosed membrane proteins from the basolateral domain are not exposed to the reducing agent. Next, the cells were incubated at

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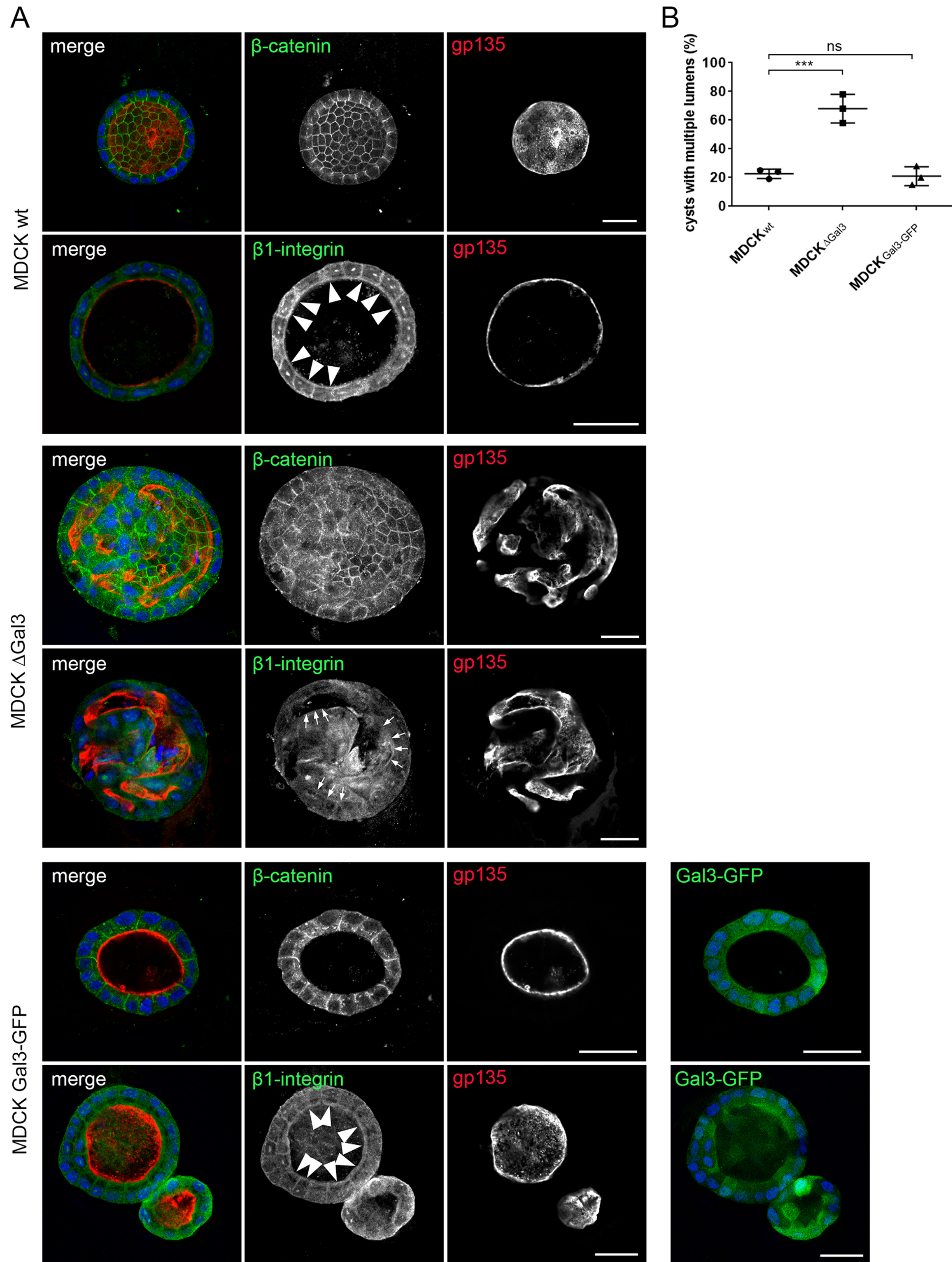

Fig. 4. Apical β1-integrin is required for proper cyst development. (A) $MDCK_{wt}$, $MDCK_{\Delta Gal3}$ and $MDCK_{Gal3\text{-}GFP}$ cells were cultured in Matrigel for 7 days. Polarity of cysts was analysed by confocal microscopy. Cysts were stained for β1-integrin (green), β-catenin (green) and gp135 (red). Arrowheads indicate apical β1-integrin staining. Arrows highlight apical domains with absent β1-integrin staining. Nuclei are shown in blue. Scale bars: 25 µm. (B) Quantification of cysts with multiple lumens in $MDCK_{wt}$, $MDCK_{\Delta Gal3}$ and $MDCK_{Gal3\text{-}GFP}$ cells. Mean±s.d., $n=3$. For each cell line, 10 cysts per experiment were categorized. Cysts with more than one black lumen encircled by gp135 were classified as 'multi-lumen'. Statistical significance was tested by one-way ANOVA; $***P<0.001$; ns, not significant.

