Soluble Frizzled-related proteins promote exosome-mediated Wnt re-secretion

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Summary

Wnt is a secreted signaling protein, or morphogen, that participates in various aspects of embryogenesis, tissue homeostatics and tumorigenesis. Disruption of any Wnt signaling components can lead to mis-regulation in cell-cell communication, tissue patterning, and limb development. Therefore, the transduction of the Wnt signal is tightly controlled by various modulators at multiple levels. Wnt is post-translated with palmitoleic and believed to act at both short and long range from the producing to the responding cells by generating a concentration gradient. Due to the lipid modification, after secretion into the extracellular space, members of Wnt family require carriers to form complexes that shied their hydrophobic lipid adduct. For example, Wnt3a associates together to form a homo-trimer, a basic unit of a higher molecular weight complex. Alternatively, in cell culture supernatant, the heterodimers of Wnt with a serum component, afamin, are detectable. Wnt3a complexes with homotrimers or heterodimers with afamin in conditioned media can be dissociated efficiently when co-expressed with sFRP2, leading to the formation of Wnt3a/sFRP2 heterodimers. This dissociation was also observed in *Xenopus* embryo to expand the diffusion range of Wnt proteins. In addition, various models have been proposed to provide the explanation of how these Wnt signaling molecules propagate in extracellular space with lipid adduct, affecting the trafficking, diffusion, and solubility. For instance, progress has been made in explaining how secreted Wnts are carried on the extracellular spreading membranous deliverers like exosomes, on filopodium-like protrusions and morphogen binding proteins on cell surface. However, little is known about whether and/or how these different transport systems and extracellular Wnt complexes relate to one another. In this study, using Wnt3a as a model, I addressed the question of whether various forms of morphogens are independently involved in their signaling in tissues or whether these forms affect one another in secretion and/or extracellular trafficking.

First of all, by establishing of an assay culture system to recover Wnts re-secreted on exosome, I found that re-secretion of Wnt3a on exosome is mediated by members of Frizzled-related proteins family, sFRPs. I detected a significant increase in the level of Wnt3a ligands re-secreted on exosome in the presence of sFRP1 and sFRP2, but not sFRP3, sFRP4 or exFzd8. In addition, co-culture of GFP-Wnt3a expressing L cells with sFRP2 expressing HEK293 cells or microinjection of mRNA of GFP-Wnt3a and sFRP2 observed many GFP-positive puncta, clearly localized along

cell boundaries of sFRP1 and sFRP2-expressing cells. Taking advantage of live imaging, I could directly visualize the movement of mCherry-Wnt3a after incorporation into cells, by comparing colocalization with CD63, an exosome marker, fused with BFP and pH-sensitive green fluorescent protein, pHluorin.

Interestingly, taking the advantage of non-invasive approach, Analytical Ultra Centrifugation with a fluorescence detection system, AUC-FDS, which enables to examine protein complexes from cultured media without any requirement of detergent, I could measure the interaction of Wnt3a and Wnt5a, but not Wnt11 with sFRP2 secreted in culture supernatant. Since complex-forming ability is correlated with a specificity of sFRP-induced cell surface accumulation and exosome-mediated re-secretion of Wnt proteins, I hypothesized that heterodimer formation under natural conditions is involved in the accumulation of Wnt on cell membranes, which seems to be followed by exosome-mediated re-secretion.

Furthermore, our results obtained from western blotting revealed the involvements of membrane proteins in sFRP2-mediated exosomal re-secretion of Wnt. The level of Wnt3a incorporated into the cells in the presence of sFPR2 was not reduced when endocytosis was blocked at 4^{o}C. These surprising results suggest the binding of Wnt ligands occurred before internalization even. Surprisingly, heparan sulfate proteoglycan, HSPG, but not co-receptors LRP5/6, is crucial for the attachment of Wnt3a on the cell surface for further Wnt internalization and re-secretion of exosome. While Frizzled receptors are required for Wnt activity and re-secretion of Wnt on exosome, their effect is not specific for sFRP2-mediated exosomal re-secretion of Wnt3a. GFP-Wnt3a puncta on boundaries of sFRP2 expressing cell and the level of Wnt3a recovered in exosome fraction were dependent on HSPG, because they disappeared with expression of a membrane-bound form of Heparinase, an enzyme could cleave HS chain of HSPG both in vivo and in vitro.

Based on these above results, I propose that Wnt heterodimerization with its carrier protein, sFRP2, enhances Wnt accumulation at sugar chains on HSPGs on the cell surface, leading to increased endocytosis and exosome-mediated Wnt re-secretion.

Table of Contents

List of figures

I. Introduction

1.1 Wnt signaling

Wnt is secreted signaling protein, or morphogen, that participates in various aspects of embryogenesis, tissue homeostasis and tumorigenesis, … The Wnt family comprises of 19 protein members in mammals. Wnt proteins can activate mainly in 2 different pathways including canonical Wnt/β-catenin signaling and non-canonical Wnt signaling (Figure 1).

1.1.1 Canonical Wnt signaling

In the absence of Wnt ligands, the destruction complex comprised of the scaffolding protein Axin, Glycogen Synthase Kinase 3β (GSK3β) and Adenomatous Polyposis Coli (APC), will capture cytosolic β-catenin. CK1 and GSK3β will phosphorylate β-catenin for ubiquitination and degradation by proteasome (Clevers & Nusse, 2012; Willert & Nusse, 2012).

On the other hand, upon the binding of Wnt ligand to the Frizzled receptor (Fzd) and low-density lipoprotein-related protein 5/6 (Lrp5/6) co-receptors, the canonical Wnt/β-catenin signaling pathway is initiated, resulting in the phosphorylation of cytoplasmic protein Disheveled (Dvl). Subsequently, this phosphorylated Dvl in turn recruits the multiprotein destruction complex for translocation to the intracellular domain of LRP5/6 co-receptor. Phosphorylated Dvl, LRP5/6 co-receptor and Axin associate together to form signalosome (Fiedler *et al*, 2011; Bilic´, *et al*, 2007), resulting in the dissociation of the destruction complex, preventing the phosphorylation and degradation of β-catenin. Accumulation of β-catenin in cytoplasm will translocate to the nucleus, then bind to TCF/LEF transcriptional factors to activate the transcription of target genes (Figure 1A).

The Wnt/β-catenin signaling pathway plays numerous crucial roles during embryogenesis and homeostasis. Disruption of any Wnt signaling components can lead to mis-regulation in cell-cell communication, tissue patterning, and limb development, ... (Van Amerongen & Nusse, 2009; Clevers *et al*, 2014). Therefore, the transduction of the Wnt signal is tightly controlled by various modulators at multiple levels. In the extracellular context, Wnt activity is regulated by secreted proteins such as sFRPs, Wif-1, and Dkks, which act as antagonists to regulate ligand-receptor

interactions (Cruciat & Niehrs, 2013). Numerous studies have demonstrated the importance of the small family of four secreted growth factors known as R-spondins (Rspon1 to 4), which act as regulators of not only Wnt/beta-catenin but also PCP signaling. All four members of the R-spondin family can potentiate the Wnt/beta-catenin signaling pathway by stabilizing and amplifying the levels of the Wnt receptor Fzd and the co-receptor LRP5/6 at the plasma membrane. This amplification process involves the association of a ternary complex, in which the furin-like domain (FU1) of R-spondin binds to E3 ubiquitin ligases ZNRF3/RNF43, whereas FU2 domain interacts with leucine-rich repeat-containing G-protein coupled receptors (LGRs). This interaction induces the clearance of E3 ligases from the cell membrane, subsequently preventing the ubiquitination, internalization, and degradation of Wnt receptors caused by E3 ligases.

1.1.2 Non-canonical Wnt signaling

Different to canonical Wnt pathway which is dependent on the regulation of β-catenin level in the cytoplasm, non-canonical Wnt pathway acts independently of β-catenin, "Wnt non-canonical planar cell polarity (PCP)" signaling pathway and Wnt/calcium pathway to regulate cell polarity and behavior. In this section, I mainly discuss about the Wnt/PCP pathway (Figure 1B).

This PCP pathway is also initiated upon the binding of Wnt ligands (Wnt5, Wnt11) to Fzd receptor on cell membrane. Instead of resulting in the accumulation of β-catenin in the cytoplasm, the Wnt-Fzd complex activates downstream of a series of signaling to provide directional information both locally and globally during skin development, hair and cilia polarized localization and orientation, cell movement in mammals (Yang & Mlodzik, 2015; Devenport, 2014). PCP signaling pathway is essential for controlling body axis elongation through convergence-extension during vertebrate gastrulation and neurulation, regulating the orientation of cell division (Borovina *et al*, 2010). In mammals, beside to the fundamental roles, PCP could be additionally required for the orientation of sensory hair cells in the inner ear, follicles and cilia in epithelium.

Basically, the PCP core proteins comprise of 6 proteins interacting with each other to form 2 separate PCP complexes: Frizzled-Disheveled-Flamingo and Van Goh-Prickle complexes (Yang & Mlodzik, 2015). These complexes localize asymmetrically at opposing sides of each epithelial cell. In which, the binding of Wnt ligand to the transmembrane Fzd receptor on the cell membrane

causes recruitment of cytosolic Dvl for phosphorylation (Gao *et al*, 2011). Instead of resulting in an accumulation of β-catenin in the cytoplasm, this phosphorylated Dvl allows to activate the downstream of GTPases Rho and Rac or JNK signaling cascades, leading to the re-agreement of membrane and cytosolic proteins (Boutros *et al*, 1998; Yamanaka *et al*, 2002).

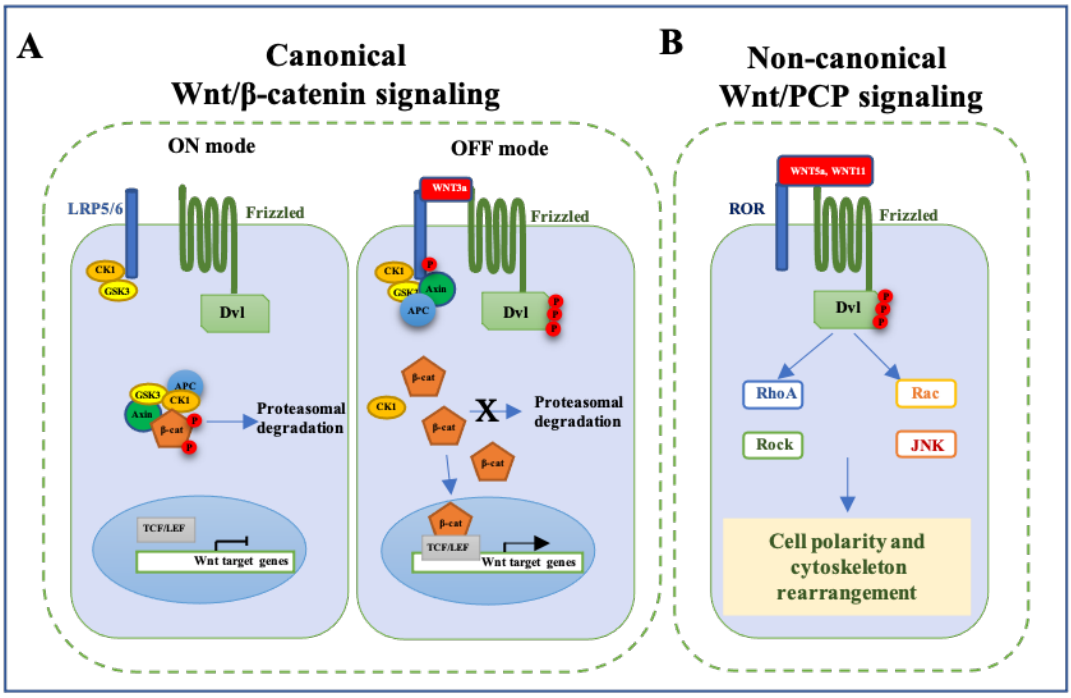


Figure 1: Overview of Wnt signaling pathway.
A. Cononical Wnt/β-catenin signaling mediates Wnt target gene expression by regulating the degradation and accumulation of β-catenin in cytoplasm. **B**. Non-canonical Wnt/PCP signaling regulates cell polarity and cytoskeleton rearrangement.

