

RESEARCH ARTICLE

Basolateral secretion of Wnt5a in polarized epithelial cells is required for apical lumen formation

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ABSTRACT

Wnt5a regulates planar cell polarity in epithelial cells, but it remains to be determined whether Wnt5a and its receptors are sorted apically or basolaterally, and how Wnt5a signaling is involved in apical and basolateral polarization. We found that Wnt5a was secreted basolaterally in polarized kidney epithelial cells. The basolateral secretion of Wnt5a required Wntless (Wls), clathrin and adaptor protein 1 (AP-1). Wnt5a receptors were also localized to the basolateral membranes, but their sorting did not require Wls. Wnt5a-induced signaling was stimulated more efficiently at the basolateral side than the apical side of epithelial cells. Knockdown of Wnt5a delayed apical lumen formation of the epithelial cyst, and these phenotypes were rescued by wild-type Wnt5a, but not by a Wnt5a mutant that is secreted apically. Although apoptosis was not required for apical lumen formation in a wild-type cyst, apoptosis was necessary for eliminating luminal cells in a Wnt5a-depleted cyst. These results suggest that Wnt5a and its receptors are sorted to their correct destination by different mechanisms and that the basolateral secretion of Wnt5a is necessary for apical lumen formation in the epithelial cyst.

KEY WORDS: Epithelial cells, Glycosylation, Polarity, Lumen, Wnt5a

INTRODUCTION

The generation and maintenance of epithelial cell polarity requires the correct delivery of newly synthesized and endocytosed proteins to apical or basolateral membrane domains (Yeaman et al., 1999). To establish apical-basolateral polarity, cells must sense their environment, which can be mediated by the interaction of cells with the extracellular matrix, including basement membrane proteins. Cells also recognize soluble factors, such as morphogens and growth factors, and activate signaling pathways to support the orientation of their polarity (Bryant and Mostov, 2008). As an example of a soluble factor, Wnt5a promotes the induction of single-cell polarization in rat intestinal epithelial cells (IEC6 cells) (Gon et al., 2013). Wnt5a is a member of the Wnt family, which is a large family of secreted molecules that play an important role in developmental

processes; defective Wnt signaling in postnatal life causes disease in humans (Kikuchi et al., 2011; Logan and Nusse, 2004; Polakis, 2007). Our previous study showed that when IEC6 cells were seeded on thick Matrigel containing basement membrane proteins, a single cell was polarized cell autonomously, with an F-actin cap forming on the opposite side to Matrigel; knockdown of Wnt5a inhibited this phenotype (Gon et al., 2013). In cancer cells, Frizzled-2 (Fz2), a Wnt5a receptor, forms a complex with integrin $\alpha 2$, and Dvl, a cytoplasmic protein that mediates Wnt5a signaling, interacts with focal adhesion kinase (FAK), thereby enhancing cell-to-substrate adhesion (Matsumoto et al., 2010). Thus, Wnt5a and its receptors appear to sort to the basolateral membranes of polarized epithelial cells and might be required for the formation of apical-basolateral polarity.

Wnt proteins are post-translationally modified with oligosaccharides and lipids during their synthesis (Harterink and Korswagen, 2012; Komekado et al., 2007; Kurayoshi et al., 2007; Takada et al., 2006; Willert et al., 2003). Our previous study also revealed that the majority of Wnt3a and Wnt11 are secreted basolaterally and apically, respectively, in polarized Madin-Darby canine kidney (MDCK) epithelial cells (Yamamoto et al., 2013). The basolateral traffic of Wnt3a requires adaptor protein 1 (AP-1) and clathrin, which are known to be important for the sorting of basolateral membrane proteins (Boehm and Bonifacino, 2001; Deborde et al., 2008; Fölsch, 2005). It is generally thought that glycosylation of secretory proteins is necessary for their folding in the endoplasmic reticulum (ER) or exit from the ER (Spiro, 2002). Wnt3a and Wnt11 were glycosylated at multiple Asn residues, and their glycosylation profiles differed (Yamamoto et al., 2013). The complex-type glycan attached to Wnt11 at the N-terminal region is unique, and glycosylation is necessary for the apical secretion of Wnt11.

In addition, Wnt3a and Wnt11 are modified with palmitoleic acid at a conserved Ser residue (Takada et al., 2006; Yamamoto et al., 2013). The lipidation of Wnt3a and Wnt11 occurs in the ER, is catalyzed by porcupine and is essential for their exit from the ER. Wntless (Wls), which plays a crucial role in the trafficking of Wnt, recognizes palmitoleic-acid-modified Wnts in the ER and cycles between the plasma membranes and the ER through the Golgi (Port and Basler, 2010; Yu et al., 2014). Wls itself is trafficked to the basolateral membranes and its recycling is not required for the apical sorting of Wnt11 from the trans-Golgi (Yamamoto et al., 2013). Crystal structural analyses have revealed that the lipid modification of *Xenopus* Wnt8 inserts into the groove of the extracellular domain of mouse Fz8, suggesting the necessity of lipid modification of Wnts for the recognition and activation of receptors (Janda et al., 2012). Thus, post-translational modifications have important roles in the secretion and action of Wnts, and determination of Wnt5a modifications

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using mass spectrometry is necessary for the understanding of Wnt5a signaling.

Low-density lipoprotein receptor-related protein 6 (LRP6), receptor tyrosine kinase-like orphan receptor 2 (Ror2) and receptor-like tyrosine kinase (Ryk) function as co-receptors for Wnt3a, Wnt5a and Wnt11, respectively (Kim et al., 2008; Nishita et al., 2010; Yamamoto et al., 2006), although their cross-reactivity with other Wnts has also been reported (Lu et al., 2004; Yoshikawa et al., 2003). To transmit signals efficiently, these receptors need to be sorted to the same location as their specific Wnts. However, the mechanism underlying how they are sorted to their specific destinations is largely unknown. To elucidate the common and unique mechanisms in the polarized secretion of Wnt family members, we examined the polarized sorting of Wnt5a and its receptors. Here, we show the basolateral trafficking mechanisms of Wnt5a and its receptors. Furthermore, we examine the roles of Wnt5a polarized secretion in apical lumen formation of the epithelial cyst, and we demonstrate that basolaterally but not apically secreted Wnt5a promotes cell-to-substrate adhesion and activates Rac, thereby enhancing apical lumen formation without apoptosis.

RESULTS

Polarized secretion of Wnt5a

MDCK cells are commonly used in apical and basolateral polarization experiments with two-dimensional (2D) and 3D cultures. There are two types of MDCK cells: MDCK type I (MDCK I) and MDCK type II (MDCK II). The difference between the two types of MDCK cells is based on low (MDCK I) and high (MDCK II) passages of parent cell lines (Dukes et al., 2011). In addition, MDCK I cells have higher trans-epithelial electric resistance than MDCK II cells because they lack claudin-2 expression (Furuse et al., 2001). In MDCK I cells, *Wnt4*, *Wnt5a* and *Wnt7a* mRNAs were expressed at higher levels than *Wnt1*, *Wnt3*, *Wnt5b* and *Wnt11* mRNAs, whereas *Wnt5a* mRNA expression was lower than *Wnt4* and *Wnt7a* mRNA expression in MDCK II cells (supplementary material Fig. S1A). Western blot analysis revealed that endogenous Wnt5a was detected in MDCK I cells, but not in MDCK II cells (supplementary material Fig. S1B). When MDCK cells were cultured as a monolayer on a filter support (2D culture) (Gottardi et al., 1995), podocalyxin (an apical membrane marker) and transferrin receptor (TfR, a basolateral membrane receptor) were clearly sorted to apical and basolateral membranes, respectively (Fig. 1A). Under these conditions, the majority of endogenous Wnt5a in MDCK I cells was secreted basolaterally (Fig. 1A). To show the trafficking of Wnt5a more clearly, Wnt5a was stably expressed in MDCK I and MDCK II cells (MDCK I/Wnt5a and MDCK II/Wnt5a; supplementary material Fig. S1C). Exogenously expressed Wnt5a was secreted basolaterally in both cell lines (Fig. 1B).

These findings were confirmed using MDCK II cysts in 3D Matrigel culture. When Wnt5a was transiently expressed in MDCK II cells and the cells were permeabilized, Wnt5a was detected in the cytoplasm but not in the apical lumens, whereas when the cells were stained without permeabilization, Wnt5a was detected on the basal membranes (Fig. 1C). In MDCK II/Wnt5a cells with permeabilization, Wnt5a was observed in the immediate vicinity of basal membranes but not in the apical lumen (Fig. 1C). In subsequent experiments, we overexpressed Wnt5a and Wnt receptors in MDCK II cells to examine its trafficking mechanisms, and knocked down endogenous Wnt5a in MDCK I cells to examine its function.