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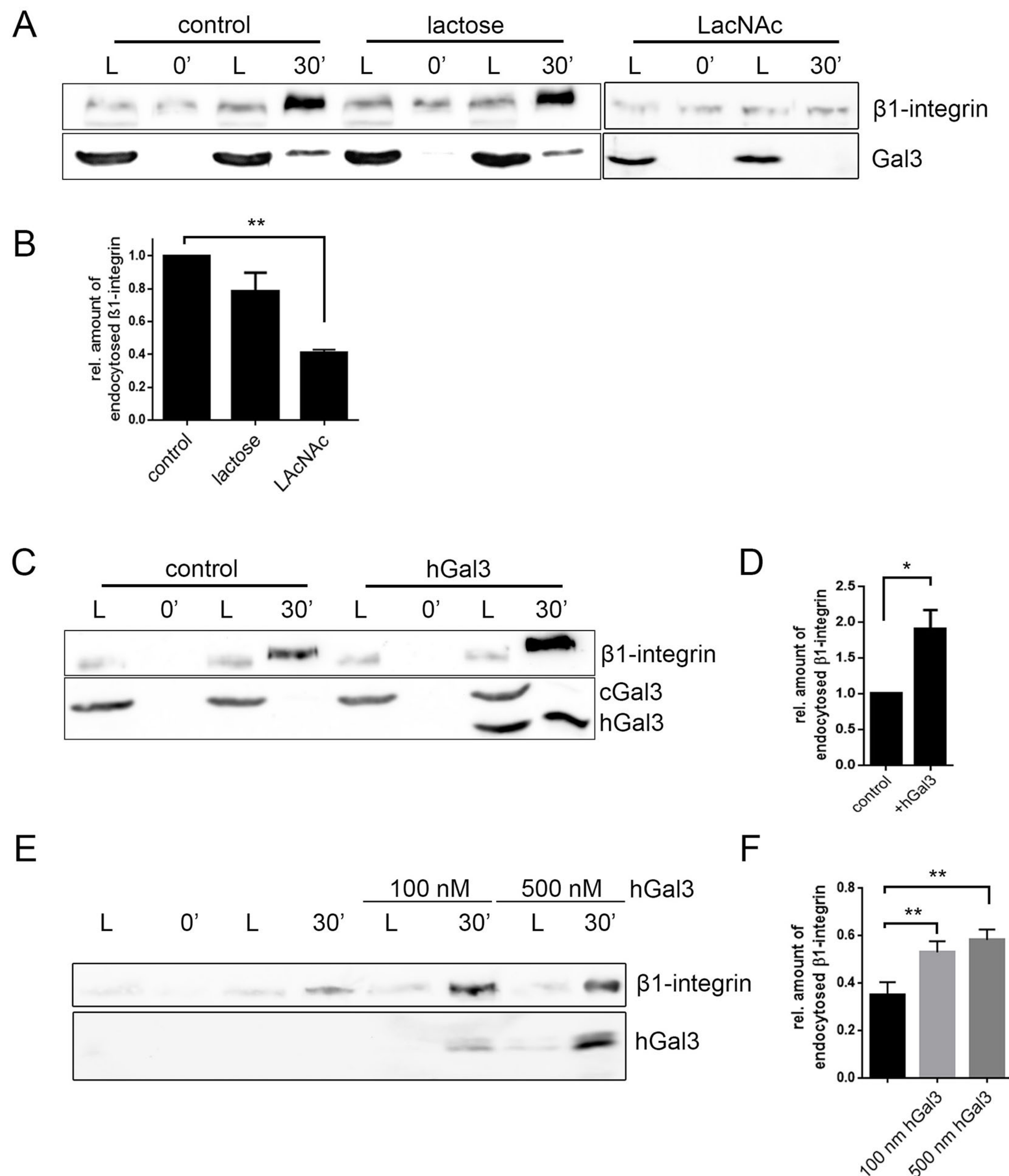

Fig. 5. Galectin-3 drives apical endocytosis of β1-integrin. (A,B) Apical membrane proteins of polarized $\mathrm{MDCK}_{\mathrm{Gal3\text{-}YFP}}$ cells labelled with NHS-SS-biotin. Endocytic uptake was allowed for 0 or 30 min in the absence or presence of 50 mM lactose or 25 mM LacNAc. Thereafter, biotin label of non-internalized apical membrane proteins was removed by glutathione. Biotinylated proteins were isolated with neutravidin beads and analysed by immunoblotting (A) and amount of neutravidin-retrieved β1-integrin was normalized to integrin levels in the corresponding lysates (B). 0 min values were subtracted and amounts of endocytosed β1-integrin after 30 min are shown. Control was set to 1. Mean±s.d., *n*=3. Statistical significance was tested with one-way ANOVA; $^{**}P<0.01$. (C,D) Reversible biotinylation of apical membrane proteins as described in A. Endocytosis was allowed for 0 or 30 min in the absence or presence of 1.5 µM hGal3. Amounts of neutravidin-retrieved β1-integrin were normalized to tubulin levels in the corresponding lysates (not shown). 0 min values were subtracted and amounts of endocytosed β1-integrin after 30 min are shown (B). Control was set to 1. Mean±s.d., *n*=4. Statistical significance was tested with Student's *t*-test; $^{*}P<0.05$. (E,F) Reversible biotinylation of apical membrane proteins from polarized $\mathrm{MDCK}_{\Delta\mathrm{Gal3}}$ cells as described in A. Endocytic uptake was allowed for 0 or 30 min in the absence or presence of 100 nM or 500 nM hGal3. $\mathrm{MDCK}_{\mathrm{Gal3\text{-}YFP}}$ cell control was set to 1. Mean±s.d., *n*=3. Statistical significance was tested with one-way ANOVA; $^{**}P<0.01$.

37°C for up to 60 min to allow transcytosis from the basolateral to the apical membrane compartment. Residual proteins exclusively at the basolateral (Fig. 7A,C) or at the apical and basolateral cell surface (Fig. 7B,D) were reduced and debiotinylated by glutathione. Following basolateral reduction, precipitation of biotinylated proteins with neutravidin beads and immunoblot analysis revealed no significant alterations in biotinylated β1-integrin. This suggests that basolaterally internalized β1-integrin is inefficiently recycled to the basolateral cell surface within 60 min of internalization. Reduction of the apical and the basolateral medium slightly but insignificantly decreased levels of biotinylated β1-integrin. This loss was dramatically increased in the presence of externally added recombinant human galectin-3. The effect only became apparent if the apical and the basolateral media were reduced, which indicates that basolaterally internalized β1-integrin attains glutathione sensitivity by being exposed at the apical membrane. Thus, we

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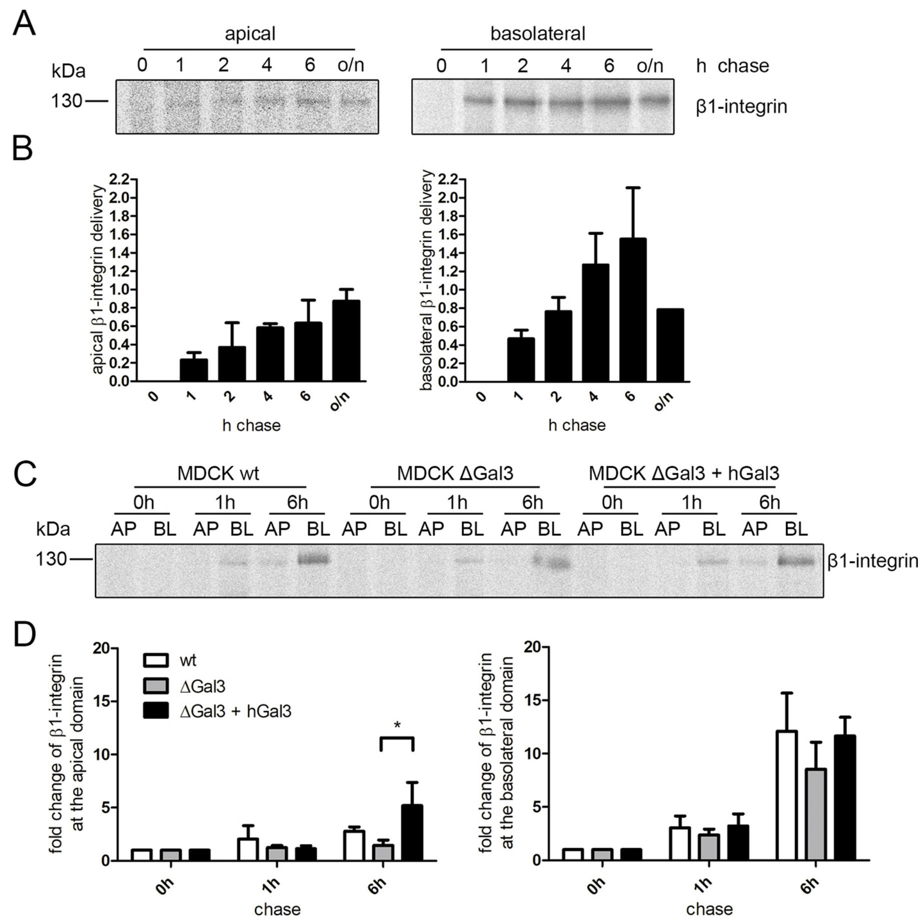