Figure 1 Overview of Wnt signaling pathway.

A. Cononical Wnt/β-catenin signaling mediates Wnt target gene expression by regulating the degradation and accumulation of β-catenin in cytoplasm. **B**. Non-canonical Wnt/PCP signaling regulates cell polarity and cytoskeleton rearrangement.

1.2 Various forms of secreted Wnt

Due to post-translational modification with palmitoleic acid, after secretion into the extracellular space, members of Wnt protein family require carriers to form complexes that shield their hydrophobic lipid modification.

Using a non-invasive approach, analytical ultracentrifugation with a fluorescent detection system, AUC-FDS) (Nelson *et al*, 2016; Schuck *et al*, 2002; Zhao *et al*, 2014), without any purification and addition of detergents, has revealed that after secretion, Wnt3a associates together to form a homo-trimer. These homo-trimers act as the basic unit of a higher molecular weight complexes and efficiently shield the lipid modification from the aquatic environment. Interestingly, these Wnt large complexes are less mobile but retain activity (Takada *et al*, 2018). In addition to this comprehensive size distribution analysis of Wnt3a, the Takagi group also reports the heterodimers of Wnt with a serum component, afamin (Mihara *et al*, 2016), which maintain an active and water-soluble form of hydrophobic Wnt proteins after isolation.

Notably, there is no detectable monomer of secreted Wnt detectable in condition as close as possible to the native environment. An appreciable amount of monomeric Wnt3a was detected only in the presence of detergents, such as CHAPS, during the purification to maintain solubility and activity (Karl Willert *et al*, 2003).

Another long-time reported Wnt carrier is members of the secreted Frizzled-Related Protein (sFRP) family. Wnt3a complexes with homotrimers or heterodimer with afamin in conditioned media can be dissociate efficiently when co-expressed with sFRP2, leading to the formation of Wnt3a/sFRP2 heterodimers. This dissociation was also observed in *Xenopus* embryo to expand the diffusion range of Wnt proteins. Similarly, SWIM, a soluble protein in Drosophila, was considered as a mediator of Wingless trafficking by their interaction on cell surface of Wnt source cells, enabling monomeric Wingless to diffuse through wing discs (Mulligan *et al*, 2012). In this paper, I mainly focus on the heterodimer of Wnt and sFRP.

1.3 Wnt morphogen transport systems

Wnt is morphogen believed to act at both short and long range from the producing to the responding cells by generating a concentration gradient. Various models have been proposed to provide the explanation on how these Wnt signaling molecules propagate in extracellular space with lipid adduct, affecting the trafficking, diffusion, and solubility (Figure 2).

1.3.1 Extracellular membranous deliverers

Progress has been made in explaining how secreted Wnts are carried on the extracellular spreading membranous deliverers (Figure 2B).

Lipoproteins, large and globular complexes with a central core of hydrophobic lipids, allow the lipid-modified Wnt proteins to anchor for intracellular transport (Neumann et al, 2009). In Drosophila, Wingless, a homologue of Wnt, was found to release and anchor to lipoproteins and lipoprotein particles called argosomes in the developing wing epithelium. In mammalian cell culture systems, it was suggested that Wnt3a released by lipoproteins in culture medium remains active and its lipid modifications are required for their association.

In many cases, Wnt ligands can reach receiving cells by releasing and spreading on extracellular vesicles (Evs) to mediate the delivery of active proteins from one cell to another, especially on exosomes (Gross *et al*, 2012; Luga *et al*, 2012). Exosomes are secreted EVs originating from endosome fused into multivesicular body (MVB) and released into extracellular space. By biochemically recovering extracellular Wnts in exosome fraction, there is a growing realization that active Wnt ligands are secreted and placed on exosomes from Drosophila and mammalian cells through Ykt6-dependent exosome secretion. Thus, extracellular membranous particles provide a potential delivery system for the long-range transfer of hydrophobic morphogens (Gross *et al*, 2012; Linnemannstöns *et al*, 2020; Witte *et al*, 2020).

In addition, the finding of endosomal system-mediated Wnt secretion on exosome also leads to several studies of Wnt “re-secretion”. In the polarized Drosophila imaginal disc epithelium, after being secreted from producing cells, Wingless is trafficked to the apical side of epithelial cells, then internalized and undergoes transcytosis, and is released from the basolateral side for inducing activity or diffusion in imaginal discs (Witte *et al*, 2020; Linnemannstöns *et al*, 2020).

1.3.2 Filopodium-like protrusions

One alternative to exosome is specialized signaling filopodia. Filopodium-like protrusions have also been reported to associate with the Wnt morphogen for further delivering Wnt ligands in long distance from source cells (Figure 2C). Filopodia are actin-based cell extensions from cell

membrane and can carry several different Wnts. In *Xenopus* fibroblast cell culture (Holzer *et al*, 2012), Wnt2b was reported to be mobilized on cell protrusions. Additionally, recent studies with imaging analyses revealed that Wnt8a accumulates as clusters on the cell membrane and determines the position of new filopodia. As a result, Wnt8a is transported on the tip of actin-containing filopodia to the membrane of the receiving cells, subsequently, activates the Wnt signaling pathway (Stanganello *et al*, 2015; Stanganello & Scholpp, 2016).

1.3.3 Morphogen binding proteins on cell surface

Another model suggestion for the transport mechanism of Wnt proteins is through specialized morphogen binding proteins on the cell surface. One of the long-term focused candidates is Heparan Sulfate Proteoglycans (HSPGs) (Figure 2D). HSPGs are comprised of a core protein, including members of 2 major families, glypican and syndecan, attached to the cell membranes, with another component, heparan sulfate (HS) GAG chains. Several lines of evidence show that HSPGs regulate not only the concentration of Wnt ligands at the cell surfaces but also maintain the solubility and signaling activity. For instance, Dally and Dally-like proteins in Drosophila can interact with Wingless and transfer them to Fzd receptors. Loss of function of these membrane-bound HSPG results in a decrease in extracellular Wingless level and signaling activity. Both in vivo and in vitro suggest the requirement of both glypican core proteins and HS chain in the Wnt binding, accumulation and positively regulating Wnt signaling.

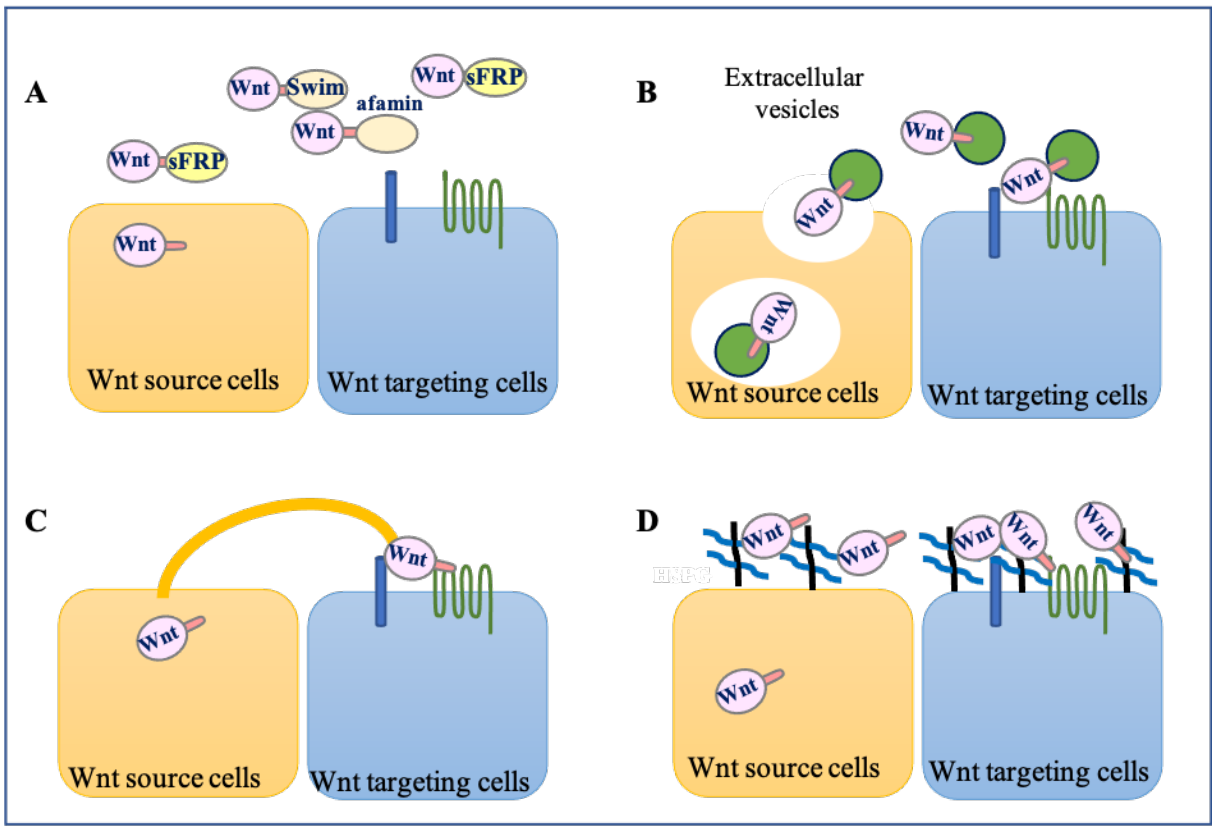


Figure 2 Various models for the intercellular transport of Wnt proteins.

To shield the hydrophobic lipid adduct for long range transport, several models of Wnt transport in extracellular space have been proposed.

A. Wnt binding proteins. **B**. Extracellular vesicles. **C**. Filopodia-like cell protrusion. **D**. Later diffusion

1.4 Secreted-Frizzled-related protein family

In this study, I mainly focus on the significance of Wnt and sFRP complexes in mediating Wnt transport in extracellular space.