Wls is essential for the secretion of Wnts, and is trafficked to the basolateral membranes by AP-1 and clathrin from the trans-Golgi network (Port and Basler, 2010; Yamamoto et al., 2013). Consistent with these observations, knockdown of Wls completely suppressed Wnt5a secretion in polarized MDCK II/Wnt5a cells, although the polarized distribution of podocalyxin and TfR was not impaired (Fig. 1D; supplementary material Fig. S1D). Clathrin and AP-1A, AP-1B and AP-4 sort cargo proteins to the basolateral membranes in epithelial cells (Boehm and Bonifacino, 2001; Deborde et al., 2008; Fölsch, 2005; Gravotta et al., 2012; Simmen et al., 2002). Knockdown of clathrin or μ 1A and μ 1B (also known as AP1M1 and AP1M2, respectively), which are medium subunits of AP-1A and AP-1B, respectively, disturbed the polarized sorting of TfR but not that of podocalyxin (Fig. 1E; supplementary material Fig. S1D,E). Under these conditions, the basolateral secretion of Wnt5a was severely suppressed (Fig. 1E). However, the depletion of μ 4 (also known as AP4M1; a medium subunit of AP-4) did not affect the polarized distribution of Wnt5a, podocalyxin and TfR (Fig. 1F; supplementary material Fig. S1D). μ 1B-HA expression rescued the phenotypes observed in μ 1A- and μ 1B-depleted cells (Fig. 1G), excluding small interfering (si)RNA off-target effects. These results suggest that Wls, clathrin and AP-1 are important for the sorting of Wnt5a to the basolateral membrane.

Post-translational modification of Wnt5a

Because post-translational modifications of secretory proteins are important for their trafficking, we determined the glycan profiles of Wnt5a. Wnt5a has four possible *N*-glycosylation sites; namely, Asn-X-Ser/Thr, including N114, N120, N312 and N326 (Kurayoshi et al., 2007). Tryptic peptides of purified Wnt5a were subjected to nano-flow liquid chromatography followed by matrix-associated laser desorption/ionization mass spectrometry (MALDI-MS). About 74.3% of the entire Wnt5a amino acid sequence was covered; N114, N312 and N326 were glycosylated with high-mannose, hybrid and high-mannose-type oligosaccharides, respectively, but N120 was not glycosylated (supplementary material Table S1). Further analyses of tryptic peptides using nano-flow liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) confirmed that glycans were attached to N114, N312 and N326 of Wnt5a (Fig. 2; supplementary material Table S2).

When all glycosylation sites were mutated, Wnt5a was never secreted (Kurayoshi et al., 2007). To investigate the role of each glycan of Wnt5a in secretion, various combinations of mutations were added to the Wnt5a glycosylation sites (supplementary material Fig. S2A). Wnt5aN114Q/N120Q (N120 is glycosylated when N114 is mutated), Wnt5aN312Q, Wnt5aN326Q or Wnt5aN312Q/N326Q showed similar secretion levels to wild-type Wnt5a. By contrast, Wnt5aN114Q/N120Q/N312Q or Wnt5aN114Q/N120Q/N326Q completely impaired secretion. These results show that glycosylation at N114 alone, but not glycosylation at N312 or N326 alone, is sufficient for Wnt5a secretion, thereby suggesting that glycosylation at N114 is important for maintaining the overall folded structure of Wnt5a.

A lipid-modified peptide was also observed among the peptides identified by MALDI-tandem mass spectrometry (MALDI-MS/MS) (supplementary material Table S1). The peptide containing S244, observed at *m/z* 1455.9 (supplementary material Fig. S2B), was identified as the peptide with the monounsaturated fatty acid (C16:1), palmitoleic acid. The site of lipidation was clearly

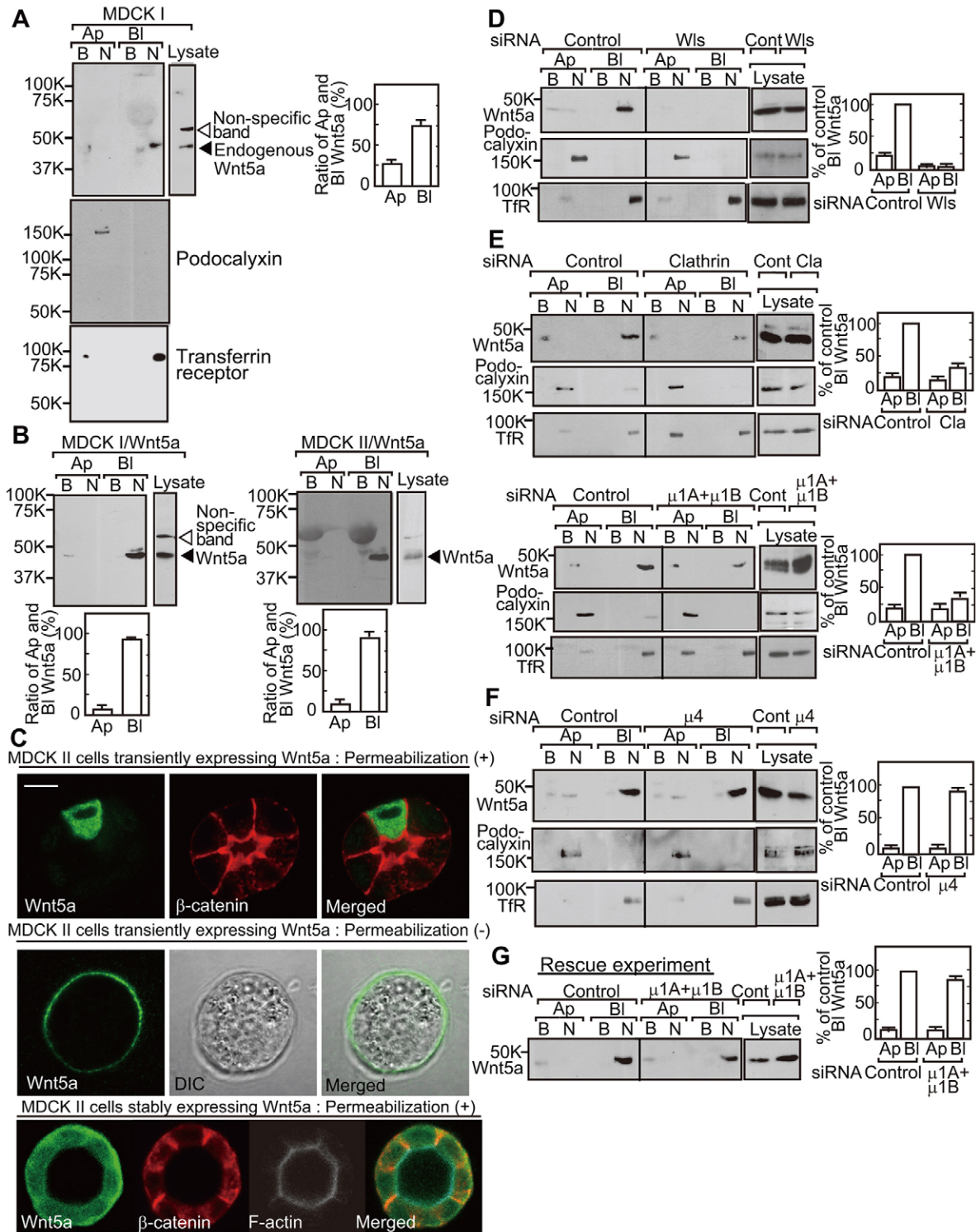


Fig. 1. See next page for legend.

identified as S244 by MALDI-MS/MS (supplementary material Fig. S2C), indicating that Wnt5a is modified with palmitoleic acid as are Wnt3a and Wnt11 (Takada et al., 2006; Yamamoto et al., 2013). In addition, Wnt5aS244A failed to be secreted (supplementary material Fig. S2D), confirming that the modification with palmitoleic acid is essential for Wnt secretion.

Addition of glycans to the N-terminal region of Wnt5a induces its apical secretion

We have previously shown that the complex-type glycan attached to N40 of Wnt11 is essential for its apical secretion, and that galectin-3, a galactose-binding lectin, is required for the apical secretion of Wnt11 (Yamamoto et al., 2013). Given that Wnt5a lacks a glycosylation site in the N-terminal region, Leu68 was

Fig. 1. Polarized secretion of Wnt5a. (A) MDCK I cells seeded on a Transwell polycarbonate filter were subjected to the apical-basolateral sorting assay. Soluble Wnt5a was detected in the precipitates using Blue–Sephadex from the apical media, and membrane-associated Wnt5a was observed in the precipitates using Neutravidin–agarose from the basolateral membranes. Wnt5a signals were quantified using NIH Imaging and the results were expressed as the ratio of Wnts between apical (Ap) and basolateral (Bl) fractions. Podocalyxin and transferrin receptor (TfR) were used as apical and basolateral membrane protein markers, respectively. Results are shown as means \pm s.e.m. from four independent experiments. B, precipitation using Blue–Sephadex; N, precipitation using Neutravidin–agarose. (B) MDCK I/Wnt5a and MDCK II/Wnt5a cells were subjected to the apical-basolateral sorting assay. (C) MDCK II cells transiently or stably expressing Wnt5a were cultured in 3D Matrigel, and the cysts with (+) or without (–) permeabilization were stained with the indicated antibodies or Alexa-Fluor-633-conjugated phalloidin to visualize F-actin, or observed by relief-contrast microscopy (DIC). F-actin and β -catenin were used as apical and basolateral markers, respectively. Scale bar: 10 μ m. (D) MDCK II/Wnt5a cells were transfected with control (cont) or Wls siRNA, and the cells were then subjected to the apical-basolateral sorting assay. The Wnt5a signal was precipitated using Neutravidin–agarose in the control basolateral fraction, which was set to 100%. (E,F) MDCK II/Wnt5a cells were transfected with siRNAs for clathrin (Cla) (E), μ 1A and μ 1B (E) or μ 4 (F), and the cells were subjected to the apical-basolateral sorting assay. (G) MDCK II/Wnt5a cells stably expressing μ 1B–HA were transfected with siRNAs for μ 1A and μ 1B, and the cells were then subjected to the apical-basolateral sorting assay.