Fig. 6. Newly synthesized β1-integrin is transported to the apical and basolateral membrane domains. (A,B) MDCK cells were pulse labelled with [^{35}S]methionine and chased for the indicated time intervals. β1-integrin was isolated from the respective membrane domains by surface biotinylation and immunoprecipitation. Amount of newly synthesized β1-integrin at the cell surface was analysed by radiography (A). The fractions of β1-integrin retrieved from the apical and basolateral membrane were derived from maximal apical or basolateral β1-integrin delivered, respectively (B). Mean±s.d., *n*=3. (C,D) $MDCK_{wt}$ and $MDCK_{\Delta Gal3}$ cells were metabolically labelled, chased and analysed as in A. $MDCK_{\Delta Gal3}$ cells were incubated with 1.5 μM hGal3 during pulse and chase periods (C). Apical and basolateral surface β1-integrin levels were normalized to tubulin-normalized β1-integrin lysate levels, at 0 h of each cell line, respectively (D). Fold change of β1-integrin at the apical (left) and basolateral (right) membrane domain is shown. The amount of surface β1-integrin at the two membrane domains was calculated and 0 h values of each domain and each condition were set to 1. Mean±s.d., *n*=3. Statistical significance was tested for 6 h values using one-way ANOVA and Bonferroni's post test; $^{*}P<0.05$.

conclude, that galectin-3 can promote transcytosis of β1-integrin from the basolateral to the apical plasma membrane. We also analysed the influence of galectin-3 in the transcytotic passage of basolateral polypeptides in polarized $MDCK_{\Delta Gal3}$ cells by confocal microscopy. Here, basolateral proteins were labelled with reducible biotin conjugates followed by a 4 h time interval to internalize and redistribute this protein pool in the absence or presence of hGal3 (Fig. 8). Following basolateral reduction to remove residual biotin at the basolateral cell surface, cells were fixed and biotinylated proteins were visualized using streptavidin-Alexa488. These experiments revealed that biotin appeared at the apical cell surface if hGal3 had been added to the cells. In the absence of hGal3, or in control experiments without internalization, streptavidin-Alexa488 fluorescence intensities did not significantly increase, which suggests that galectin-3 promotes the redistribution of basolateral polypeptides to the apical membrane domain. Considered together, our data show that galectin-3 directs newly synthesized and basolaterally endocytosed β1-integrin to the apical membrane domain of epithelial cells.

DISCUSSION

In this study, we show that galectin-3 modulates the apical localization of β1-integrin in polarized epithelial cells. Endogenous galectin-3 is sufficient to support a minor pool of apical β1-integrin. Knockout of galectin-3 results in a decreased surface expression of apical β1-integrin, and perturbations in cell proliferation and cyst formation. Conversely, moderate overexpression of galectin-3 elevates apical β1-integrin delivery but does not affect cell proliferation and correct cyst formation.

Integrins function as αβ-heterodimeric receptors to convey polarity cues from the extracellular matrix (Yu et al., 2005) and it has been previously demonstrated that β1-integrins are not exclusively localized on the basolateral surface but are also located on the apical surface of subconfluent and fully polarized MDCK cells (Praetorius and Spring, 2002; Schwimmer and Ojakian, 1995; Zuk and Matlin, 1996). We found that galectin-3 interacts with α2β1-integrin at the apical membrane domain of MDCK cells. This interaction is facilitated by the presence of sphingolipids and the integrity of cholesterol-rich membrane microdomains, which are strongly enriched at the apical membrane (Simons and van Meer, 1988). A glycosphingolipid-enriched membrane microenvironment likewise facilitates the uptake of galectin-3 and β1-integrin into clathrin-independent carriers (Lakshminarayan et al., 2014). Moreover, endogenous or externally supplied galectin-3 sorts newly synthesized as well as internalized β1-integrin to the apical cell surface. The question of whether β1-integrin can be redistributed to the apical cell surface by extracellular interaction partners has been addressed previously (Zuk and Matlin, 1996). Apical overlay of MDCK cells with the extracellular matrix component type I collagen did not stimulate delivery of additional newly synthesized integrin to apical plasma membranes. However, interaction of β1-integrin with collagen on the apical membrane drives the formation of tubulocysts (Zuk and Matlin, 1996). Evidence for a link between tubulogenesis and galectin-3 expression originally came from Bao and Hughes (1995). This corresponds to the observation that ricin-resistant MDCK cells, which fail to transfer galactose residues during synthesis of glycoconjugates for later interaction with galectin-3, undergo