There are 5 sFRPs (sFRP 1-5) in humans, secreted and capable to regulate Wnt signaling by direct binding and prevent the binding of Wnt to the Frizzle receptors. The structure analysis reveals three structural units. Besides an amino terminal signal peptide, all sFRP members contain a coiled cysteine rich domain (CRD) with 10 conserved cysteine residues and similar sequence with CRD of the Fzd transmembrane receptor. This domain is required for the formation of Wnt and sFRP heterodimer and Wnt activity. In addition, sFRPs also have a carboxyl terminus netrin-like domain

(NTR)(Bányai & Patthy, 1999; Chong *et al*, 2002). Based on sequence homology analyses, while sFRP1, 2 and 5 form a subgroup, sFRP3 and 4 are classified to another one (Figure 3). The protein structure analyses could provide explanations for biological function of sFRPs during embryonic development (Bovolenta *et al*, 2008; Esteve *et al*, 2011)

Initially, sFRPs were believed to act as antagonists of Wnt/β-catenin signaling pathway. For example, Frzb, an orthogo of sFRP3 in *Xenopus*, was first identified as a Wnt inhibitor and preventing Wnt8 function in posterization (Leyns & Bouwmeester, 1997; Wang & Krinks, 1997). However, besides reported inhibition function, sFRP1 and sFRP2 also could promote the Wnt/β-catenin signaling pathway in a dose-dependent manner. These conflicting results suggest that sFRP members could act as multi-functional mediators to regulate Wnt signaling, by either binding directly to Wnt ligands or Frz receptors. Interestingly, microinjection of mRNA of Frzb clearly showed an expansion of the distribution range of Wnt8 in Xenopus embryo (Mii & Takada, 2020; Mii & Taira, 2011, 2009), suggesting another function of sFRPs in the regulation of Wnt signaling range (Wawrzak *et al*, 2007).

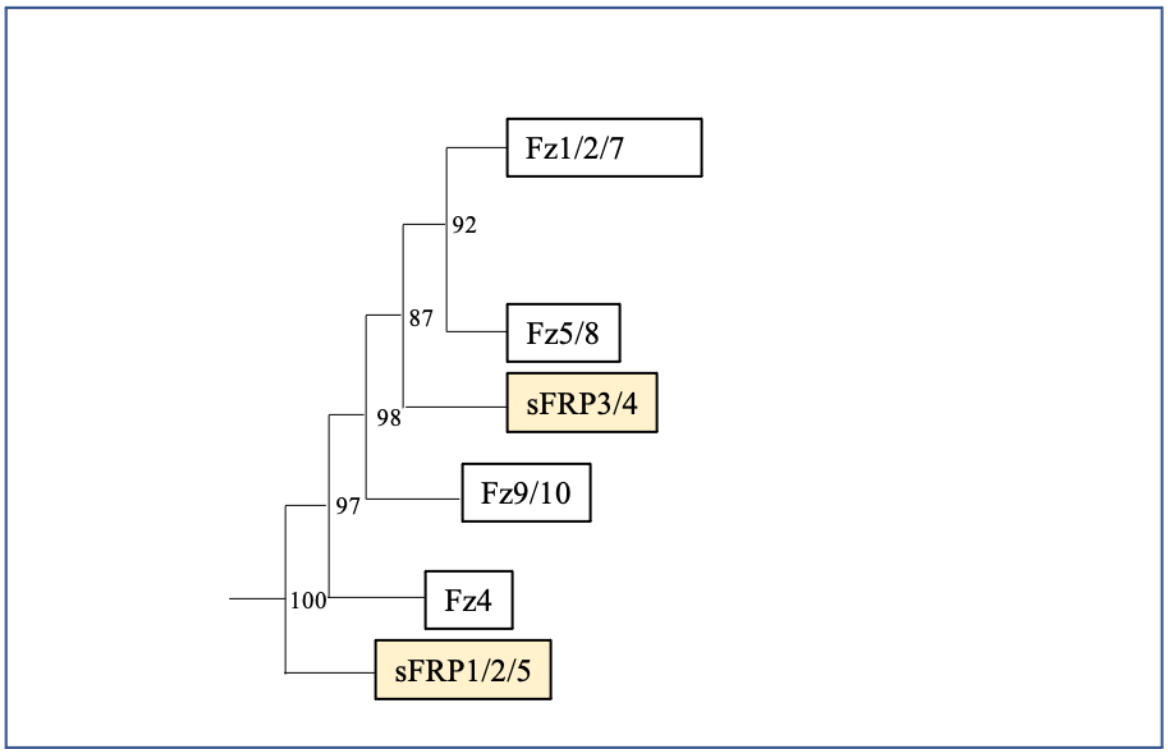


Figure 3 The phylogenic tree of sFRP family proteins.

Amino acid sequence analyses show that sFRP1, sFRP2 and sFRP5 form a subgroup while sFRP3 and sFRP4 belong to another one.

1.5 Aims of this study

As discussed above, the post-translational modifications make Wnt ligands become hydrophobic. This lipid adduct restricts the extracellular level, the diffusion and spreading of Wnt ligands, then affects the signaling range. Although various models have been proposed to explain how lipid-modified Wnts could be solubilized to spread over long distance in aquarius environment, little is known about whether these extracellular transport systems and various forms of Wnt morphogens are independently involved or related to another in their signaling.

In this study, I focused on addressing the question whether exosome-mediated Wnt re-secretion is affected by formation of different Wnt extracellular complexes, by establishment of an assay culture cell system to recover Wnts re-secreted on exosome.

II. Material and Methods

2.1 Cell culture and transfection

L and HEK293 cells were kindly provided by Dr. Masatoshi Takeichi (RIKEN), and the MDCK-II cell line was kindly provided by Dr. Tetsuhisa Otani (NIPS). *Fzd 1-10* KO HEK293 cells were a generous gift from Dr. Benoit Vanhollebeke (UNI, Belgium)(Eubelen *et al*, 2018), and SuperTopFlash/HEK293 cells were kindly provided by Dr. Tadasuke Tsukiyama (Hokkaido Univ) (Tsukiyama *et al*, 2015).

MDCK, L and HEK293 cells were cultured in DMEM or DMEM:Ham's F12 (1:1) medium supplemented with 8.3% FBS and antibiotics. L cells stably expressing FLAG-GFP-tagged mouse Wnt3a, FLAG-GFP-tagged mutant Wnt3a (GFP-Wnt3a(C77A)), FLAG-tagged mouse Wnt3a, FLAG-tagged mutant Wnt3a (Wnt3a(C77A)), non-tagged Wnt3a, non-tagged Wnt5a, and HEK293 cells stably expressing mouse sFRP2-FLAG, or mouse Fzd8-CRD-MycHis (exFzd8) were established previously (Takada *et al*, 2018; Shibamoto *et al*, 1998; Tsukiyama *et al*, 2015) and maintained in culture containing 400 μg/mL G418 or 4 μg/mL Blasticidin S. L cells stably expressing FLAG-mCherry-tagged mouse Wnt3a under the control of the PGK promoter, HEK293 cells stably expressing FLAG-mClover-mouse Wnt5a and mouse sFRP1-FLAG and sFRP3-FLAG under control of the CMV promoter, mouse Wnt11-mClover under control of the PGK promoter, and mouse sFRP4-FLAG under control of the Tet-on promoter, were established by transfection of plasmid constructs with GeneJuice transfection reagent (Millipore), or the calcium phosphate method, followed by drug screening with 400 μg/mL G418, 1200 μg/mL G418, or 6 μg/mL Blasticidin S and by Western blotting after picking colonies.

HEK293 cells stably expressing both sFRP2-FLAG and pHluorin-M153R-CD63-mTagBFP were established by transfection of plasmid constructs with GeneJuice transfection reagent into sFRP2 producing HEK293 cells, followed by drug screening with 1000 μg/mL G418 and picking colonies. pHluorin-M153R-CD63-mTagBFP was generated by substituted mScarlet to mTagBFP from original published construct (Addgene #172118), then cloned into pCS2 neo.

2.2 SDS-polyacrylamide gel electrophoresis and Immunoblotting

To examine protein levels in samples prepared in this study, Western blotting was carried out by following a standard protocol. Briefly, samples were diluted in 2x SDS-PAGE sample buffer composed of 4% SDS, 20% glycerol, 0.1M Tris-HCl (pH6.8), 9% 2-mercaptoethanol, and 0.01% bromophenol blue, heated at 95°C for 10 min and loaded on 10%, or 12% acrylamide gels for electrophoresis. Proteins were transferred to a PVDF membrane and blocked in 5% skim milk, before being incubated with primary antibodies.

2.3 Preparation of exosomes

To prepare exosome-depleted cultured supernatant (CS), mouse L cells stably expressing FLAG-GFP-tagged, FLAG-tagged or non-tagged Wnt3a were cultured with parental HEK293 cells or HEK293 cells stably expressing sFRP2-FLAG, sFRP3-FLAG, sFRP4-FLAG, or exFzd8-MycHis at a 9:1 ratio and an initial density of 2 x 10^6 cells on 100-mm-culture plates for 2 days. Then, medium was removed, and cells were rinsed twice with PBS (-) or DMEM containing 4% exosome-free FBS and incubated with fresh DMEM containing 8% exosome-free FBS for 2 more days. The CS was then collected and processed by centrifugation.

To examine the effect of sFRP2 on re-secretion of Wnt3a on exosomes, cells were treated with exosome-depleted CS from GFP-Wnt3a/L cells co-cultured with parental HEK293 cells or HEK293 cells expressing sFRP1, sFRP2, sFRP3, or exFzd8. Mouse L cells stably expressing FLAG-GFP-tagged, FLAG-tagged or non-tagged Wnt3a were cultured with parental HEK293 cells or HEK293 cells stably expressing sFRP1-FLAG, sFRP2-FLAG, sFRP3-FLAG, or exFzd8-MycHis at a 9:1 ratio in cell number and an initial density of 2 x 10^6 cells on 100-mm-culture plates for 2 days. Then, medium was removed, and cells were rinsed twice with PBS (-) or DMEM containing 4% exosome-free FBS and incubated with fresh DMEM containing 8% exosome-free FBS for 2 more days. The CS was then collected and exosome-depleted CS from these cultures was prepared by the following procedure (Théry *et al*, 2006). The CS was first centrifuged at 1,500 x *g* for 10 min, then 10,000 x *g* for 30 min to remove cellular debris, and supernatant was recovered by further centrifugation at 100,000 x *g* for 18 h. This was used as exosome-depleted CS. Then

exosome-depleted CS was added to the culture of secondary cells, including MDCK, L, and HEK293 cells. I refer to this as Re-secretion assay #1.

Alternatively, secondary cells were treated with exosome-depleted CS (input S100 fraction) from GFP-Wnt3a/L mixed with that from sFRP2-, sFRP3-, sFRP4-, exFzd8-expressing or control HEK293 cells in a 4:1 ratio. I refer to this as Re-secretion assay #2. In both Re-secretion assay #1 and #2, the CS of secondary cells was collected 24 h after treatment, and processed using the following ultracentrifugation: 1,500 x *g* for 10 min, 10,000 x *g* for 30 min (the supernatant was referred to as S10 (2nd), 100,000 x *g* for 90 min (recovered supernatant was referred to as S100 (2nd) and the exosome-containing pellet was termed P100 (2nd)).