mutated to Asn to generate the amino acid sequence N68-X-S70, which is a possible glycosylation site (Fig. 3A). Wild-type Wnt5a secreted from MDCK II/Wnt5a cells showed different migration

patterns by SDS-PAGE upon treatment with peptide: N-glycosidase F (PNGase F), which cleaves N-linked glycans, and upon treatment with endoglycosidase H_f (Endo H_f), which cleaves high-mannose and hybrid-type glycans, but not the complex-type glycan (Fig. 3B). This indicates that Wnt5a is heterogeneously glycosylated, which is consistent with the results of mass spectrometric analyses. Wnt5aL68N secreted from MDCK II/Wnt5aL68N cells (supplementary material Fig. S1G) exhibited lower mobility by SDS-PAGE than wild-type Wnt5a owing to the addition of glycan, and the amount of Endo H_f-resistant glycan increased (Fig. 3B), indicating that the complex type was attached to Wnt5aL68N. In contrast to wild-type Wnt5a, Wnt5aL68N was secreted apically in polarized MDCK II/Wnt5aL68N cells (Fig. 3B). Basally secreted Wnt5a was associated with membranes (see ‘N’ in Fig. 3B), whereas apically secreted Wnt5a was present in the medium (see ‘B’ in Fig. 3B), suggesting that Wnt5a might interact with the basement membranes. Wnt5aL68N in the conditioned medium induced a mobility shift of Dvl2 in NIH3T3 cells at a similar efficiency to that of control Wnt5a conditioned medium (Fig. 3C), suggesting that Wnt5aL68N is active.

Kifunensine and deoxymannojirimycin (dMM) inhibit ER- α -mannosidase I and Golgi- α -mannosidase I, respectively (Elbein et al., 1990; Fuhrmann et al., 1984). Gnt-I (also known as MGAT1), which is one of six N-acetylglucosamine transferases in vertebrates, initiates the formation of hybrid- and/or complex-type glycan (Rademacher et al., 1988). Treatment of MDCK II/Wnt5aL68N cells with kifunensine and dMM changed the

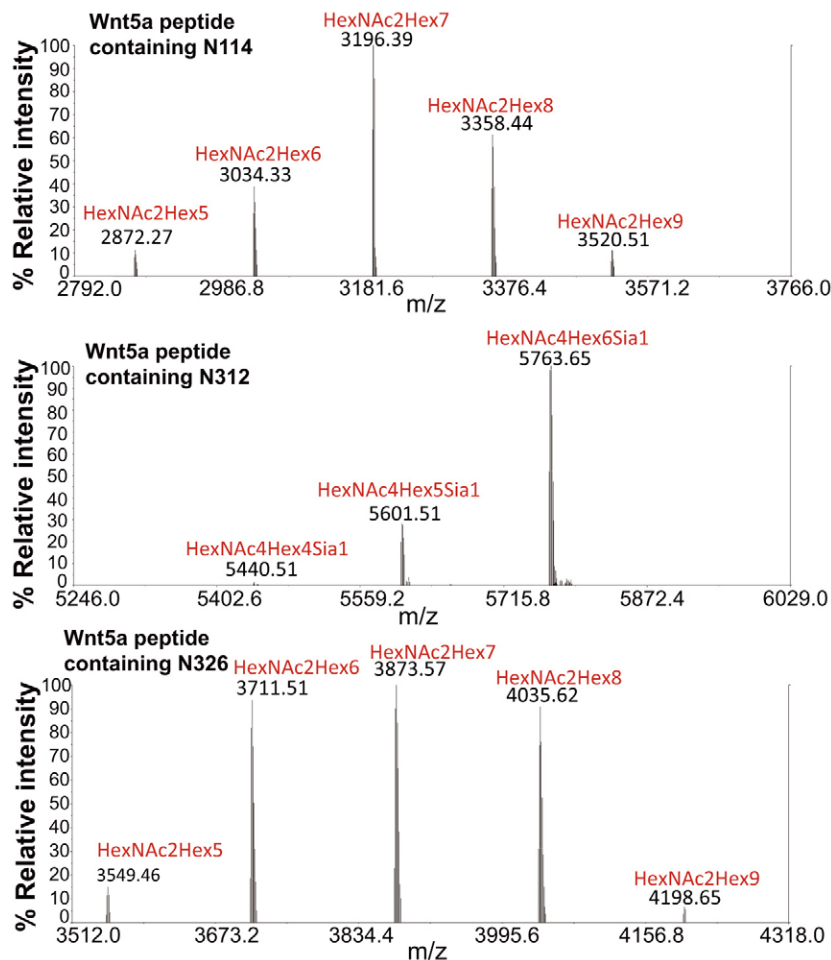


Fig. 2. Determination of the glycan profiles of Wnt5a. Wnt5a glycopeptides that eluted after 32.1 (N114), 36.5 (N312) and 28.6 min (N326) from nano-flow liquid chromatography were analyzed by ESI-MS. The mass spectra were deconvoluted to singly charged ions.