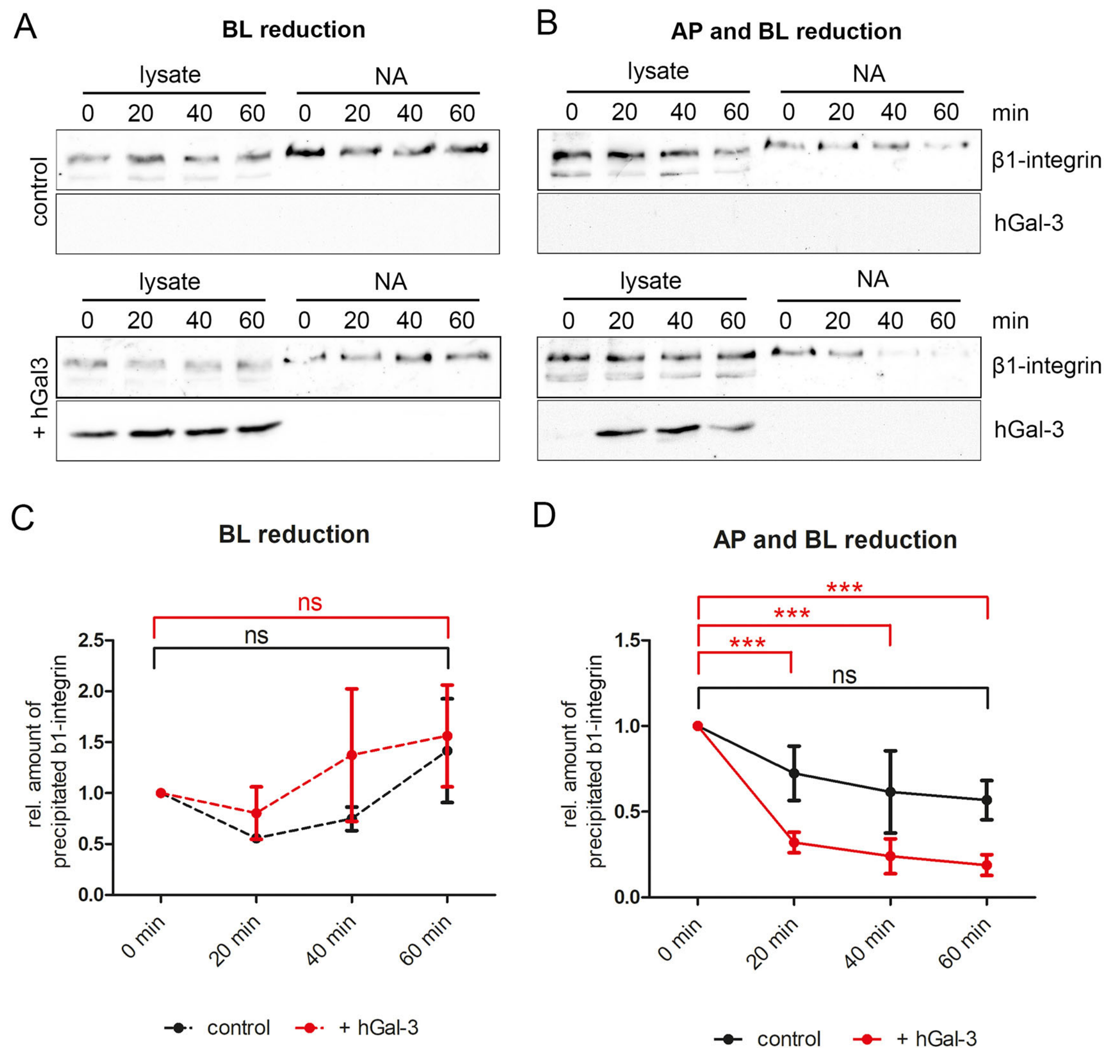

Fig. 7. β1-integrin is transcytosed from the basolateral membrane to the apical domain in the presence of galectin-3. (A,B) Basolateral membrane proteins of polarized MDCK$_{\Delta Gal3}$ cells were biotinylated with NHS-SS-biotin. Endocytosis of biotin-labelled membrane proteins was allowed for 15 min. Biotin label of non-internalized proteins was removed by glutathione. Cells were incubated at 37°C in the absence (control, top) or presence of 1.5 µM hGal3 (bottom) for the indicated periods. Subsequently, the basolateral (A) or the apical and basolateral membrane domains (B) were incubated with glutathione prior to precipitation with neutravidin beads (NA). Isolated proteins were analysed by immunoblotting. (C,D) Quantification of A and B, respectively. Relative amounts of precipitated β1-integrin were normalized to tubulin levels in the corresponding lysates (not shown). Mean±s.e.m., *n*=3. Statistical significance was tested with one-way ANOVA and Bonferroni's post-test; ****P*<0.001.

enhanced cystogenesis and abnormal morphogenesis (Bao and Hughes, 1999). Consequently, organ culture analysis of mouse metanephrons revealed that galectin-3 modulates ureteric bud branching (Bullock et al., 2001). As a result of the binding affinity of galectin-3 to laminins and β1-integrins it was speculated that extracellular galectin-3 could modulate metanephric growth by crosslinking laminins to membrane-bound receptors. Our data point to an alternative mode of action for galectin-3 by redistribution of integrin receptors and by their enhancement at the apical cell surface.

Previous studies revealed that at the apical membrane interaction of collagen I with β1-integrins induces activation of Rac1, which is required for collagen overlay-induced tubulocyst formation (Yu et al., 2005). Accordingly, expression of dominant-negative Rac1N17 caused inversion of polarity from MDCK cells grown in 3D culture so that the apical cell surface was misoriented towards the extracellular matrix (O'Brien et al., 2001). Similar effects on cell polarity were observed when the Rac1 opponent RhoA was activated during MDCK cell cyst development (Yu et al., 2008). Consequently, alterations in the polarized distribution of integrins would affect tubulocyst formation and cyst development, as evidenced by knockout of galectin-3 in this study.

We also reported that the absence of galectin-3 influences the stabilization of centrosomes and primary cilia, with effects on epithelial morphogenesis (Koch et al., 2010). Hence, perturbations in the microtubule architecture of galectin-3-depleted cells may explain how the lectin participates in epithelial morphogenetic events. Recent observations showing that galectin-3 plays crucial roles in the maintenance of the microtubule-organizing centre are consistent with this interpretation (Clare et al., 2014). In addition to this role in cytoskeletal organization, our findings suggest that galectin-3 influences morphogenetic events in epithelia by intracellular sorting, recycling and transcytosis of newly synthesized and endocytosed β1-integrin. Several components of the membrane trafficking machinery are known to be involved in the recycling of integrins. As recently published, plasma membrane