To examine the possibility that sFRP2 enhances direct incorporation of GFP-Wnt3a into exosomes independently of cells, GFP-Wnt3a incorporation into exosomes was examined in cell-free conditions, in which, exosome-depleted CS (input S100 fraction) of GFP-Wnt3a/L and that of sFRP2-expressing HEK293 or control HEK293 were mixed with S10 of parental MDCK cells, from which exosomes had not yet been removed. These were further incubated at 37°C for 24 h. Then, mixtures were centrifuged to obtain P100 fraction.

2.4 Xenopus microinjection

This study was performed in accordance with Guidelines for Animal Experimentation of the National Institutes of Natural Sciences, with the approval of the Animal Care and Use Committee (IACAC) of the National Institutes of Natural Sciences. Microinjection experiments in *Xenopus laevis* eggs were carried out according to standard methods (Hazel L. Sive *et al*, 2000; Mii & Taira, 2009), as follows. Eggs for fertilization were obtained from gonadotropin (ASKA Pharmaceutical) injected female frogs. Artificial fertilization was done using testis homogenate, followed by de-jellying using 4% L-cysteine (pH 7.8) and incubated at 17°C in 0.1x Steinberg's solution. mRNAs synthesized with an mMESSAGE mMACHINE™ SP6 Transcription SP6 kit (Invitrogen) were microinjected into the animal caps of ventral blastomeres at the 4-cell stage and observed at the gastrula stage. mRNAs were injected at a final concentration as follows: FLAG-GFP-mWnt3a; 500 pg/embryo, mRuby; 100 pg/embryo, sFRP1, sFRP2, sFRP3, or sFRP4; 1000 pg/embryo, Lyn-mBFP; 100 pg/embryo, Heparinase III; 400 pg/embryo.

To collect exosomes secreted from *Xenopus* embryos, 30 embryos injected with FLAG-GFP-Wnt3a together with or without sFRP2 were dissociated in 1.2 mL of modified phosphate buffer (50mM Na Phosphate, 35mM NaCl, 1mM KCl) with EDTA at stage 11.5. This 1.2 mL of embryo-dissociated buffer was then incubated for 5 h at 17℃ and followed by the exosome preparation protocol to obtain the P100 fraction, as described above.

For immunostaining, microinjected embryos were treated using a protocol described previously (Mii *et al*, 2017). Fixed embryos were incubated with HepSS-1 (mouse monoclonal antibody, IgM; prepared in-house) or NAH46 (mouse monoclonal antibody, IgM; prepared in-house) in TBST overnight at 4℃, washed 3 times with TBS and incubated with Alexa Fluor 647-conjugated secondary antibody.

2.5 Chemical inhibitor

MDCK cells grown to 80% confluency were treated with S100 of GFP-Wnt3a with or without sFRP2 and 10 μM GW4869 (an inhibitor of MVB formation) (Sigma-Aldrich D1629) or DMSO for 24 h before preparation of exosome fractions.

2.6 Tracking of mCherry-Wnt3a in receiving cells

The dual-fluorescence reporter allows us to track cytosolic CD63-positive structures with pH-insensitive blue fluorescence under acidic condition, especially MVB. Dual blue and pH-sensitive green fluorescence can be detected when MVB fuses to plasma membrane and exosomes are released into neutral environment. To monitor Wnt3a movement in neighboring cells, I took advantage of this novel construct to track mCherry-Wnt3a localization during MVB formation, fusion, and exosome secretion. L cells stably expressing mCherry-Wnt3a were co-cultured with HEK293 cells stably producing sFRP2 and pHluorin-M153R-CD63-mTagBFP on glass bottomed plates at a ratio of 3:2 for 1 day before observation with confocal microscopy. To inhibit MVB formation, 12 h after culture, cells were treated with DMEM medium containing 10 μM GW4869 or DMSO, observation was done 24 h further after chemical inhibitor treatment.

2.7 Analytical ultracentrifugation

All analyses conducted by analytical ultracentrifugation with a fluorescence detection system (AUC-FDS) were carried out using a procedure described previously (Takada *et al*, 2018). CSs for AUC-FDS analysis was prepared with FluoroBrite DMEM (Gibco) with 8% FBS from either GFP-Wnt3a/L, GFP-Wnt3a (C77A)/L, mClover-Wnt5a/HEK293, or Wnt11-mClover/HEK293 cells with sFRP2-, sFRP3-, or sFRP4-expressing or parental HEK293 cells. CSs were collected from confluent culture, centrifuged at 1,500 x *g* for 10 min, 10,000 x *g* for 30 min to remove cell debris, and then subjected to analytical ultracentrifugation (AUC; Beckman Coulter) with a fluorescence detection system (FDS; AVIV Biomedical). All AUC experiments were conducted using Beckman 12-mm charcoal-filled Epon double-sector centerpieces at 20 °C and at a rotor speed of 42,000 rpm. Acquired data were analyzed using a *c*(*s*) model of SEDFIT (Schuck *et al*, 2002). Resulting sedimentation coefficient distributions were transformed to standard conditions of water at 20 °C by considering density and viscosity. Density was measured using a density meter (Anton Paar DMA4500), and viscosity of FluoroBrite DMEM with 8% FBS was measured with a Lovis 2000ME viscometer.

2.8 Wnt internalization

Cells at 80% confluency were treated with exosome-depleted CS collected from co-culture or mixture of those from individual cultures stably expressing cell lines for indicated periods at 37℃ in CO_2 incubator, prior to being washed twice with PBS (-), before being lysed with SDS-PAGE sample buffer. For assays performed at 4℃, cells and exosome-depleted CS were pre-cooled on ice for 10 min before treatment, kept at 4℃, and then collected after the indicated time point.

2.9 Genome editing with the CRISPR/Cas9 system

Lrp5/6 dKO cells were generated in MDCK cells by electroporation with Cas9-gRNA RNP complexes, as described previously (Otani *et al*, 2019). For *Lrp5* targeting, gRNA1 - 5’ GATGAAGCTGAGCTTGGCAT 3’, gRNA2 - 5’ GCTGAGCACTTGAATATCCA 3’, gRNA3 - 5’ GGTCAAGGTCCTGCCAGAAG 3’ were used. For *Lrp6* targeting, gRNA1 - 5’GAGAATGCTACAATTGTAGT 3’, gRNA2 – 5’ GTGGACTTTGTGTTTGGTCA 3’, gRNA3 – 5’ GGATCTAAGGCAATAGCTCT 3’ were used. Potential target cells were screened by

Western blotting using LRP5 and LRP6 antibody, and finally confirmed by Sanger sequencing of genomic PCR products.

2.10 Wnt3a activity assay

To monitor activity of Wnt3a in *Lrp5*-KO, *Lrp6*-KO, or *Lrp5/6* dKO MDCK cells and *Fzd1-10* KO HEK293 cells, I transfected pre-plated cells with plasmids expressing firefly luciferase, which is inducible under control of a promoter containing 8 tandem repeats of the TCF/LEF1 binding sites (SuperTopFlash reporter) (Shimizu *et al*, 2012), and a *Renilla* luciferase-expressing vector under control of the TK promoter, which served as a transfection internal control. Lipofectamine LTX Plus (Thermo Fisher Scientific) was used to transfect MDCK cells and GeneJuice (Millipore) was used to transfected HEK293 cells. At 12 h post-transfection, culture medium was changed to conditioned medium of Wnt3a/L or control cells. Cells were harvested after another 12 h and luciferase activity was monitored using a Dual-Luciferase® Reporter Assay System (Promega).
Activity of fluorescently tagged Wnt5a and Wnt11 was monitored by examining activity antagonistic to Wnt3a/β-catenin signaling. A mixture of CSs of Wnt3a expressing L cells and those of parental HEK293, or HEK293 cells expressing Wnt5a or Wnt11 was used to treat SuperTopFlash (STF)/HEK293 cells that stably contain the firefly luciferase reporter gene under control of a promoter with 8 tandem repeats of TCF/LEF1 binding sites (Tsukiyama *et al*, 2015). Twelve hours after treatment, cell lysates were harvested and luciferase activity was monitored using a Luciferase Assay System (Promega).

Wnt/β-catenin signaling activity of exosome fractions was monitored by adding the P100 exosome fractions to STF/HEK293 cells. After incubation for 24 h, cell lysates were collected and firefly luciferase activity was monitored as described above.

2.11 Microscopy and image processing

Culture cells and embryos were observed with a Leica confocal microscope (SP8) with 40x objective and images were processed using ImageJ. To examine the effect of sFRP on Wnt3a distribution, 2 x 10^5 cells of FLAG-GFP-Wnt3a/L cells and sFRPs-FLAG/HEK293 cells were cultured in a ratio 3:2 for 2 days on glass-bottomed dishes pre-treated with a collagen mixture (Cellmatrix). Cells were fixed with a fixation solution for amphibian, MEMFA, for 10 min, and

then washed 3x with TBS. For tracking of GFP-Wnt3a internalization in the presence of sFRP2, living cells were treated with FM 4-64 FX (Invitrogen: F34653) at a final concentration of 5mμg/mL and monitored by time course imaging.

2.12 Quantification and statistical analysis

Error bars indicate s.e. Statistical significance was calculated by One-way Anova, Two-way Anova or Student's T-test where appropriate. Differences were considered statistically significant at $p \leq 0.05$

III. Results

3.1 sFRP2 increases exosomal re-secretion of Wnt3a

3.1.1 Effect of different Wnt-binding proteins on exosome-mediated re-secretion of GFP-Wnt3a

As described in the Introduction, in the imaginal disc of *Drosophila*, it has been proposed that secreted Wnt proteins are taken up by cells and then released again into the extracellular space (Yamazaki *et al*, 2016; Witte *et al*, 2020; Linnemannstöns *et al*, 2020). Furthermore, Wnt re-secretion is dependent on an endosomal recycling mechanism required for exosome-mediated secretion (Witte *et al*, 2020; Linnemannstöns *et al*, 2020). To examine the impact of several Wnt binding proteins on exosome-mediated re-secretion, I established an assay system to investigate exosomal re-secretion of Wnt (Figure 4). First, exosome-depleted culture supernatant (CS) was prepared by ultracentrifugation at 100,000 x *g* from L cells expressing GFP-Wnt3a. Then, secondary cells were treated with this supernatant to follow endocytosis of Wnt3a and release of Wnt3a via exosomes. I refer to this protocol, in which exosomal re-secretion of Wnt can be examined, as Re-secretion assay #1 (Figure 4A). In this protocol, the amount of Wnt3a re-secreted on exosomes was monitored by recovery in the pellet after ultracentrifugation of the culture supernatant at 100,000 x *g* (referred to as P100; Figure 4A). Release of exosome-mediated Wnt3a by monitoring GFP-Wnt3a in the P100 fraction was detectable 24 h after addition of GFP-Wnt3a CS (Figure 4B, C, D). This release was decreased by treatment with GW4869, a chemical that inhibits formation of multivesicular bodies (MVB; Figure 4E, F) (Trajkovic *et al*, 2008). Since endocytosed proteins are incorporated into exosomes via MVBs, this result indicates that this method monitors exosome-mediated re-secretion of Wnt ligands.