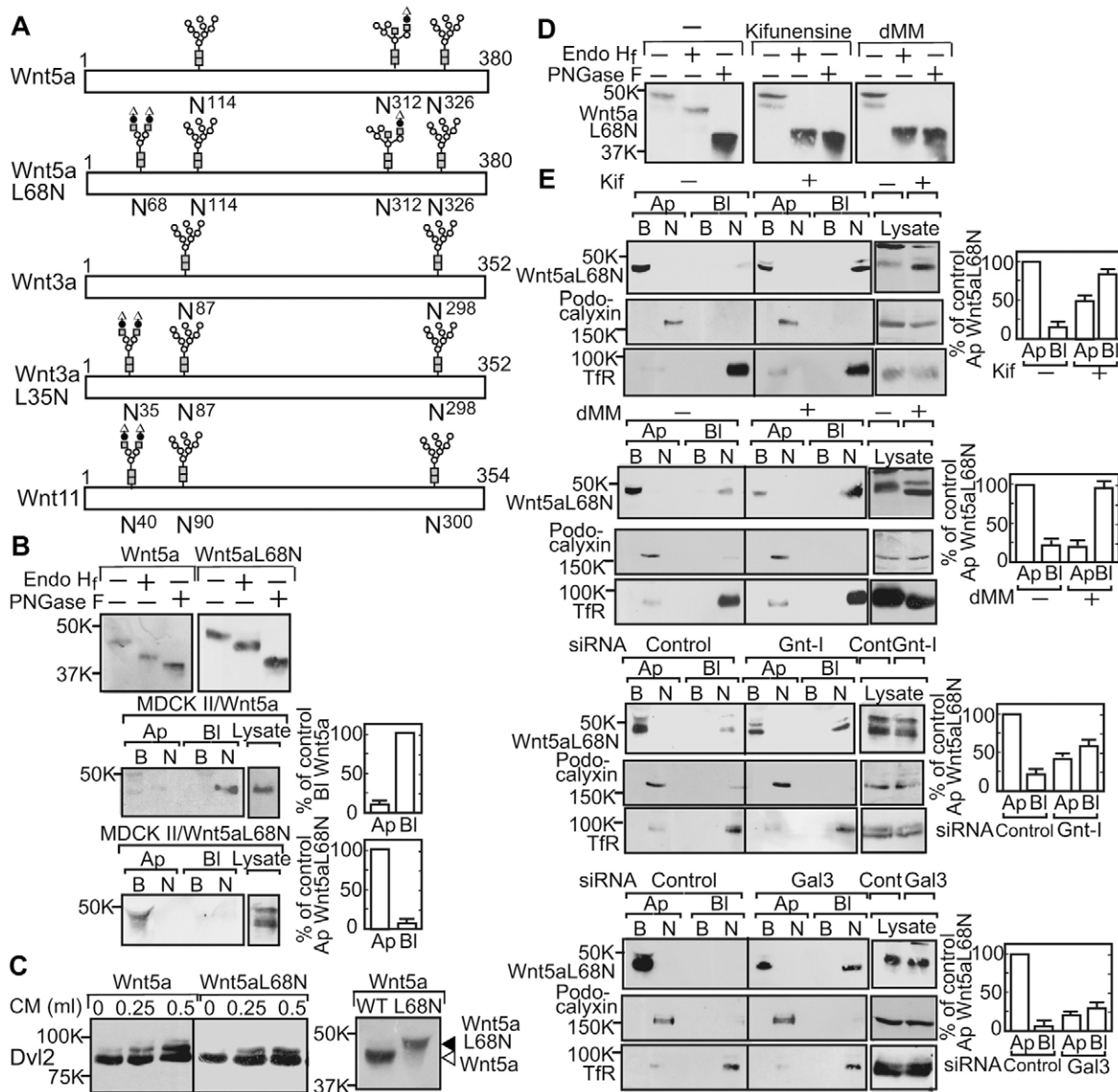


Fig. 3. Addition of glycans to the N-terminal region of Wnt5a induces its apical secretion. (A) Glycan profiles of Wnt5a, Wnt5aL68N, Wnt3a, Wnt3aL35N and Wnt11 are shown. (B) Wnt5a or Wnt5aL68N in conditioned medium from MDCK II cells was precipitated using Blue–Sephacrose, and the precipitates were treated with Endo H_r or PNGase F, and then probed with anti-Wnt5a antibody (upper panels). MDCK II/Wnt5a or MDCK II/Wnt5aL68N cells were subjected to the apical-basolateral sorting assay (middle and lower panels). The signal of Wnt5a was precipitated using Neutravidin–agarose in the control basolateral (Bl) fraction and the signal of Wnt5aL68N was precipitated using Blue–Sephacrose in the control apical (Ap) fraction was set to 100%. B, precipitation using Blue–Sephacrose; N, precipitation using Neutravidin–agarose. (C) NIH3T3 cells were stimulated with the indicated amounts of Wnt5a- or Wnt5aL68N-containing conditioned medium (CM) from L cells for 2 h, and lysates were then probed with anti-Dvl2 antibody (left two panels). Conditioned media (20 μl) containing Wnt5a or Wnt5aL68N are shown in the right panel. (D) MDCK II/Wnt5aL68N cells were treated with kifunensine (kif) or deoxymannojirimycin (dMM), and Wnt5aL68N in conditioned medium was then precipitated using Blue–Sephacrose. The precipitates were treated with Endo H_r or PNGase F and then probed with anti-Wnt5a antibody. (E) MDCK II/Wnt5aL68N cells were treated with kif or dMM, or transfected with Gnt-I or galectin 3 (Gal3) siRNA, and the cells were subjected to the apical-basolateral sorting assay. cont, control. Results are shown as means ± s.e.m. from four independent experiments.

sensitivity of Wnt5aL68N to Endo H_r (Fig. 3D) and inhibited its apical secretion (Fig. 3E). siRNAs for Gnt-I and galectin-3 inhibited the apical secretion of Wnt5aL68N (Fig. 3E; supplementary material Fig. S1F). By contrast, these manipulations did not affect the polarized distribution of podocalyxin and Tfr (Fig. 3E). Therefore, prevention of the maturation of the hybrid or complex-type glycan from the high-mannose-type glycan on Wnt5aL68N by these manipulations would impair its apical secretion.

Similar to Wnt5a, Leu35 was mutated to Asn in Wnt3a (Wnt3aL35N), and Wnt3aL35N was additionally modified with

the complex-type glycan and secreted apically (Fig. 4A; supplementary material Fig. S1H). Treatment with kifunensine, dMM and siRNAs for Gnt-I and galectin-3 inhibited the apical secretion of Wnt3aL35N (Fig. 4B). Wnt3aL35N induced the accumulation of β-catenin to a similar degree as wild-type Wnt3a (Fig. 4C). These results suggest that the addition of complex-type glycan to the N-terminal region is sufficient for Wnt5a and Wnt3a to be secreted apically without losing their activities. Therefore, one amino acid substitution at the appropriate region of Wnts might be sufficient for Wnts to change their sorting destination.

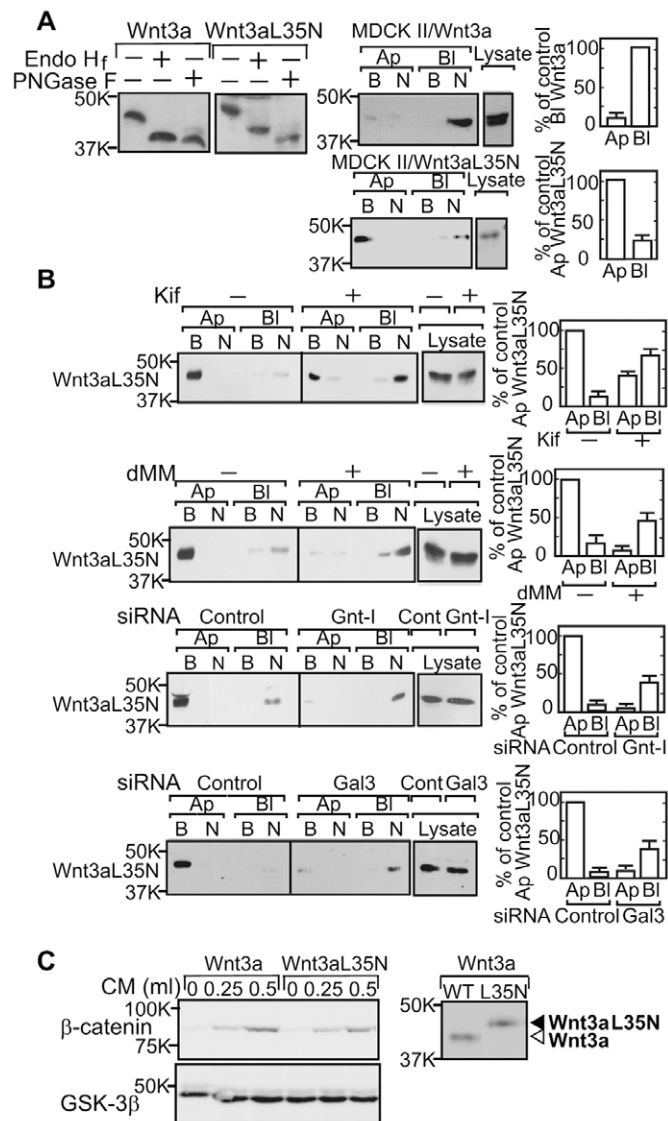


Fig. 4. Addition of the glycosylation site at the N-terminal region is sufficient to change the polarized secretion of Wnt3a. (A) Wnt3a or Wnt3aL35N in conditioned medium from MDCK II cells was precipitated using Blue–Sepharose, and the precipitates were treated with Endo H_f or PNGase F and then probed with anti-Wnt3a antibody (left panels). MDCK II cells expressing Wnt3a or Wnt3aL35N were subjected to the apical-basolateral sorting assay (right panels). The signal of Wnt3a precipitated using Neutravidin–agarose in the control basolateral (Bl) fraction or the signal of Wnt3aL35N precipitated using Blue–Sepharose in the control apical (Ap) fraction was set to 100%. (B) MDCK II/Wnt3aL35N cells were treated with kif or dMM, or transfected with Gnt-I or galectin3 (Gal3) siRNA, and the cells were then subjected to the apical-basolateral sorting assay. Results are shown as means ± s.e.m. from four independent experiments. (C) L cells were stimulated with the indicated amounts of wild-type Wnt3a- or Wnt3aL35N-containing conditioned medium (CM) for 2 h, and lysates were then probed with anti-β-catenin and anti-GSK-3β antibodies (left two panels). Wild-type Wnt3a and Wnt3aL35N in conditioned medium (20 μl) are shown in the right panel. GSK-3β was used as a loading control.

Polarized localization of Wnt receptors

There are ten Frizzled (Fz) family members, which function as Wnt receptors (Kikuchi et al., 2011; Schulte and Bryja, 2007). Although the binding specificity between Wnts and Fzs is not

clear, it has been shown that Fz2 mediates Wnt3a and Wnt5a signaling and that Fz7 functions as the Wnt11 receptor (Sato et al., 2010; Uysal-Onganer and Kypta, 2012). To determine the polarized localization of Fzs, MDCK II cells stably expressing FLAG–Fz2 or FLAG–Fz7 were generated (MDCK II/FLAG–Fz2 and MDCK II/FLAG–Fz7, respectively). The majority of FLAG–Fz2 was localized to the basolateral membranes, whereas FLAG–Fz7 was localized to both apical and basolateral membranes evenly (Fig. 5A–C). Knockdown of Wls did not affect the polarized localization of either Fz (Fig. 5B,C; supplementary material Fig. S1D). The polarized localization of FLAG–Fz2 was impaired in clathrin-depleted cells and cells depleted of both μ1A and μ1B (μ1A/μ1B-depleted), but not in μ4-depleted cells (Fig. 5B; supplementary material Fig. S1D,E). These results suggest that Fz2- or Fz7-loaded vesicles destined to the plasma membranes do not contain Wls, and that the basolateral localization of Fz2 requires the clathrin and AP-1 complex.

Single transmembrane receptors, including LRP6, Ror2 and Ryk, function as co-receptors of Wnt3a, Wnt5a and Wnt11, respectively (Kim et al., 2008; Nishita et al., 2010; Yamamoto et al., 2006). Although endogenous LRP6 and exogenously expressed Ror2–GFP were primarily located at the basolateral membranes of MDCK II cells (Fig. 5A,D,E), Ryk–GFP was evenly distributed in both the apical and basolateral membranes (Fig. 5A,F). Knockdown of Wls did not affect the localization of these co-receptors (Fig. 5D–F; supplementary material Fig. S1D). The basolateral membrane localization of LRP6 and Ror2–GFP was disturbed in clathrin- and μ1A/μ1B-depleted cells but not in μ4-depleted cells (Fig. 5D,E; supplementary material Fig. S1D,E).

Consistent with the polarized localization of the receptors, stimulation of polarized MDCK II/FLAG–Fz2 cells with recombinant Wnt5a at the basolateral side activated Rac1 more efficiently than that at the apical side (Fig. 5G). Recombinant Wnt3a induced LRP6 phosphorylation and the mobility shift of Dvl2 at the basolateral side more efficiently than the apical side (Fig. 5G).