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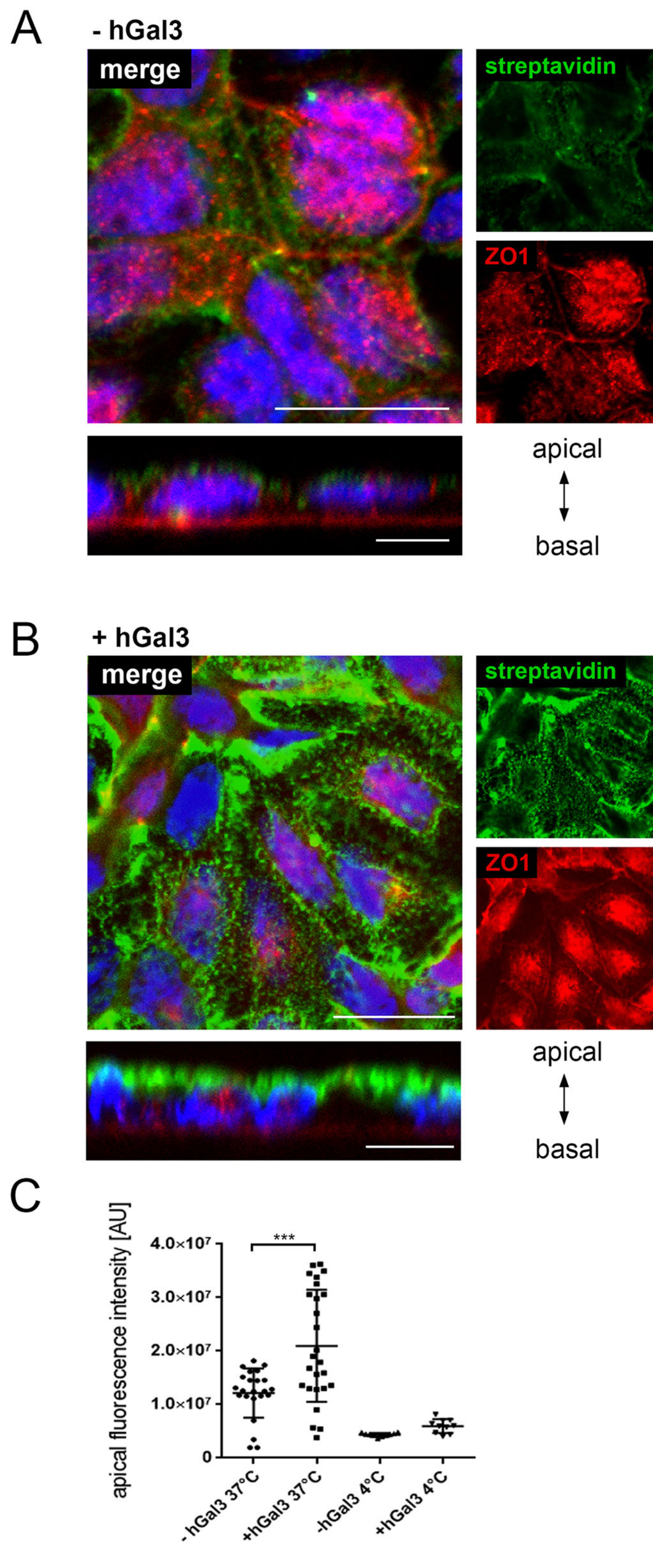

Fig. 8. Increased transcytosis from the basolateral membrane to the apical domain in the presence of galectin-3. Basolateral membrane proteins of polarized MDCK$_{\Delta Gal3}$ cells were biotinylated with NHS-SS-biotin. Endocytosis of biotin-labelled membrane proteins was allowed for 15 min. Biotin label of non-internalized proteins was removed by glutathione. Transcytosis of biotin-labelled membrane proteins was allowed for 4 h at 37°C in the absence (A) or presence of 1.5 µM hGal3 (B). Biotin label of non-transcytosed proteins at the basolateral membrane was removed by glutathione. Subsequently, the cells were fixed, permeabilized and immunostained for ZO1 (red). Biotinylated polypeptides were visualized by streptavidin-546 staining. Nuclei are shown in blue. Scale bars: 10 µm. (C) Quantification of A, B and control experiments performed at 4°C to block endocytic uptake of biotinylated proteins. Mean±s.d., *n*=3. Statistical significance was tested with Student's *t*-test; ****P*<0.001.

levels of these proteins are regulated by the WASH (WASP and SCAR homologue) complex, which is important for recycling integrins (Buckley et al., 2016), or by the novel retromer-independent endosomal cargo recycling complex 'retriever' (McNally et al., 2017). Intracellularly, integrins can be diverted from degradative endosomal pathways into recycling endosomes by γ-adaptin ear-containing Arf-binding protein-3 (GGA3) and sorting nexin 17 (SNX17)-mediated transport (Bottcher et al., 2012; Ratcliffe et al., 2016; Steinberg et al., 2012). Furthermore, the SNX17 interaction partner low-density lipoprotein receptor-related protein-1 (LRP1) binds integrins at the cell surface and regulates their uptake and recycling in tumour cells (Theret et al., 2017). Our study suggests that galectin-3 guides β1-integrin to the endosomal trafficking pathway for apical recycling and thereby protects it from lysosomal degradation. Recycling of β1-integrin in mouse 3T3 fibroblast cell lines involves internalization into Rab4-positive endosomes and recycling to the plasma membrane in a Rab11-dependent fashion (Roberts et al., 2001; Nader et al., 2016). Galectin-3 also enters Rab11-positive recycling endosomes (Schneider et al., 2010) and Rab11 is located on apical recycling endosomes in polarized epithelial cells (Casanova et al., 1999; Goldenring et al., 1996). Moreover, basolateral-to-apical transcytosis of the transferrin receptor in MDCK cells is dependent on Rab11 (Perez Bay et al., 2013). These endosomes are also traversed in the biosynthetic route of apical cargo in MDCK cells (Thuenauer et al., 2014). Consequently, Rab11-positive recycling endosomes seem to play a critical role in galectin-3-mediated β1-integrin sorting. Our data further indicate that β1-integrin internalized at the basolateral membrane enters a transcytotic route to the apical membrane domain and is inefficiently recycled back to the basolateral membrane. This is surprising in view of the observation that inactive β1-integrin recycles back to the plasma membrane of cancer cells via a fast-loop pathway, whereas active β1-integrin recycles with much slower kinetics (Arjonen et al., 2012). It is thus likely that we monitored internalization and transcytosis of a specific subset of β1-integrin.

In epithelial cells, recycling endosomes offer the opportunity for transcytosis of cargo from the basolateral to the apical membrane domain. Prominent examples include the polymeric immunoglobulin receptor, which transports dimeric IgA from the basolateral to the apical surface for release into the apical medium (Mostov and Deitcher, 1986). The galectin family member tandem-repeat galectin-4 is involved in basolateral to apical epithelial transcytosis of the transferrin receptor (TfR) (Perez Bay et al., 2014). Here, the lectin prevents lysosomal targeting of basolaterally internalized TfR and mediates apical trafficking in AP-1B-deficient epithelia. Galectin-4 was proposed to operate by clustering and recruitment of TfR polypeptides into specific lipid microdomains for segregation into apical transport vesicles. This function might be facilitated by the capacity of galectin-4 to bind to glycoproteins and glycolipids (Stechly et al., 2009). Similar functions by glycoprotein crosslinking into HMWCs have been described for galectin-3-mediated apical sorting (Delacour et al., 2007) or intracellular guidance (Carlsson et al., 2013) and our data also suggest that recruitment of β1-integrin into HMWCs is stabilized by galectin-3. Quantitative support for the formation of a galectin-3–integrin lattice on the surface of live cells comes from

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a recent study using single-particle tracking (Yang et al., 2017). It has also been shown that full-length galectin-3 but not N-terminally truncated Gal3C is capable of clustering integrin on membranes (Wang et al., 2017). We therefore anticipate that apical sorting of newly synthesized and basolaterally endocytosed β1-integrin likewise relies on galectin-3-mediated clustering. The galectin-3-dependent stabilization of β1-integrin HMWCs in MDCK cells strongly supports this idea. Galectin-3 and galectin-4 have been found in endosomal organelles. Here, they sort newly synthesized proteins and basolaterally internalized proteins into carriers destined for the apical membrane. It would be interesting to explore the link between galectin, glycoprotein and glycolipid clusters with the cellular transport machinery for apical cargo delivery.