Then, I examined the effect of soluble Wnt-binding proteins on exosome-mediated re-secretion of Wnt3a by examining recovery in the P100 fraction (Figure 2B, C) (Chen *et al*, 2016; Momen-Heravi *et al*, 2013). MDCK cells were treated with exosome-depleted CS (input S100 fraction) of the co-culture of GFP-Wnt3a-expressing L cells and HEK293 cells with expression of sFRP2, sFRP3, or exFzd8, as well as sFRP1. As predicted, CS prepared from co-culture with exFzd8-expressing cells reduces the level of GFP-Wnt3a in the P100 fraction, suggesting that sequestration by exFzd8 reduces GFP-Wnt3a incorporation into exosomes (Figure 4B). To our surprise, CS from co-culture with sFRP1- and sFRP2-expressing cells resulted in a significant increase in the amount

of Wnt3a, whereas that with sFRP3-expressing cells showed little effect on exosome-mediated re-secretion of Wnt3a (Figure 4B, C). Because this effect of sFRP2 was also sensitive to GW4869 (Figures 4E, F), sFRP2 likely impacts exosome-mediated re-secretion of Wnt3a. Similar results were obtained using non-tagged Wnt3a (Figure 5) and in various cell lines (Figure 6).

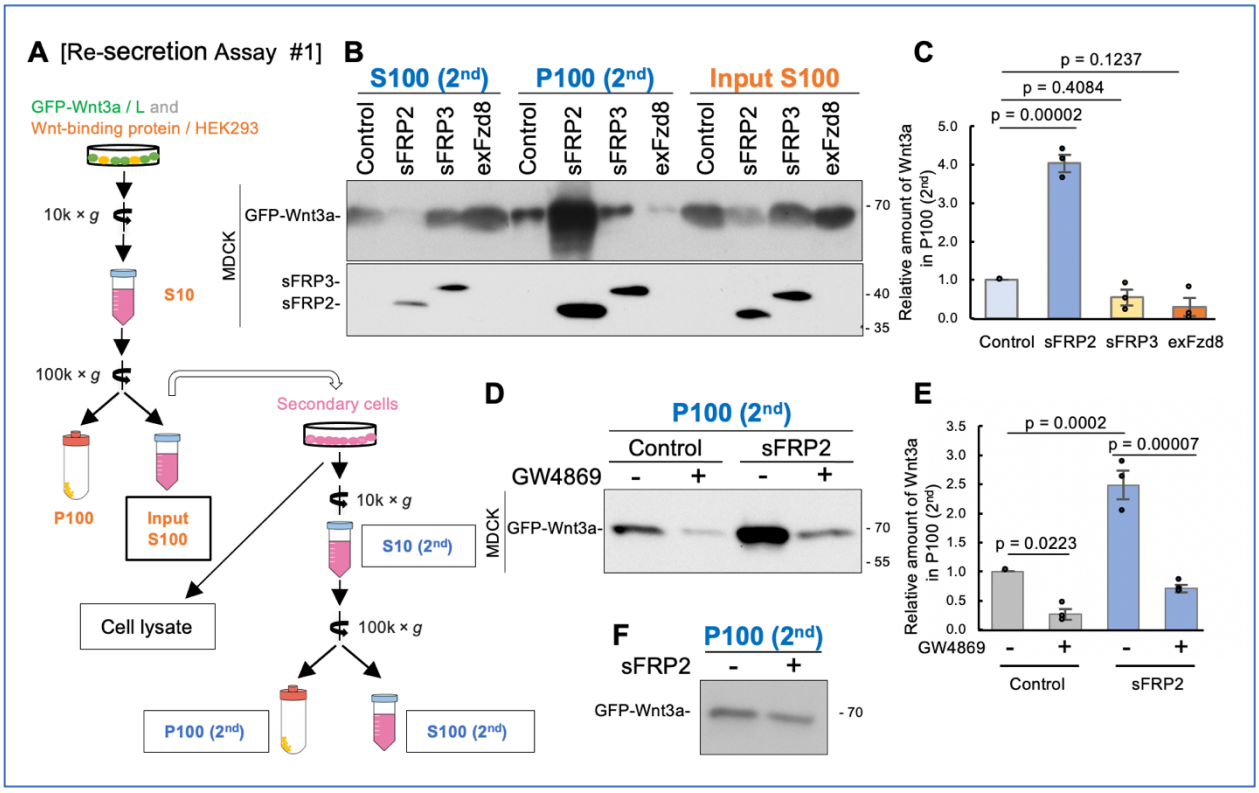


Figure 4 sFRP2 promotes Wnt3a secretion on exosomes.

A-D. Exosome-mediated re-secretion of GFP-Wnt3a from MDCK cells. The experimental procedure, referred to as Re-secretion assay, is shown in **A**. At 24 h after treatment with exosome-depleted S100 supernatant (input S100) prepared from co-culture of GFP-Wnt3a/L and HEK293 cells with or without producing sFRP1, sFRP2, sFRP3, or exFzd8, amounts of re-secreted GFP-Wnt3a from MDCK cells were examined by Western blotting after fractionation into S100 (S100 (2nd)) and P100 (P100 (2nd)) fractions by ultracentrifugation (**B, C**). Relative amounts (amount in P100 (2nd) / amount in input S100) of GFP-Wnt3a shown in **B** were examined as shown in **D**. **E, F.** The effect of MVB inhibitor on exosome-mediated re-secretion of GFP-Wnt3a. MDCK cells cultured with exosome-depleted CS (input S100) prepared from co-culture of GFP-Wnt3a cells and sFRP2-expressing cells were treated without inhibitor or with MVB inhibitor GW4689.

Amounts of GFP-Wnt3a were examined as shown in **E**. In **B, C** and **E**, S100 (2nd) and/or P100 (2nd), were subjected to Western blot to examine relative amounts of GFP-Wnt3a loaded on exosomes. Relative amounts of GFP-Wnt3a shown in (**D, F**) were quantified using Image J software and results are shown as mean ± s.e. $p < 0.05$ was considered statistically significant, using one-way ANOVA with Turkey HSD analysis.

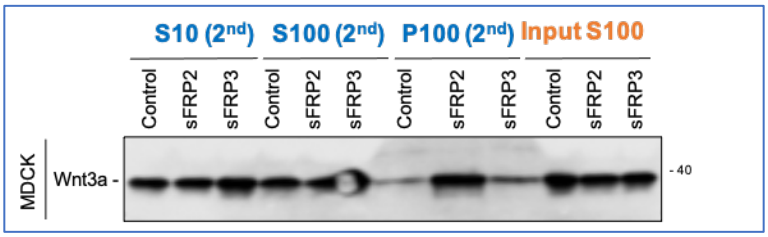


Figure 5 Effect of sFRP2 and sFRP3 on exosome-mediated re-secretion of non-tagged Wnt3a.

Experiments shown in Figure 4A were performed by replacing GFP-Wnt3a-expressing L cells with non-tagged Wnt3a-expressing L cells.

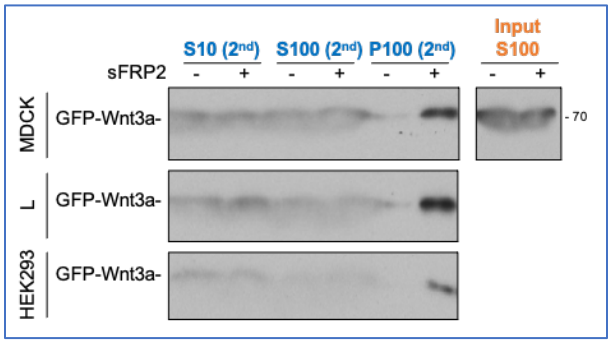


Figure 6 Increase of exosome-mediated secretion of GFP-Wnt3a by sFRP2 in various cell lines.

Experiments shown in Figure 4A were carried out utilizing various cell lines. MDCK, L, and HEK293 cells were treated with exosome-depleted co-cultured CSs (input S100) from GFP-Wnt3a-producing L cells and sFRP2-producing or control HEK293 cells, followed by P100 (P100 (2nd)) fractionation.

The effect of sFRP2 was detected with or without a GFP tag (Figure 4B, Figure 5), but CS from sFPR3, sFRP4 and exFzd8-expressing cells did not increase exosomal re-secretion of Wnt3a. Thus, the interaction between Wnt3a and sFRP2 after secretion from producing cells causes increased

Wnt3a re-secretion on exosomes. In contrast, simple incubation of exosome-depleted CS (input S100 fraction) of GFP-Wnt3a-expressing cells and that of sFRP2-expressing cells with exosome-containing medium, S10 of parental MDCK, did not increase the amount of Wnt3a in the P100 fraction (Figure 7), further suggesting that the effect of sFRP2 in increase of exosomal re-secretion of Wnt3a is cell-mediated, i.e., endocytosis and MVB-mediated re-secretion.

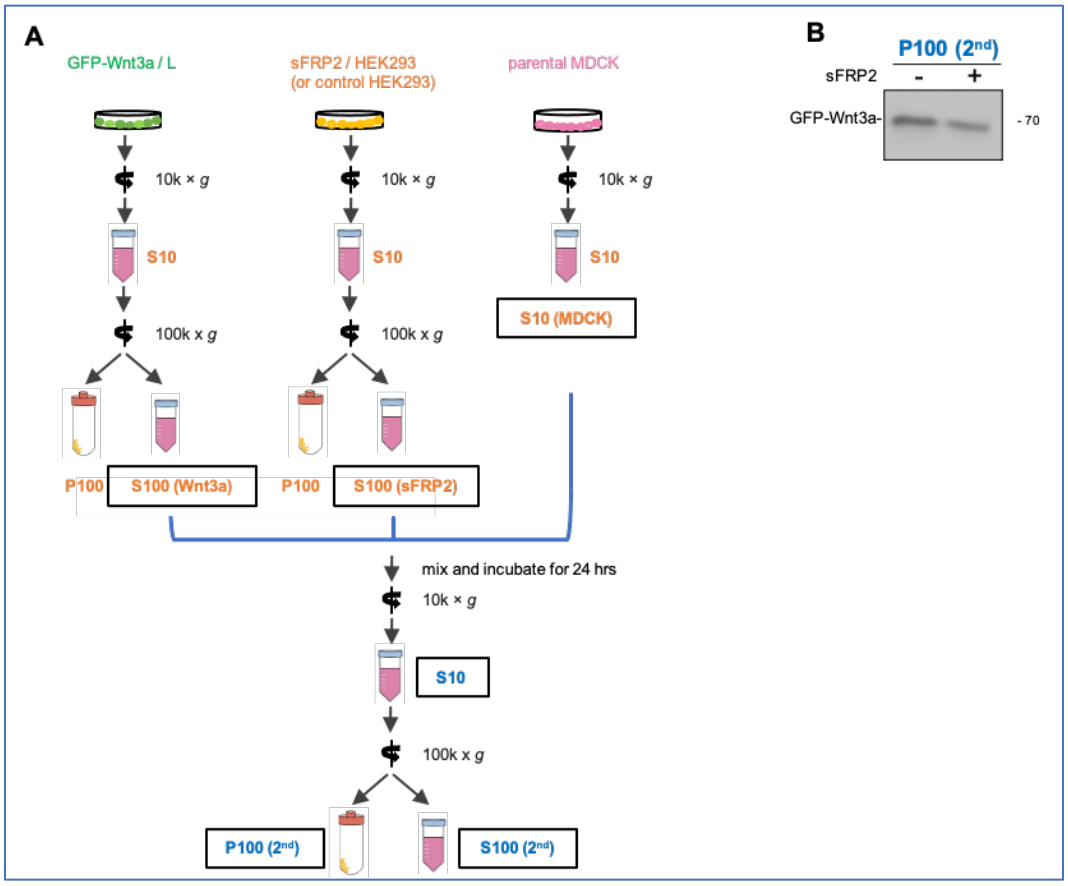


Figure 7 Exosome-mediated secretion of GFP-Wnt3a in an incubation without cell treatment.