Basolateral secretion of Wnt5a is required for apical lumen formation of MDCK cysts

The role of Wnt5a basolateral secretion in epithelial cells was examined by inhibiting endogenous Wnt5a synthesis and secretion in MDCK I cells. When MDCK I cells were cultured in 3D Matrigel, a nascent lumen was observed at day 4. Each cell in the cyst had a free, lateral and basal surface by acquiring polarity, and the cyst increased the lumen diameter at days 6–7, and cystogenesis was complete at days 8–9 (Fig. 6A).

Treatment of MDCK I cells with a Wnt secretion inhibitor IWP2 (Chen et al., 2009), caused a delay in lumen formation, so that a period of 10–11 days was required for formation of the lumen consisting of monolayer cells (Fig. 6A). At day 7, some cells were still observed in the luminal space of cysts treated with IWP2, and they did not make contact with the extracellular matrix (ECM) (Fig. 6A,B). Approximately 70% of cysts consisting of control MDCK I cells showed a dilated lumen, which was lined by a polarized monolayer cells, and of which the diameter was >50% that of the cyst (Fig. 6B). By contrast, treatment with IWP2 caused the number of cysts with dilated lumens to decrease, whereas the number of cysts with narrow lumens, which had multilayered cells and a diameter <50% that of the cyst, increased (Fig. 6B). Similar phenotypes were observed in Wnt5a-depleted cells (Fig. 6C; supplementary material Fig. S3A); however, knockdown of Wnt4 or Wnt7a did not affect apical lumen formation (supplementary material Fig. S3B). As well as

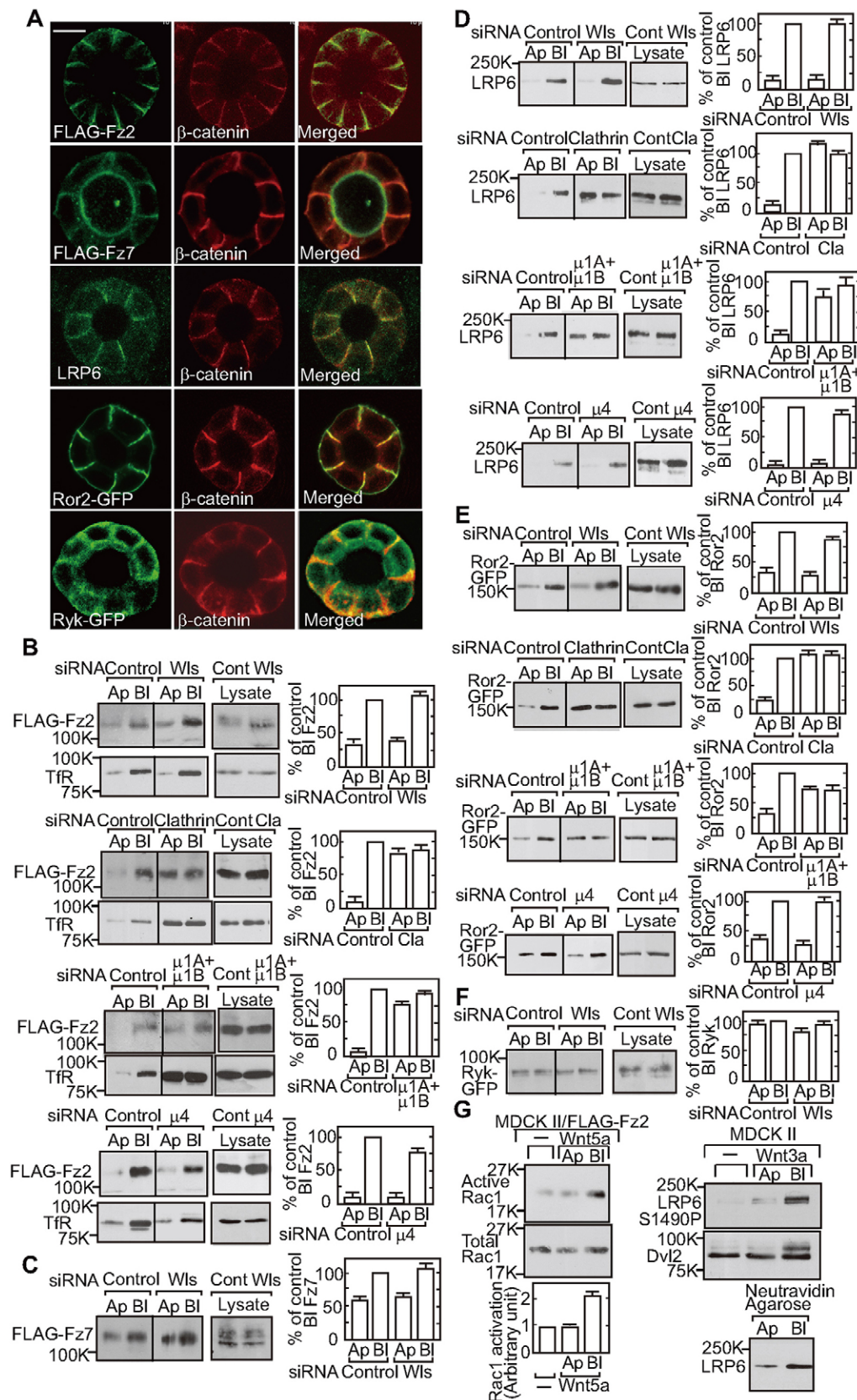


Fig. 5. The polarized localization of Wnt receptors. (A) MDCK II, MDCK II/FLAG-Fz2, MDCK II/FLAG-Fz7, MDCK II/Ror2-GFP or MDCK II/Ryk-GFP cells were cultured in 3D Matrigel, and the cysts were stained with the indicated antibodies. Scale bar: 10 μm. (B–F) MDCK II/FLAG-Fz2 (B), MDCK II/FLAG-Fz7 (C), MDCK II (D), MDCK II/Ror2-GFP (E) or MDCK II/Ryk-GFP (F) cells were transfected with siRNA for Wntless (Wls), clathrin (Cla), μ1A and μ1B, or μ4, and the cells were then subjected to the apical-basolateral sorting assay. The signal of the receptors was precipitated using Neutravidin-agarose from the control basolateral (BI) fraction, which was set to 100%. Ap, apical; cont, control. (G) MDCK II/FLAG-Fz2 cells were stimulated with Wnt5a (50 ng/ml) (left panel) or MDCK II cells were stimulated with Wnt3a (100 ng/ml) (right panel) from the apical or basolateral sides, and lysates were then subjected to the Rac activation assay or the phosphorylation assay of LRP6 and Dvl2. Results are shown as means ± s.e.m. from four independent experiments.

in MDCK II cells, apical secretion of Wnt5aL68N was observed in polarized MDCK I/Wnt5aL68N cells, and basolateral membrane localization of Ror2-GFP was observed in MDCK I/

Ror2-GFP cells (supplementary material Fig. S3C). The phenotypes induced by Wnt5a siRNA were rescued by expression of wild-type Wnt5a, but not by Wnt5aL68N