Our data further show that galectin-3-mediated intracellular sorting also modulates β1-integrin expression levels in MDCK cells. This is consistent with a study of human breast carcinoma cell lines demonstrating that galectin-3-overexpressing cells, with respect to low galectin-3-expressing cells, showed increased surface expression of α4- and β7-integrin (Matarrese et al., 2000). Concerning the molecular mechanism by which the lectin affects β1-integrin expression levels in MDCK cells, our data indicate that the integrin is restrained from lysosomal degradation by galectin-3-mediated recruitment into apical transport pathways and recycling. A related role of galectin-3 has been described for the intracellular trafficking of human serum transferrin (Carlsson et al., 2013). Here, galectin-3 guides a specific subpopulation of internalized transferrin into a fast recycling route to the plasma membrane. In analogy to our observations, the N-terminal domain of galectin-3 is required for this function, which verifies the idea of intracellular sorting by galectin-3-mediated cluster formation.

In MDCK cells, galectin-3-induced alterations in the expression level and the subcellular distribution of β1-integrin certainly affect integrin signalling and integrin-mediated adhesion. In general, molecular lattices of galectins and surface glycoproteins on the plasma membrane regulate cell proliferation and cell differentiation (Lau et al., 2007). Previous data suggest that, in addition, extracellular galectin-3 can directly influence integrin α2β1-integrin-mediated adhesion via galactoside-dependent oligomeric interactions (Friedrichs et al., 2008). It remains unclear whether the altered integrin signalling observed in this study participates in a reduced proliferation rate in the absence of galectin-3. A recent publication describes a blockade of integrin signalling pathways and inhibition of cell growth in the presence of the galectin-3 inhibitor RN1 (Zhang et al., 2017). However, integrins are a complex family of proteins consisting of two subunits associated in various combinations, and at this time, it is unknown which signalling pathways might be implicated. Future experiments will explore the dysregulation of integrin expression and cell proliferation caused by knockdown of galectin-3 in more detail.

MATERIALS AND METHODS

Cell culture, CRISPR/Cas9 gene editing and metabolic labelling

MDCK type II ($MDCK_{wt}$) and $MDCK_{\Delta Gal3}$ cells were cultured at 37°C under 5% CO_2 in minimum essential medium (MEM, Gibco) containing 10% fetal calf serum (FCS) with antibiotics and glutamine. For the generation of $MDCK_{\Delta Gal3}$ cells, galectin-3 expression was eliminated by CRISPR/Cas9 gene editing as described below. $MDCK_{Gal3\text{-}YFP}$ and $MDCK_{Gal3\text{-}GFP}$ cells were generated by transfection with corresponding expression plasmids and selection in MEM containing 10% FCS, supplemented with 0.5 mg/ml G418. Plasmid transfection and subsequent analysis of protein expression by immunoblot and fluorescence microscopy were performed essentially as described previously (Delacour et al., 2006).

For transport studies of newly synthesized proteins, MDCK cells were grown on PET filters (83.3930.041, Sarstedt) for 5-7 days. Cells were washed twice with PBS and incubated with methionine-free MEM for 1 h at 37°C. For biosynthetic labelling, 60 µCi [^{35}S]methionine was added to the basolateral medium and incubated for 30 min. Medium was replaced by culture medium (MEM containing 10% FCS). Cells were then further incubated at 37°C for different periods of time.

If indicated, polarized MDCK cells were treated for 10 h prior to the onset of the experiment with 10 µM fumonisin or 50 mM MβCD (Alfalah et al., 2002). Endocytic recycling was blocked by inclusion of 0.3 mM or 0.6 mM primaquine (Woods et al., 2004). For 3D cultures, trypsinized MDCK cells were resuspended in pure Matrigel (BD Biosciences) at a final concentration of 2×10^4 cells/ml. 30 µl of Matrigel cell suspension was added to precooled 1.2 mm coverslips. MDCK cysts were grown for 7 days and medium was renewed daily. For proliferation experiments, cells were stained with Trypan Blue and live cells were counted by Countess, Thermo Fisher Scientific.

DNA constructs

Plasmid pSpCas9n(BB)-2A-Puro (PX462) V2.0 was from Addgene (plasmid #62987, deposited by Feng Zhang). Oligo pairs encoding the 20 nt guide sequences against canine Gal3 (5′-CAC CGC CTT ATG ACC TAC CTT TGC C-3′, 5′-AAA CGG CAA AGG TAG GTC ATA AGG C-3′) were annealed and ligated into the *Bbs*I-digested plasmid to generate pCRISPR-Cas9ΔGal3. Following transfection of pCRISPR-Cas9ΔGal3, cells were selected for 48 h with 2 µg/ml puromycin (Sigma-Aldrich). Lysates of MDCK cell clones were analysed for the presence of Gal3 by immunoblot.

Antibodies

The following antibodies were used: mouse monoclonal antibody directed against gp135 was kindly provided by George Ojakian (State University of New York Health Science Center, New York, USA). Mouse monoclonal antibodies directed against β1-integrin/CD29 (BD 610468) were purchased from BD Bioscience. Rabbit polyclonal anti-β1-integrin antibodies (GTX128839) were obtained from GeneTex. Rabbit polyclonal antibodies directed against galectin-3 were obtained from GeneTex (GTX113486) and Santa Cruz (H-160, sc-20157). In our tests, both antibodies recognized human galectin-3 with high affinity, but recognition of canine galectin-3 was significantly weaker and not sufficient to detect low amounts of canine Gal3 that oligomerize with hGal3. Rabbit polyclonal antibodies directed against α2-integrin (H-293) (sc-9089) as well as from goat against clusterin-α/gp80 (C-18) (sc-6419) were purchased from Santa Cruz. The mouse polyclonal antibody against GFP (JL-8) (632380) was obtained from Clontech, polyclonal rabbit antibodies directed against β-catenin (C2206) were purchased from Sigma and polyclonal goat antibodies against villin (C-19) (sc-7672) was obtained from Santa Cruz. Primary antibodies were diluted 100-fold for immunofluorescence and 2000-fold for immunoblotting. HRP-conjugated secondary antibodies against mouse (170-6516) or rabbit (170-6515) were obtained from Bio-Rad. HRP-conjugated secondary antibody against goat were purchased from Santa Cruz (sc-2020). Alexa-labelled secondary antibodies were purchased from Thermo Fisher.