A. Schematic figure represents the experimental procedure in free-cell condition. Separately prepared exosome-depleted CSs (S100) of GFP-Wnt3a/L and that of sFRP2-expressing (or control) HEK293 cells were mixed with S10 of parental MDCK cells, which still maintains secreted exosomes. This mixture was incubated in tubes at 37ºC for 24 h, followed by P100 fractionation. B. Western blot shows the level of GFP-Wnt3a recovers in P100 fraction.

3.1.2 Visualization of Wnt3a movement after incorporation into cells

Next, I directly visualized the movement of Wnt3a after incorporation into cells (Figure 8). The movement of mCherry-Wnt3a was examined by comparing with CD63 fused with BFP and pH-sensitive green fluorescent protein, pHluorin. Since pHluorin is not detectable at low pH, as in cytoplasm, but is detectable after secretion, I can distinguish intracellular and extracellular CD63 by fluorescence (Figure 8A-R) (Sung *et al*, 2020). Given that CD63, an exosome marker, is also enriched in the exosome secretion pathway, including MVB, this tool allows us to judge the incorporation of Wnt3a into the exosome secretion pathway (Figure 8A). In this experiment, HEK293 cells expressing BFP and pHluorin-fused CD63 were surrounded by mCherry-Wnt3a expressing L cells and sFRP2 was supplied by expression in fluorescent CD63-expressing HEK293 cells. Strikingly, under these conditions, most incorporated mCherry-Wnt3a was co-localized with cytosolic CD63 (Figure 8E, I, E', I'), which is detectable only by BFP fluorescence. In particular, many mCherry-Wnt3a proteins were assembled in huge CD63-positive cytosolic structures (purple arrowhead in Figure 8I'). These structures appeared to be MVBs because they were sensitive to MVB inhibitor, GW4869 (Figure 8K-R).

In addition, the presence of many small cytosolic CD63 and mCherry-Wnt3a double-positive vesicles suggests that mCherry-Wnt3a is trafficked via CD63-positive endosomes (pink arrowhead in Figure 8I'). Furthermore, exosomal re-secretion of mCherry-Wnt3a was also detectable because a small amount of mCherry-Wnt3a protein was co-localized with extracellular CD63, which was detectable by pHluorin fluorescence (yellow arrowheads in Figure 8I'). Time lapse imaging analysis showed the color change of CD63 and mCherry-Wnt3a double-positive puncta from magenta to yellow, indicating that the double-positive intracellular puncta were secreted (Figure 8S, T). Taken together, sFRP2 specifically increases re-secretion of Wnt3a on exosomes in various cell lines.

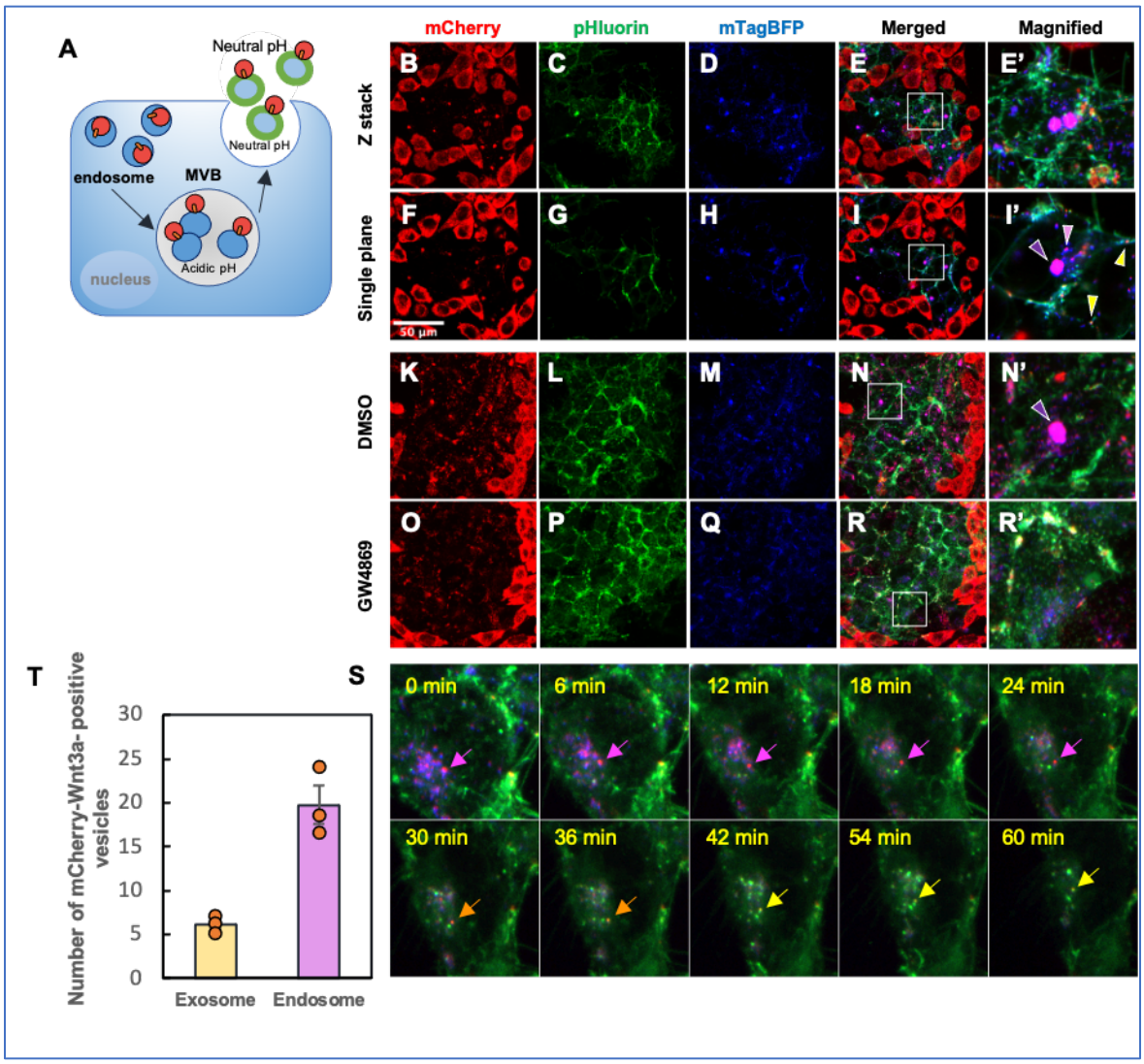


Figure 8 Visualization of Wnt3a moverment after incorporation into cells

A-R. Distribution of mCherry-Wnt3a and pHluorin-M153R-CD63-mTagBFP in sFRP2-expressing cells. To examine the localization and movement of Wnt3a after incorporation into the secondary cells, mCherry-Wnt3a producing L cells were co-cultured with HEK293 cells in which sFRP2 and pHluorin-M153R-CD63-mTagBFP were expressed. Since pHluorin is not fluorescent in acidic conditions, like cytoplasm, intracellular (BFP only) and extracellular (double-positive BFP and GFP) CD63 can be detected by different colors (**A**). Cells were inoculated 1 day before observation. mCherry-Wnt3a images (**B**, **F**), extracellular CD63 images detected by pHluorin (**C**, **G**), total CD63 images detected by mTagBFP (**D, H**), and merged images (**E, I**) of mCherry-Wnt3a and intracellular and total CD63 are shown. Z-stacked images (**B-E**) and images at single confocal planes (**F-I**) are shown. Magnified images of the area surrounded by white lines in **E** and **I** are

indicated in **E'** and **I'**, respectively. Of note, most of incorporated mCherry-Wnt3a coexisted with intracellular CD63 in large puncta (purple arrowheads), probably MVB and small puncta (pink arrowhead), probably endosomes. In addition, extracellular coexistence of mCherry-Wnt3a and CD63 was detected (mCherry, pHluorin and mTagBFP triple positive puncta; indicated by yellow arrowheads). **K-R**. Effect of MVB inhibitor to distribution of mCherry-Wnt3a and pHluorin-M153R-CD63-mTagBFP. Distribution of mCherry-Wnt3a and pHluorin-M153R-CD63-mTagBFP was examined with or without MVB inhibitor, GW4869. Experimental procedure was as same as to that shown in (**B**) to (**I**) except the addition of GW4869. **S**. Time-lapse analysis of mCherry-Wnt3a puncta in pHluorin-M153R-CD63 expressing cells with 6 min intervals. Note that mCherry-Wnt3a coexisted with CD63 gradually changes color, showing that this mCherry-Wnt3a is re-secreted with CD63. **T.** Quantification shows the number of mCherry-Wnt3a-positive vesicles normalized by the number of cells after co-culture of mCherry-Wnt3a/L and pHluorin-CD63-mTagBFP co-expressing sFRP2/HEK293 cells. Puncta double-positive for mCherry and mTagBFP indicate mCherry-Wnt3a-containing endosomes (magenta column) while those triple-positive for mCherry, GFP, and mTagBFP indicate mCherry-Wnt3a-containing exosomes (yellow column). Data are expressed as means ± s.e, in 3 different images processed by maximum intensity projection.

3.2 sFRP2 increases cell surface attachment of Wnt3a

To investigate how sFRP2 increases re-secretion of Wnt3a on exosomes, I next examined amounts of GFP-Wnt3a proteins in lysates of secondary cells after treatment with exosome-depleted CS (input S100 fraction) according to Re-secretion assay (Figure 4A). The amount of GFP-Wnt3a was clearly increased in MDCK (Figure 9A), L, and HEK293 cells (Figure 9B) in the presence of sFRP2 at 37℃. However, because sFRP2 increased the amount of GFP-Wnt3a even at 4℃, this increase is not primarily due to an increase in endocytosis. Rather, this result shows that the increase already occurred before endocytosis, probably at attachment of GFP-Wnt3a to cell surfaces (Figure 9A).