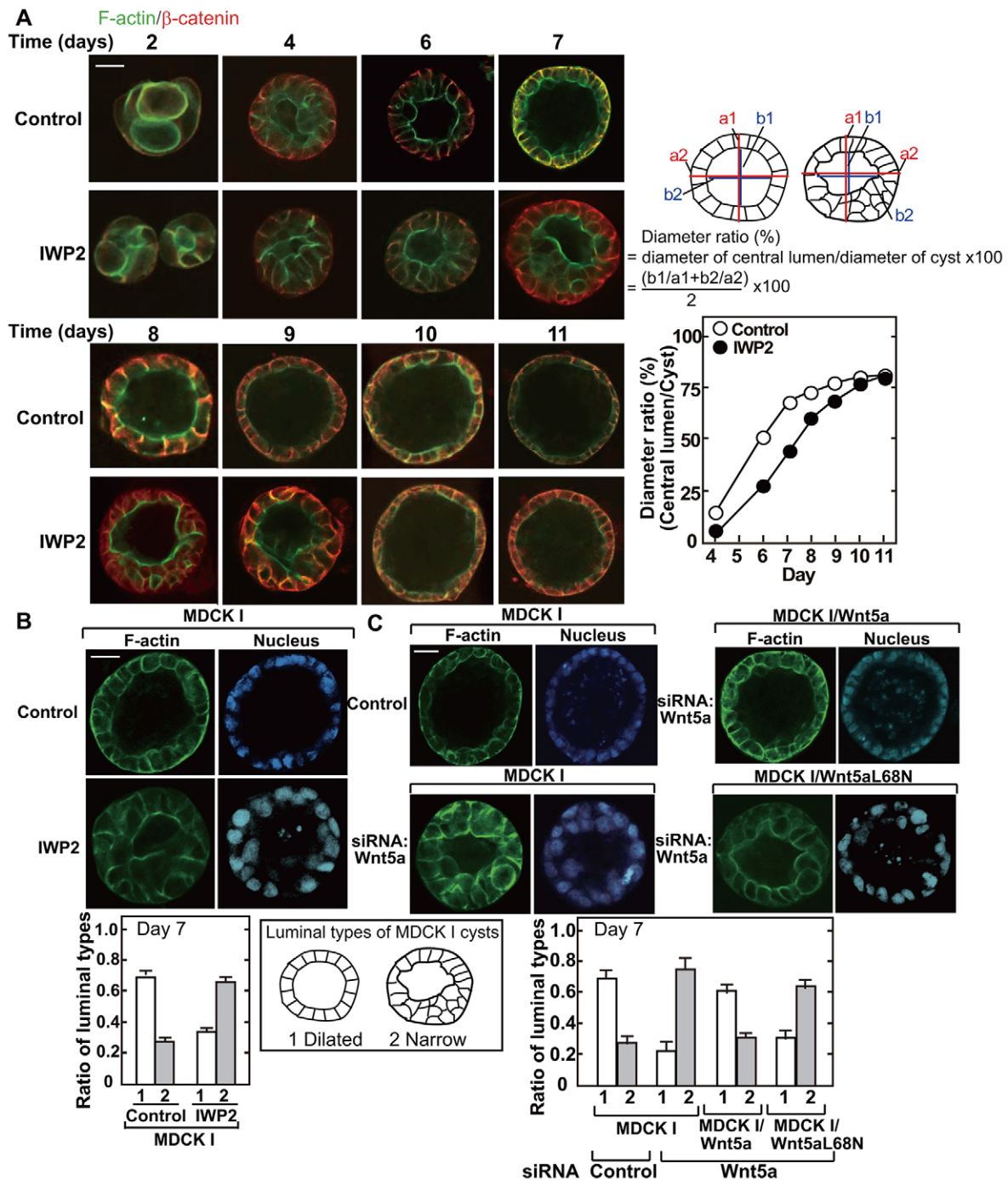


Fig. 6. Basolateral secretion of Wnt5a is required for the apical lumen formation of MDCK cyst. (A) MDCK I cells cultured in 3D Matrigel were treated with or without 5 μ M IWP2 for the indicated days, and the cysts were then stained with anti- β -catenin antibody and Alexa-Fluor-488-conjugated phalloidin to visualize F-actin. The diameter ratio between the apical lumen and the whole cyst was calculated using the following formula: diameter ratio (%) = diameter of central lumen (blue line)/diameter of the cyst (red line) $\times 100$. The mean of the two diameter ratios in the cysts at the indicated days were plotted (right panel). (B) MDCK I cells cultured in 3D Matrigel were treated with or without 5 μ M IWP2 for 7 days, and the cysts were then stained with Alexa-Fluor-488-conjugated phalloidin (green) and DRAQ5 (blue) to visualize the nucleus. Schematic images of MDCK I cyst with a dilated lumen (1) or narrow lumen (2) are shown. The cysts at day 7 were classified by their luminal types, and the number of cysts of each type was counted. Results are expressed as the ratio of luminal types, and are shown as the means \pm s.e.m. from four independent experiments. (C) MDCK I, MDCK I/Wnt5a, or MDCK I/Wnt5aL68N cells were transfected with control or Wnt5a siRNA, and the cysts were then stained with phalloidin and DRAQ5. The cysts at day 7 were classified by their luminal types. Scale bars: 20 μ m.

(Fig. 6C; supplementary material Fig. S3A). These results indicate that Wnt5a signaling from the basolateral side is required for efficient apical lumen formation in MDCK I cells.

Apoptosis is required for apical lumen formation in Wnt5a-depleted cells

Treatment of MDCK I cells with IWP2 or depletion of Wnt5a suppressed the phosphorylation of FAK and paxillin at days 5–7

in 3D Matrigel culture (Fig. 7A), suggesting a reduction in cell-to-substrate adhesion. These manipulations also reduced Rac activity (Fig. 7B). Decreases in FAK activity, paxillin phosphorylation and Rac activity due to Wnt5a depletion were rescued by expression of wild-type Wnt5a, but not by Wnt5aL68N (Fig. 7A,B). When MDCK I cells were treated with NSC23766 (a Rac GEF inhibitor), some cells were observed in the luminal region at days 5–9, resulting in a delay of lumen formation, similar to the treatment with IWP2 and Wnt5a siRNA (Fig. 7C).

Some lumen formation models, including hollowing and cavitation, which require directional vesicle trafficking and luminal cell death, respectively, have been proposed (Datta et al., 2011). In the control cysts, very few luminal cells underwent apoptosis, whereas cysts in which Wnt5a signaling was suppressed had apoptotic cells, as determined by staining with the apoptotic marker, cleaved caspase-3 (Fig. 7C,D). Control MDCK I cysts formed a clear apical lumen with a few cells, even in the presence of the general caspase inhibitor QVD-Oph, but Wnt5a-depleted or IWP2-treated MDCK I cysts showed a decreased formation of apical lumen surrounded by monolayer cells at day 11 in the presence of QVD-Oph (Fig. 7E). Thus, apoptosis might be required for apical lumen formation in Wnt5a signaling-deficient cells. Taken together with the report that knockdown of integrins impairs apical lumen formation (Myllymäki et al., 2011), these results suggest that Wnt5a signaling promotes lumen formation by the enhancement of cell-to-substrate adhesion through integrins.

DISCUSSION

Polarized secretion of Wnt5a

It is believed that the trafficking of some apically sorted proteins is dependent on glycosylphosphatidylinositol-anchor-interacting signals or glycan-mediated signals, whereas basolaterally sorted proteins are delivered by the interaction of cytoplasmic trafficking signals with sorting machineries such as clathrin and APs (Boehm and Bonifacino, 2001; Deborde et al., 2008; Fölsch, 2005; Vagin et al., 2009). In this study, we determined the profiles of glycans attached to Wnt5a by ESI-MS/MS. N114 and N326 of Wnt5a were modified with high-mannose-type glycans. Both Asn residues are conserved in most Wnts, and modification with high-mannose-type glycan was also observed at the conserved Asn residues of Wnt3a and Wnt11 (Yamamoto et al., 2013). By removing both high-mannose-type glycans, the secretion of Wnt3a, Wnt5a and Wnt11 was impaired, suggesting that these glycans are necessary for maintaining the overall folded structure. Wnt5a was also modified with hybrid-type glycan at N312, although it was not essential for secretion because the Wnt5aN312Q mutant displayed secretion comparable to that of wild-type Wnt5a. The glycosylation site is unique in Wnt5a and Wnt5b, but the role of glycosylation at this site remains unknown.

Wnt5a and Wnt3a were secreted apically following the addition of the glycosylation site at their N-terminal region (Wnt5aL68N and Wnt3aL35N). The complex-type glycan was attached to the glycosylation site, and their apical secretion depended on galectin-3, similar to that of Wnt11. Therefore, Wnts that are modified with the complex-type glycan at the N-terminal region might be secreted apically by galectin-3. It is noteworthy that a single amino acid substitution of Wnt is able to change its trafficking route without causing it to lose activity.

The Wnt5a basolateral secretion is likely to be mediated by the same mechanism as that of Wnt3a (Yamamoto et al., 2013), because its sorting requires clathrin and AP-1, which are common machineries for the basolateral sorting of membrane proteins (Boehm and

Bonifacino, 2001). Given that Wls is also trafficked to the basolateral membrane, and is recognized by palmitoleic-acid-modified Wnts (Port and Basler, 2010; Yamamoto et al., 2013), Wls should localize to Wnt5a- and Wnt3a-loaded vesicles.

Polarized trafficking of Wnt receptors

Drosophila Fz1 (dFz1) and dFz2 act as receptors for wingless, a *Drosophila* Wnt homolog. Both receptors mediate the β -catenin-dependent pathway, and dFz1 also functions in the β -catenin-independent pathway, especially the planar cell polarity (PCP) pathway (Strutt, 2003). dFz1 is concentrated at apical junctions in the wing imaginal disc, whereas dFz2 is distributed throughout the apical and basolateral membranes (Wu et al., 2004). The C-terminal cytoplasmic amino acid sequence of dFz2 prevents it from accumulating at the apical junctions, and the apical localization of dFz1 is necessary for the activation of the PCP pathway. These results suggest that it is the localization of the receptors that determines pathway specificity.

Our study showed that Fz2 mainly localized to the basolateral membranes in MDCK II cells, whereas Fz7 was relatively evenly distributed throughout the apical and basolateral membranes. Fz2 acts as a common receptor for Wnt3a and Wnt5a (Sato et al., 2010), and Fz7 functions as a receptor for Wnt11 (Kim et al., 2008; Yamanaka and Nishida, 2007). LRP6 and Ror2, which are receptors for Wnt3a and Wnt5a, respectively (Nishita et al., 2010; Yamamoto et al., 2006), were also trafficked to the basolateral membranes using clathrin and AP-1. Ryk, which mediates Wnt11 signaling (Kim et al., 2008), was localized to both the apical and basolateral membranes. Because Wnt3a and Wnt5a are secreted basolaterally and Wnt11 is secreted apically, the localization of their receptors in polarized MDCK II cells is understandable. The signaling pathways of Wnt3a and Wnt5a were strongly activated when both Wnts stimulated the basolateral side, indicating that localization of their receptors is functionally coupled to the polarized secretion of Wnts. Thus, it is likely that the appropriate Wnt receptors are sorted to the same polarized fractions as the corresponding Wnts, thereby leading to efficient activation of their signals.