Production and labelling of recombinant proteins

Recombinant human galectin-3 was expressed and purified as previously reported (Straube et al., 2013). Recombinant canine apo-transferrin was purchased from Sigma. For the iron loading of apo-transferrin, 50 µM iron citrate was generated by incubation of iron chloride overnight at room temperature with 100-fold sodium citrate. Apo-transferrin was dissolved in 1 M $NaHCO_3$, pH 8, and incubated with 50 µM iron citrate for 1 h at room temperature. Conjugation of recombinant proteins to biotin and Alexa Fluor 555 was performed essentially as described in the manual using succimidyl ester derivatives (A20187, Thermo Fisher).

Immunoblot and immunoprecipitation

For preparation of cell lysates, the cells were washed with PBS^{++} (PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$), collected in lysis buffer (25 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1% Triton X-100, 0.5% NP40, pH 7.5) and incubated at 4°C for 30 min on a rotating platform.

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After centrifugation for 15 min at 13,000 rpm, the supernatants were either used for further experimental procedures or separated by SDS-PAGE using the Hoefer-Mini-VE system (Amersham Pharmacia Biotech) and transferred to nitrocellulose membranes. The membranes were blocked in 5% skimmed milk powder in PBS for 1 h and incubated overnight at 4°C with specific antibodies. Detection was performed with HRP-conjugated secondary antibody visualized by ECL (Thermo Fisher Scientific) and an Intas Gel Imager CCD camera. The results were quantified using LabImage 1D software (see below).

For immunoprecipitation, Protein-G Sepharose (PGS) beads were coated with specific antibodies or non-specific IgG (IgG from human serum, Sigma) by incubation on a rotating platform overnight at 4°C. Cell lysates were precleared by addition of IgG-coated PGS beads. Precipitations were performed using PGS beads coated with specific antibodies at 4°C for 1.5 h or overnight.

Secretion and velocity sedimentation assay

The secretion of galectin-3 was analysed by replacement of the apical and basolateral MDCK cell medium by FCS-free medium and medium-collection for 4 h. Galectin-3 content was assessed by immunoblotting. The preparation of total membranes and velocity sedimentation were performed essentially as previously described (Delacour et al., 2007).

Lectin binding and proteomic analysis

To identify and analyse galectin-3 interaction partners at the plasma membrane, MDCK cells were seeded on PET filter insets and incubated for at least 5 days. The integrity of the cell monolayer was assessed using the leak test (Klumperman et al., 1991) and by measurements of the transepithelial resistance (Zink et al., 2012). 1.5 µM hGal3-biotin was added to the apical or basolateral compartment for 30 min at 4°C. Incubation of hGal3-biotin was performed in PBS^{++}, 50 mM lactose or at pH 4.5, as indicated in the figure legends. Cells were then lysed and biotinylated hGal3 and associated proteins were isolated with neutravidin beads (NA, Thermo Scientific) and separated by SDS-PAGE. Samples were either analysed by western blotting as described above or by mass spectrometry. For mass spectroscopy analysis, samples were lysed in 25 mM Tris-HCl, 50 mM NaCl, 0.5% sodium deoxycholate with 0.5% Triton X-100, pH 8, and SDS gels were stained by InstantBlue Protein Stain (Expedeon). Excised protein bands were analysed by MALDI-TOF (Ultraflex II, Bruker) in collaboration with Stefan Baumeister, Protein Analytics Facility, Marburg. Samples that were analysed by immunoblot were lysed in 20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100. For microscopy analysis, 1.5 µM hGal3-Alexa555 was incubated at the apical membrane for 30 min at 4°C. Thereafter, cells were fixed and immunofluorescence detection was performed as described below.

Surface biotinylation

Distribution of membrane proteins at the plasma domains was assessed by surface biotinylation. Therefore, filter grown cells were incubated with Sulfo-NHS-Biotin (21217, Thermo Fisher Scientific) to label surface proteins. To specifically label apical or basolateral membrane proteins, biotin label was applied to the apical or basolateral chamber, respectively. After several washing steps with PBS^{++}/0.1 M glycine and PBS^{++} the cells were lysed in 20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100. For uptake experiments, cells were transferred to 37°C before lysis. Cells labelled with reducible Sulfo-NHS-SS-Biotin (21331, Thermo Fisher Scientific) were incubated with reduction buffer to remove biotin from non-internalized biotinylated surface proteins. Together with BSA, the cell lysates were incubated with neutravidin beads (Thermo Scientific, cat no. 29200) to select the biotinylated surface proteins. After repeated washing steps, proteins were eluted with Laemmli buffer, separated by SDS-PAGE and analysed by western blotting.

Internalisation assays

To assess uptake of surface proteins labelled with the reducible NHS-SS-biotin conjugate, cells were transferred to 37°C before lysis and then incubated at 37°C for 30 min in the presence or absence of 50 mM lactose, 25 mM LacNAc or 1.5 µM hGal3. Subsequently, cells were incubated with reduction buffer (25 mM reduced L-glutathione, 90 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 60 mM NaOH, 10% FCS) to remove biotin from non-internalized biotinylated surface proteins. Cell lysates were incubated with 0.2% (w/v) BSA and neutravidin beads to isolate biotinylated and internalized surface proteins. After extensive washing, the proteins were eluted with Laemmli buffer, separated by SDS-PAGE and analysed by western blot analysis.

For endocytosis experiments with recombinant, Alexa-labelled hGal3 and cTf, surface binding to the apical or basolateral membrane domain of filter-grown polarized MDCK cells was performed at 4°C for 30 min. Cells were incubated at 37°C for 30 min to allow internalization. 10 min after induction of endocytosis, cells were washed with 150 mM lactose or 0.2% acetic acid to remove proteins remaining at the cell surface. Subsequently, cells were fixed and analysed by confocal microscopy.

For the endocytosis of recombinant hGal3 from the apical and basolateral membrane, cells were kept on ice and 5 µM recombinant hGal3 was added to filter-grown MDCK cells to the apical or basolateral domains, respectively, then endocytosis was allowed at 37°C. 20 µl from the cell lysates and 30 µl samples from the apical or basolateral medium were then analysed by immunoblotting.