I also examined the effect of sFRP2 on distribution of Wnt3a using light microscopy. When sFRP2-expressing HEK293 cell colonies were surrounded by GFP-Wnt3a-expressing L cells, many GFP-positive puncta were clearly localized along cell boundaries of sFRP2-expressing cells

(Figure 9D, G). In contrast, this boundary localization was scarcely observed when control HEK293 and sFRP3-, exFzd8-expressing cells were used instead of sFRP2-expressing cells (Figure 9C, E, F). Furthermore, by time-lapse imaging, movement of GFP puncta from cell surfaces into cells was observed in sFRP2-expressing HEK293 cells, showing that endocytosis of GFP-Wnt3a occurs specifically in these cells (Figure 9D, G, H). These biochemical and imaging results suggest that sFRP2 enhances the association of Wnt3a with cell surfaces (Figure 9), which likely follows an increase in endocytosis (Figure 8).

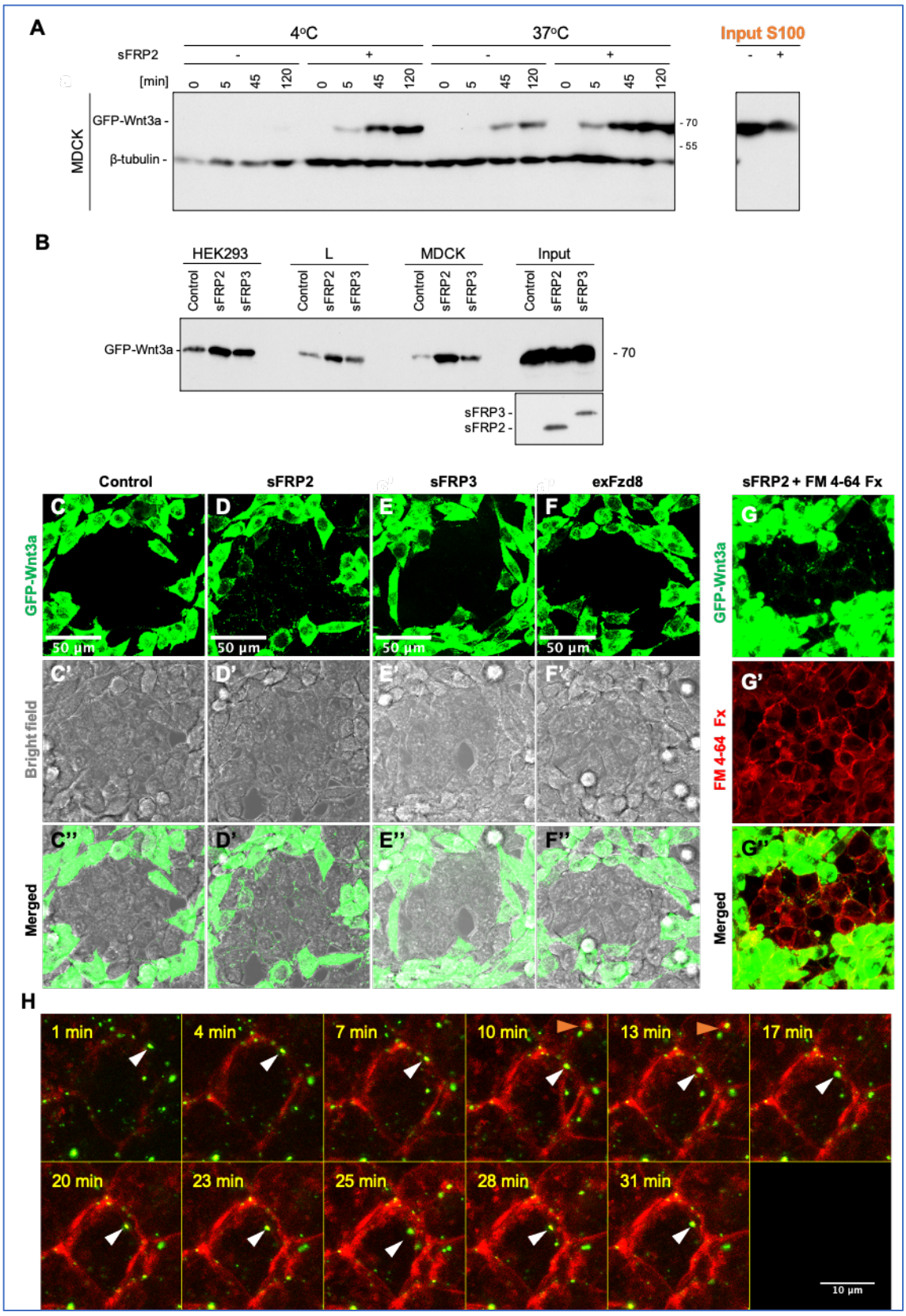

A
4°C
37°C
Input S100
sFRP2
[min]
MDCK
GFP-Wnt3a
β-tubulin
- 70
- 55
B
HEK293
L
MDCK
Input
Control
sFRP2
sFRP3
GFP-Wnt3a
- 70
sFRP3
sFRP2
Control
sFRP2
sFRP3
exFzd8
sFRP2 + FM 4-64 Fx
GFP-Wnt3a
Bright field
Merged
FM 4-64 Fx
50 µm
H
1 min
4 min
7 min
10 min
13 min
17 min
20 min
23 min
25 min
28 min
31 min
10 µm

Figure 9 sFRP2 specifically promotes attachment of Wnt3a on cell surfaces

A. Western blot detection of GFP-Wnt3a in lysates of MDCK cells collected at different times after treatment of the CS of co-culture of GFP-Wnt3a/L cells with sFRP2-expressing or control HEK293 cells. Experiments were carried out at 4ºC or 37ºC. β-tubulin was detected as an internal control. **B**. Western blot detection of GFP-Wnt3a in lysates of HEK293, L and MDCK cells collected after treatment of the CS of co-culture of GFP-Wnt3a/L cells with sFRP2, sFRP3-expressing or control HEK293 cells. Experiments were carried out at 37ºC. **C-G.** Visualization of GFP-Wnt3a in sFRP2-, sFRP3-, exFzd8-expressing, or control cells. To examine Wnt3a distribution in the presence of sFRP2 sFRP3, or exFzd8, GFP-Wnt3a-producing L cells were co-cultured with sFRP2-, sFRP3-, or exFzd8-expressing or control HEK293 cells for 2 days before observation. All images were processed by maximum intensity projection. Three independent sets were observed for each co-culture combination. GFP fluorescence images (**C-F**), bright field images (**C'-F'**), and their merged images (**C''-F''**) are shown. Images in the area where sFRP, exFzd8-expressing or control cells were surrounded by GFP-Wnt3a expressing cells are shown. In addition, FRP2-expressing cells and surrounding GFP-Wnt3a/L cells were stained with FM 4-64 Fx, a lipophilic probe that fluoresces upon binding to cell membranes and quickly endocytosed (GFP-Wnt3a image in **G**, FM 4-64 image shown in red in **G'**, and merged image in **G"**). **H.** Time-course images of GFP-Wnt3a co-stained with FM 4-64 at indicated time-point from Supplementary Movie 2. The movement of GFP fluorescent puncta with FM 4-64 in sFRP2-expressing HEK293 cells was tracked. In **G, G', G'',** cells were fixed 1 min after treatment with FM 4-64. Many puncta overlap with FM 4-64 signals following a short treatment. White and orange arrowheads in **G** indicate movement of GFP-Wnt3a puncta incorporate into cells.

3.3 Membrane proteins are involved in Wnt3a re-secretion

Given that sFRP2 increases cell-surface binding of Wnt3a, sFRP2 is likely to enhance Wnt3a interactions with membrane proteins that maintain Wnt3a on the cell surface. Thus, I next examined the effects of several membrane proteins on exosome-mediated re-secretion of Wnt3a utilizing Re-secretion assay #1. Since HSPG captures soluble ligands, including Wnt, on the cell surface, I investigated the involvement of HSPG by expressing a membrane-tethered form of Heparinase III (HepIII) in secondary cells. The increase in exosome-mediated re-secretion of Wnt3a by sFRP2 was almost negated by HepIII in MDCK cells (Figure 10A), indicating that

glycan chains on HSPG are required for the sFRP2-dependent increase in exosomal re-secretion of Wnt3a.

I next investigated whether Wnt receptors (Frizzleds, Fzd) are involved in exosomal Wnt release under the influence of sFRP2. Mice and humans express 10 members of the Fzd family (Wang *et al*, 2006; Van Amerongen & Nusse, 2009). All *Fzd* genes had already been knocked out in HEK293 cells and reported by Vanhollebeke group (Eubelen *et al*, 2018), I utilized this KO line (*Fzd1-10* KO*)* in our study (Figure 10B, C). In *Fzd1-10* KO cells, exosomal release of GFP-Wnt3a was clearly reduced with or without sFRP2 (Figure 10C), although a certain amount of GFP-Wnt3a was still released on exosomes.

To investigate whether Wnt/β-catenin signaling affects exosomal Wnt release, I asked whether LRP5 and LRP6, which act as co-receptors for Wnt3a on the cell membrane (He *et al*, 2004; Kelly *et al*, 2004; Kathleen I. Pinson *et al*, 2000) in activation of Wnt/β-catenin signaling, are required for the sFRP2-mediated increase in re-secretion of Wnt3a on exosomes. I generated *Lrp5* and *Lrp6* double knock-out (KO) MDCK cells using CRISPR/Cas9-mediated genome editing (Otani *et al*, 2019)(Figure 11A) and examined their contributions to re-secretion of Wnt3a on exosomes. However, no significant change was detected in exosome-related Wnt3a release mediated by sFRP2 (Figure11C, D), although Wnt/β-catenin signaling was significantly reduced in double KO cells (Figure 11B). Thus, sFRP2 increases exosome-mediated Wnt re-secretion independently of Wnt/β-catenin signaling, or even if this signaling is involved, some mechanism that bypasses it may exist.