Our results are consistent with the findings that Ror2 is localized to the basal sides of the embryonic midgut epithelium (Yamada et al., 2010), and that LRP6 is present in the basolateral region of *Xenopus* outer ectodermal cells (Huang and Niehrs, 2014). Wls is essential for the trafficking of Wnts (Port and Basler, 2010), but it is not required for the apical and basolateral sorting of Wnt receptors. Therefore, Wnt-loaded vesicles could be distinct from vesicles containing their receptors, which probably prevents intracellular activation of Wnt signaling by the co-existence of ligands and receptors in the same vesicles. An alternative possibility might be that Wnts and Wnt receptors are loaded in the same vesicle containing Wls and that there is a mechanism to inhibit their binding.

Regulation of apical lumen formation by basolateral secretion of Wnt5a

We have shown previously that Wnt5a is involved in the single-cell polarization of rat intestinal epithelial IEC6 cells through Rac1 activation (Gon et al., 2013), and that Wnt5a promotes cell-to-substrate adhesion in cancer cells (Matsumoto et al., 2010). Other studies have demonstrated that Rac1 activation at the basolateral side is required for the polarization of MDCK cysts (Yagi et al., 2012), and that knockdown of integrins impairs apical lumen formation in MDCK cells (Myllymäki et al., 2011).

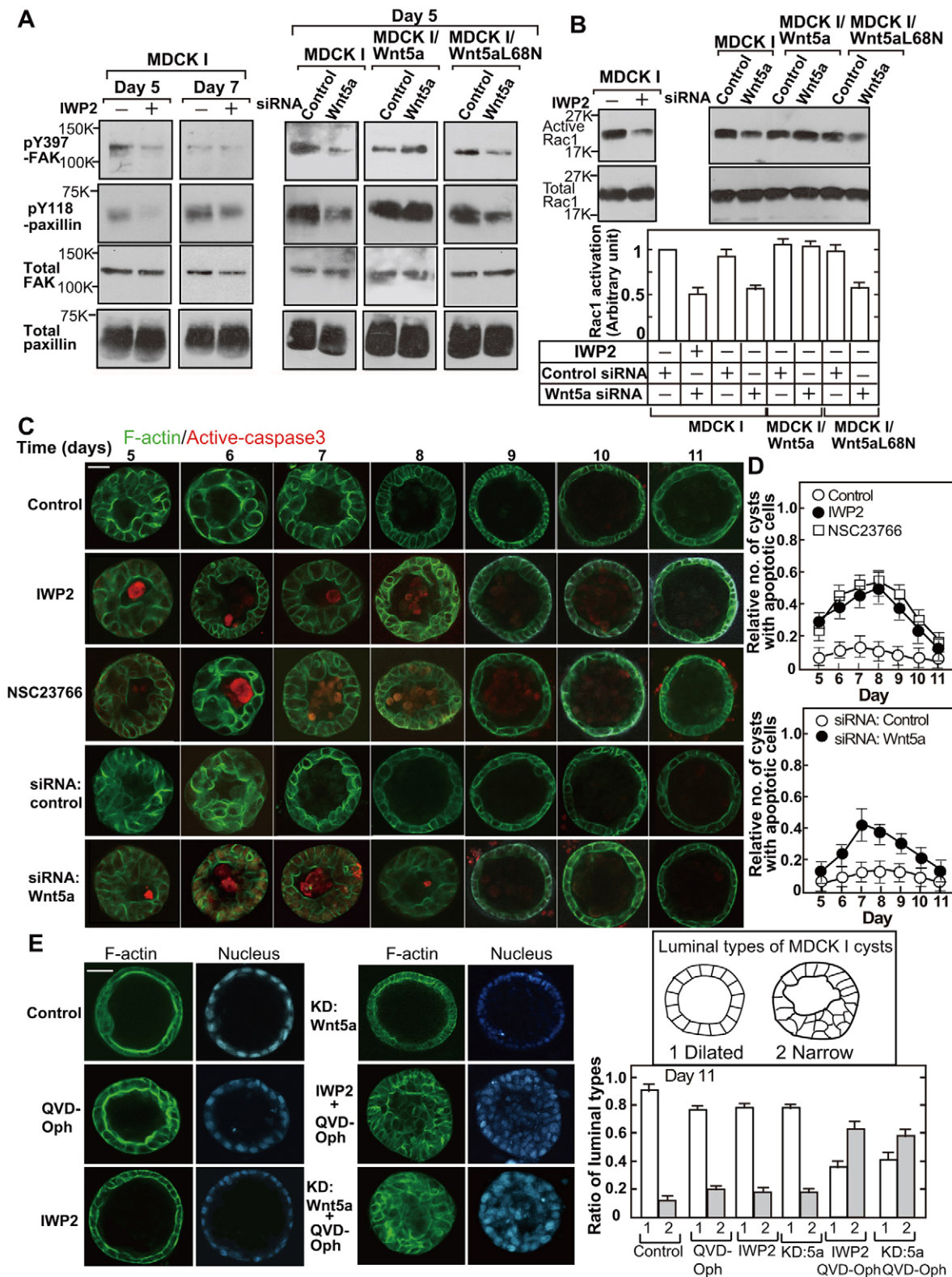


Fig. 7. See next page for legend.

However, it is not clear how these cellular events are linked to form apical lumen in polarized epithelial cells.

Lumen formation relies on the coordination of cell-to-substrate recognition, apical and basal polarization, and lumen expansion (Datta et al., 2011). Thus, cell adhesion to ECM is the first event

for lumenogenesis. Wnt5a secretion enhances cell-to-substrate adhesion in 3D culture. During MDCK cystogenesis in type I collagen gel, Rac1 activities are necessary for orientation of apical polarization through assembly of the basolateral laminin (O'Brien et al., 2001). Because our 3D culture was performed

Fig. 7. Apoptosis is required for the apical lumen formation in Wnt5a-depleted cells. (A) MDCK I, MDCK I/Wnt5a, or MDCK I/Wnt5aL68N cells were cultured in Matrigel in the presence or absence of IWP2 (left two panels) or transfected with control or Wnt5a siRNA (right three panels) for 5 or 7 days, and cell lysates at day 5 or 7 were then probed with anti-pY397-FAK and anti-pY118-paxillin antibodies. FAK and paxillin were used as loading controls. (B) MDCK I, MDCK I/Wnt5a or MDCK I/Wnt5aL68N cells were cultured in the presence or absence of IWP2, or were transfected with control or Wnt5a siRNA for 7 days, and cell lysates at day 7 were then subjected to the Rac activation assay. (C) MDCK I cells in 3D culture were treated with 5 μ M IWP2 or NSC23766, or transfected with control or Wnt5a siRNA for the indicated days, and the cysts were then stained with anti-active caspase3 antibody (red) and Alexa-Fluor-488-conjugated phalloidin (green). (D) Quantification of cysts having apoptotic cells in the lumen from 5–11 days in panel C. Results are shown as the means \pm s.e.m. from three independent experiments. (E) MDCK I cells treated with IWP2 or transfected with Wnt5a siRNA were incubated with 10 μ M QVD-Oph for 11 days, and the cysts were stained with phalloidin and DRAQ5. The cysts at day 11 were classified by their luminal types, and the number of cysts of each type was counted. Results are shown as the means \pm s.e.m. from four independent experiments. Scale bars, 20 μ m.

using Matrigel-containing laminin, Wnt5a-dependent Rac activation in MDCK I cells would not be required for laminin assembly, but would be involved in apical lumen formation. It has been reported that when apical and basolateral polarity is acquired efficiently or when cell polarity is coordinated with cell proliferation in MDCK cells, membrane separation without apoptosis in the center of the cord is sufficient for lumen formation (Martín-Belmonte et al., 2008). However, when apical and basolateral polarization is achieved slowly, apoptosis is required for clearance of the lumen. The results in this study showed that manipulations that inhibit Wnt5a signaling cause a delay in lumen formation. Apoptosis occurred to eliminate cells in the luminal space in these cysts. The phenotypes induced by Wnt5a knockdown were rescued by wild-type Wnt5a, but not by apically secreted Wnt5a. Taken together, these data suggest that polarized secretion of Wnt5a stimulates adhesion-dependent Rac activation and acquisition of apical and basolateral polarization, thereby enhancing lumen formation without apoptosis.

Wnt5a is expressed in the epithelium of the urogenital sinus, hindgut and cloacal membrane during development, and in adult uterine luminal epithelial cells (Daikoku et al., 2011; Li et al., 2011). MDCK I and IEC6 cells are good cell lines for analyzing endogenous Wnt5a roles in polarized epithelial cells. In these cells, the Wnt5a–Fz2–Ror2 axis might be involved in the establishment of apical and basolateral polarization in an autocrine manner. However, because mesenchymal cells are the primary source of Wnt5a secretion, epithelial cells that express Wnt5a receptors at the basolateral membranes might receive Wnt5a secreted from mesenchymal cells. For example, Wnt5a and Ror2 are expressed in the mesenchyme and epithelial basal region of embryonic midgut; aberrant epithelial cell clumps, which suggest polarity loss and random orientation of cells, are observed in Ror2-knockout embryonic gut (Yamada et al., 2010). In summary, Wnt5a and its receptors are trafficked to the basolateral side of polarized epithelial cells by different mechanisms, and the basolateral secretion of Wnt5a is necessary for apical lumen formation without apoptosis.