Co-internalization of recombinant hGal3-biotin and β1-integrin, was analysed by adding biotinylated hGal3 to the apical chamber of polarized MDCK cells and incubation at 10 min at 37°C (Ctrl). After removal of non-internalized lectin by washing with 250 mM lactose and PBS^{++}, cells were incubated at 37°C for the indicated periods. Thereafter, hGal3-biotin was isolated with neutravidin beads and associated β1-integrin was analysed by immunoblotting.

Biosynthetic labelling of MDCK cells

For transport studies, MDCK cells were incubated in methionine-free culture medium for 1 h prior to pulse labelling. Cells were pulse-labelled for 30 min by addition of 60 µCi [^{35}S]methionine to the basolateral chamber. Thereafter, cells were chased in culture medium containing methionine for 1-6 h and overnight. Apical and basolateral membrane domains were isolated by neutravidin pulldown, β1-integrin was precipitated using polyclonal anti-CD29 antibody (GTX128839, GeneTex, supplier xxx).

Transcytosis assays

For transcytosis experiments, basolateral membranes of $MDCK_{\Delta Gal3}$ cells were labelled with Sulfo-NHS-SS-biotin. After washing with PBS^{++} or 0.1 M glycine and PBS^{++}, internalization of basolaterally labelled surface proteins was allowed for 15 min at 37°C. Biotin label of non-internalized surface proteins was removed by incubation in reduction buffer for 45 min at 4°C. To block free SH groups, cells were incubated with 5 mg/ml iodoacetamide in PBS^{++} for 10 min. If indicated, 1.5 µM hGal3 was added to the cells and allowed to bind to the apical membrane for 15 min at 4°C. Cells were then transferred to 37°C for the indicated periods of time. Subsequently, only the basolateral or both membrane domains were incubated with reduction buffer to remove biotinylated surface proteins with basolateral origin. For fluorescence microscopy analysis, cells were fixed, permeabilized and immunostained with Alexa Fluor 647 (red)-conjugated anti-ZO1 monoclonal antibody (Zymed, cat no 40-2300). Biotinylated polypeptides were visualized by streptavidin-546 staining.

GPMV isolation and treatment

GPMVs were prepared from confluent MDCK cells as previously described (von Mach et al., 2014) and treated or not treated with 50 mM MβCD or 0.01 mg/ml proteinase K for 30 min at room temperature. Following addition of 0.4 µM Gal3-Alexa647, binding of this fluorescently tagged lectin was assessed by flow cytometry on a BD FACS Canto II (Becton Dickinson); 10,000 events were recorded for each condition.

Immunofluorescence and fluorescence microscopy

For immunofluorescence analysis, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 20 min and blocked in 5% goat serum/PBS^{++} for 1 h. Primary antibodies were incubated in goat serum/PBS^{++} overnight and secondary antibodies

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conjugated to the indicated Alexa Fluor dyes were applied in PBS^{++} for 1 h. Nuclei were stained using Hoechst 33342. Surface binding of hGal3-Alexa555 was performed at 4°C for 30 min prior to fixation of the cells. Mouse kidney samples were fixed with Carnoy (60% ethanol, 30% chloroform, 10% acetic acid) and embedded in paraffin. Paraffin sections (4 µm) were steamed for 20 min in 10 mM Tris-HCl, pH 9.0/1 mM EDTA. Antibodies were incubated in antibody diluent (Dako). Confocal images were acquired on a Leica TCS SP2 AOBS microscope using a 40× or 63× oil planapochromat objective (Leica Microsystems). Data evaluation was performed with the Leica software in combination with the Volocity imaging software package (Improvision).

Quantitative RT-PCR

For real-time (RT) PCR analysis 300,000 MDCK, $MDCK_{\Delta Gal3}$ and $MDCK_{Gal3\text{-}GFP}$ cells were seeded in triplets in 6-well plates and cultured for 5 days. RNA was isolated using the RNeasy Mini Kit (Ref 74104) from Qiagen and transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis Kit (K1621) obtained from Thermo Scientific. 200 nM of the following primers were used for RT-PCR analysis: β1-integrin forward 5′-GCGTTGCTGCTGATTTGGAA-3′, β1-integrin reverse 5′-ATTTTC-ACCCGTGTCCCATT-3′ and GAPDH forward 5′-GATTGTCAGCAAT-GCCTCCT-3′, GAPDH reverse 5′-GGTCATGGATGACTTTGGCTA-3′. The RT-PCR reaction was performed on an Mx3005P quantitative PCR system (Stratagene). PCR amplification conditions were as follows: 95°C for 15 min; 45 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s. Data evaluation was calculated using MxPro software (QPCR software, Agilent). β1-integrin gene expression was normalized to actin and then normalized to MDCK control cells with the ΔΔct method (Livak and Schmittgen, 2001).

Quantification

Band densities of western blots were measured using LabImage 1D software (INTAS). For quantitative analysis, density values were normalized as reported here and/or described in the figure legends. For secretion assays, cells were seeded on one PET filter per experiment. Apical and basolateral media were collected from the same filter. Amounts of apically and basolaterally secreted proteins were then normalized to the level of the protein in the corresponding lysate.

In experiments analysing the distribution of proteins at the apical and basolateral plasma domains, a set of two PET filters per experiment was needed, as the two membrane domains were labelled separately. Here, the amounts of apical and basolateral membrane proteins or membrane-bound proteins were normalized to either the same protein or a control protein in the associated lysate. Amounts of the analysed protein from both domains were set to 100% to calculate the surface distribution of the protein. To assess the relative amount of surface β1-integrin at the two membrane domains in different cell lines or treatments, the value of basolateral β1-integrin in the control condition was set to 1.

For β1-integrin protein expression experiments, the protein content of the lysates was measured using a Bio-Rad DC Protein Assay Kit. Equal amounts (20 µg) of lysates were resolved by SDS-PAGE. Values of band densities were not normalized. Level of $MDCK_{wt}$ β1-integrin or galectin-3 expression was set to 1.

The intensity of β1-integrin-positive fluorescence was measured from a minimum of 15 images in three experiments using ImageJ. Puncta positive for β1-integrin were counted from a minimum of 33 cells in three experiments using Volocity.

In endocytosis experiments (Fig. 5), values of neutravidin-retrieved β1-integrin bands were normalized to β1-integrin or tubulin bands from the associated lysates. 0 min values were subtracted and levels of endocytosed β1-integrin of control treated cells was set to 1.

In transport studies, kinetics of the delivery to the apical and basolateral domain were analysed separately. Band densities were normalized to the maximum value, which was set to 1. Then, 0 h background levels were subtracted from the data sets.

For transcytosis experiments (Fig. 7), neutravidin-retrieved (NA) β1-integrin levels from each point of time and condition were normalized to tubulin levels in the corresponding lysate. Levels at 0 min were set to 1.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
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