MATERIALS AND METHODS

Materials and chemicals

pPGK-neo/mouse Wnt5a, pRK5/rat Fz2 and pRK5/mouse Fz7 were provided by Dr Shinji Takada (National Institutes of Natural Sciences,

Okazaki, Japan). pEGFP-N3/mouse Ror2, p3xFLAG-CMV/rat Ryk and pCB6/ μ 1B-HA were from Drs Yasuhiro Minami (Kobe University, Kobe, Japan), Keiko Ohsawa and Shinichi Kohsaka (National Center of Neurology and Psychiatry, Tokyo, Japan) and Heike Fölsch (Northwestern University, Chicago, IL), respectively. Wnt5a was purified to homogeneity (Kurayoshi et al., 2007). Anti-Wnt5a (for immunostaining) was generated as described previously (Sato et al., 2010). Other primary antibodies used in this study are listed in supplementary material Table S3. FLAG–Fz7, Ryk–GFP and μ 1B–HA cDNAs were cloned into pCSII-CMV-MCS-IRES2-Bsd to construct lentiviral vectors (Takara Bio Inc., Shiga, Japan). pLvSIN/FLAG–Fz2 was constructed as described previously (Fumoto et al., 2012). Standard recombinant DNA techniques were used to construct pPGK-neo/Wnt5a mutants. RNA duplexes and forward and reverse primers for quantitative real-time PCR used in this study are shown in supplementary material Tables S4, S5. PNGase F and Endo H_F were from Roche Diagnostics GmbH (Mannheim, Germany) and New England Biolabs (Beverly, MA), respectively. Other materials were obtained from commercial sources.

Cell culture and transfection

MDCK I and II, L, X293T and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HEK293T cells were maintained in DMEM/Ham's F12 supplemented with 10% FBS. L or MDCK cells stably expressing wild-type Wnt5a, wild-type Wnt3a or their mutants were generated by selection with G418. To transiently express Ror2–GFP or Wnt5a, MDCK II cells were transfected with plasmids using Fugene HD (Promega, Madison, WI). MDCK II cells stably expressing FLAG–Fz7 or Ryk–GFP were generated by infection with lentiviruses. Then, the cells were selected and maintained in the same medium containing 2.5 μ g/ml blastocidin S. Three independent clones of each stable cell line were used for experiments to avoid clonal variation, and similar results were obtained.

Immunofluorescence microscopy

To visualize the intracellular localization of Wnts or their receptors in 3D Matrigel, the cells were fixed for 30 min in PBS containing 4% (w/v) paraformaldehyde (PFA) and then permeabilized with PBS containing 0.5% (w/v) Triton X-100 and 4% (w/v) BSA for 30 min. For staining of endogenous LRP6 or Ror2–GFP, cells were fixed with 100% methanol at -20° C for 30 min, washed with PBS three times and blocked for 30 min in 1% BSA in PBS containing 0.2% Triton X-100. To observe extracellularly secreted Wnt5a, MDCK II cells transiently expressing Wnt5a in 3D Matrigel were incubated with medium containing anti-Wnt5a antibody (1:100 dilution) for 1 h in a heated chamber (37 $^{\circ}$ C, 5% CO₂), washed with PBS three times and fixed for 30 min in PBS containing 4% PFA. Then, the cells were incubated with goat Alexa-Fluor-488-conjugated anti-rabbit-IgG without permeabilization. Then, the cells were stained with anti-Wnt5a, anti-LRP6, anti-GFP, anti-FLAG, anti- β -catenin or anti-active caspase 3 antibodies for 2 h. Cells were washed with PBS three times and incubated with goat Alexa-Fluor-488- or Alexa-Fluor-546-conjugated anti-mouse- or anti-rabbit-IgG, and Alexa-Fluor-488-, Alexa-Fluor-546- or Alexa-Fluor-633-conjugated phalloidin (Life Technologies, Carlsbad, CA) to visualize F-actin for 2 h. If necessary, nuclei were counterstained with a 1:1000 dilution of DRAQ5 (BioStatus Ltd, Shepshed, UK). The cells were then washed three times, mounted on slides and viewed using a confocal microscope (LSM510, Carl Zeiss, Jena, Germany).

Preparation of glycopeptides and lipopeptides of Wnt5a

Glycopeptides and lipopeptides of Wnt5a were prepared as described previously (Yamamoto et al., 2013). Briefly, after Wnt5a (1 μ g) was separated by SDS-PAGE, the protein bands were excised from the gel and were incubated with 50 nM trypsin (Promega) in 20 mM ammonium bicarbonate and 10% acetonitrile at 37 $^{\circ}$ C for 16 h. The tryptic peptides were extracted with 100 μ l of 0.1% trifluoroacetic acid in 10% acetonitrile/H₂O for glycosylation analysis, followed by 100 μ l of 0.1% trifluoroacetic acid in 60% acetonitrile/H₂O for lipidation analysis.

Nano-flow liquid chromatography/matrix-assisted laser desorption/ionization mass spectrometry

Tryptic digests of Wnt5a were injected into an Ultimate nano-LC system (Dionex, Idstein, Germany), and overall peptide identification was then carried out using a MALDI-TOF/TOF (4700 proteomics analyzer, Applied Biosystems, Framingham, MA) as described previously (Awada et al., 2010).

Nano-flow liquid chromatography/electrospray ionization mass spectrometry

To examine the glycan chains and relative abundance of each glycoform at each glycosylation site, the above tryptic digests were applied to a PicoFrit C18 column (0.075 mm×100 mm, Inertsil 3- μ m particle size, NewObjective), and the eluates were then continuously analyzed by a linear ion-trap/time-of-flight mass spectrometer equipped with a nano-flow electrospray ion source (Hitachi NanoFrontier LD, Hitachi High-Technology, Tokyo, Japan) as described previously (Kimura et al., 2013).

Apical-basolateral sorting assay

Polarized sorting of Wnt5a and Wnt receptors was examined as described previously (Yamamoto et al., 2013). MDCK I or II cells (2×10^5 cells) expressing Wnt5a, Wnt3a, their mutants or Wnt receptors were seeded on transwell polycarbonate filters (Corning Costar Quality Biological, Gaithersburg, MD) for 7 days (MDCK I cells) or 3 days (MDCK II cells). Media from the apical and basolateral sites were precipitated with Blue-Sepharose to detect Wnts secreted into the culture medium (indicated as 'B' in figures). To detect membrane cell-surface-associated Wnts or their receptors, the apical or basolateral surface membranes of the cells were selectively incubated with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce Biotechnology, Rockford, IL) for 30 min at 4°C (Sakane et al., 2012; Yamamoto et al., 2013). Biotinylated proteins were precipitated using Neutravidin-Agarose (Pierce Biotechnology), and the precipitates were probed using each antibody (indicated as 'N' in figures).

3D culture of MDCK cells

MDCK cells were suspended in Matrigel (BD Biosciences, San Jose, CA) at a density of 1×10^4 cells/ml, and 80 μ l of the cell suspension was mounted on a round coverslip as described previously (Yamamoto et al., 2013). After incubation at 37°C for 30 min to solidify the gel, the cells on the coverslip were transferred to a 24-well culture plate and incubated in 1 ml of DMEM containing 10% FBS for 7 days (MDCK I cells) or 3 days (MDCK II cells). Under the conditions, MDCK cells formed cysts. For treatment with inhibitors, 5 μ M IWP2, 5 μ M NSC23766 or 10 μ M QVD-Oph were added when plating in Matrigel, and medium was changed every 2 days.

Knockdown of mRNA in MDCK cells by siRNA

To transfect siRNA into MDCK cells expressing Wnt5a or its receptors, trypsinized MDCK cells were suspended in Opti-MEM (Life Technologies) at 10^6 cells per 100 μ l, siRNA was added (160 pmol), and cells were electroporated using NEPA21 (NEPAGENE, Tokyo, Japan) with five square pulses of 20 V of 50-ms duration (Yamamoto et al., 2013). After cells were consecutively transfected twice at 2-day intervals, knockdown cells (2×10^5 cells) were seeded on a Transwell polycarbonate filter for an additional 3 days.

Statistical analysis

Experiments were performed at least four times, and the results are expressed as means \pm s.e.m. Statistical analysis was performed using StatView software (SAS Institute, Inc., Cary, NC). Differences between the data were tested for statistical significance using Student's *t*-test. Two-tailed *P*-values less than 0.05 were considered statistically significant.

Additional methods

Methods for quantitative real-time PCR were as described previously (Sato et al., 2010; Yamamoto et al., 2013). Preparation of conditioned medium, the extracellular matrix (ECM) fraction and lysates from Wnt-producing cells were performed as described previously (Komekado et al., 2007; Kurayoshi et al., 2007).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

H.Y. designed experiments, carried out purification of Wnt proteins, performed the apical-basolateral sorting assay and immunofluorescence analyses, and wrote the manuscript. S.M. performed immunofluorescence analyses. T.K. carried out the apical-basolateral sorting assay. T.S. performed the fractionation assay. C.A. and T.T. analyzed the glycosylation and lipidation profiles of Wnt5a by mass spectrometry and provided advice. A.K. designed the experiments and wrote the manuscript.

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.163683/-DC1>

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