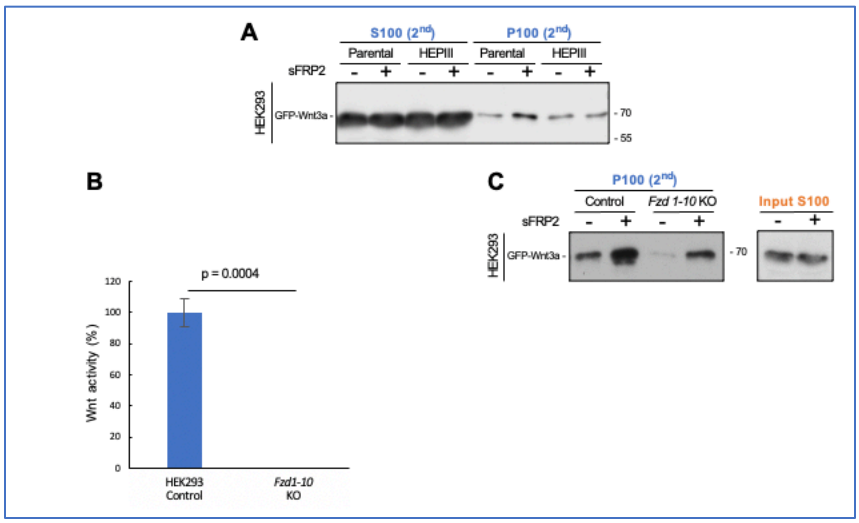


Figure 10 Effect of membrane proteins on sFRP2-induced re-secretion of GFP-Wnt3a in exosomes

A. Effect of heparinase in sFRP2-induced exosome-mediated secretion of GFP-Wnt3a. Western blot analysis of GFP-Wnt3a recovered in exosome-enriched P100 (2nd) fraction, as well as S100 (2nd) collected 24 h after treatment of exosome-depleted CS from co-culture of GFP-Wnt3a-expressing cells and sFRP2-expressing or control cells in parental or Heparinase III (HepIII) expressing HEK293 cells.

B-C. Examination of involvement of receptors of Frizzled family proteins in the increase of exosome-mediated GFP-Wnt3a re-secretion by sFRP2.

Wnt3a/β-catenin activity of *Fzd1-10* KO cells compared to control HEK293 cells was monitored by transfection of plasmids expressing SuperTopFlash reporter and *Renilla* luciferase. Reporter activity was normalized to *Renilla* luciferase activity and standardized to the activity in control or parental cells (n = 3). Results are shown as mean ± s.e. $p < 0.05$ was considered statistically significant, using Student's t test.

Fzd 1-10 KO and control HEK293 cells were treated with exosome-depleted CS (input S100) from co-culture of GFP-Wnt3a/L cells with or without sFRP2-expressing cells. P100 (2nd) fractions from these cells were subjected to Western blotting.

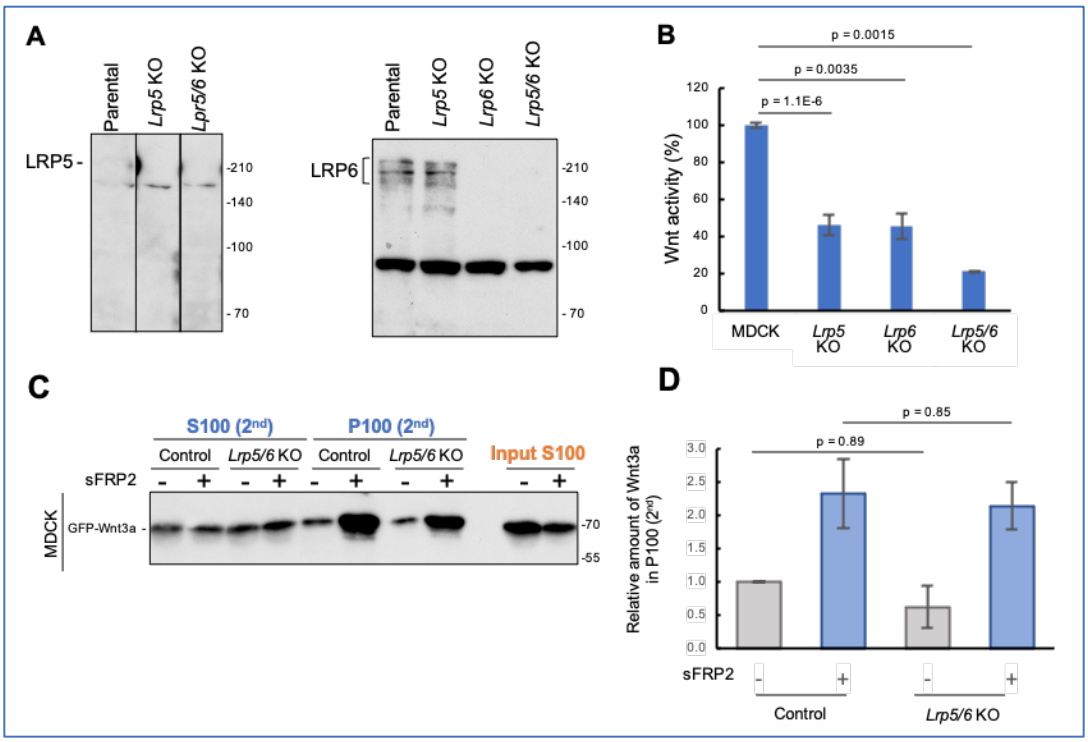


Figure 11 Examination of involvement of receptors of Frizzled family proteins in the increase of exosome-mediated GFP-Wnt3a re-secretion by sFRP2.

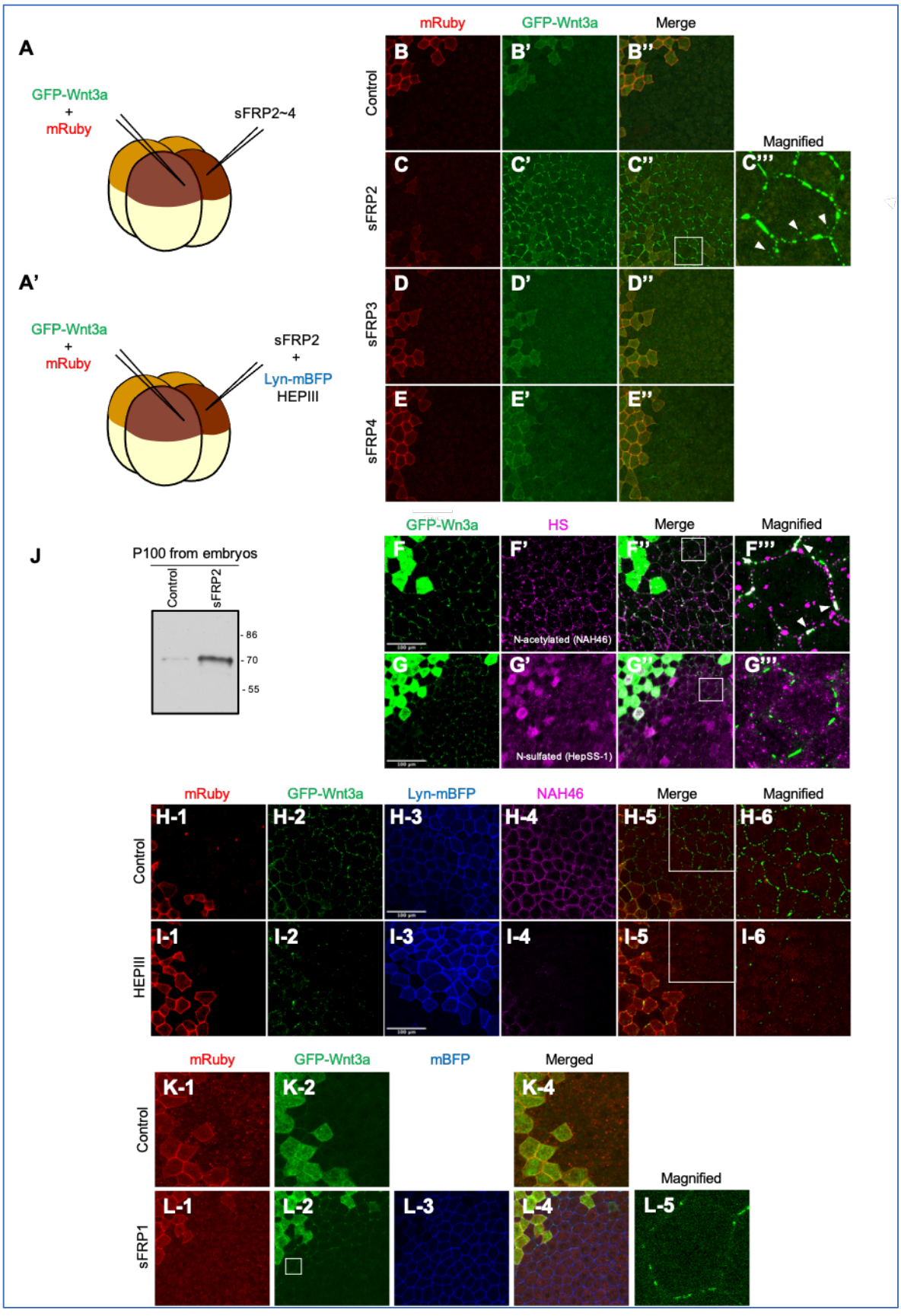
A. *Lrp5* and *Lrp6* double knock-out MDCK cells were generated by genome editing with CRISPR/Cas9 and the lack of these proteins was confirmed by Western blot analysis. LPR5 and LRP6 proteins were undetectable in double KO cells. **B.** Wnt3a/β-catenin activity of Lrp5/6 double KO cells compared to parental MDCK cells were monitored by transfection of plasmids expressing SuperTopFlash reporter and *Renilla* luciferase. Reporter activity was normalized to *Renilla* luciferase activity and standardized to the activity in control or parental cells (n = 3). $p < 0.05$ considered statistically significant, by ANOVA: One Factor with replication, followed by Turkey HSD test. *Lrp5/6* double KO and parental MDCK cells (**C**) were treated with exosome-depleted CS (input S100) from co-culture of GFP-Wnt3a/L cells with or without sFRP2-expressing cells. P100 (2nd) fractions from these cells were subjected to Western blotting. The amount of GFP-Wnt3a recovered in P100 pellets collected from CS of *Lrp5/6* double KO or parental MDCK cells was quantified with Image J and standardized against that from parental cells treated with exosome-depleted CS from co-culture of GFP-Wnt3a/L and control HEK293 cells (**D**). Data are shown as mean ± s.e. $p < 0.05$ considered statistically significant, by ANOVA: Two Factor with replication, followed by Turkey HSD test.

3.4 sFRP2 increases HSPG binding and exosomal release of Wnt3a in embryos

Previously, I showed that Wnt3a interacts with sFRP2 during diffusion in *Xenopus* embryos. In this case, sFRP2 increases Wnt3a localization on cell surfaces in embryos. To test whether this effect is specific for sFRP2, I injected GFP-Wnt3a mRNA and various sFRP mRNAs in different blastomeres at the four-cell stage and examined the effect of sFRP members on cell surface binding of GFP-Wnt3a at the gastrula stage (Figure 12A). As observed in cell culture, sFRP2 specifically increased cell-surface binding of Wnt3a in embryonic tissue. Interestingly, sFRP1 showed similar increase, but sFRP3 and sFRP4 did not (Figure 12B-E, K-L). In addition, many GFP-Wnt3a puncta were detected inside sFRP2-expressing cells (Figure 12C), indicating that endocytosis of GFP-Wnt3a was increased in the presence of sFRP2.

Since N-sulfated or N-acetylated HSPG proteins form separate clusters on cell membranes in *Xenopus* embryos (Mii *et al*, 2017), I examined the association between GFP-Wnt3a puncta and these clusters. Interestingly, localization of GFP-Wnt3a puncta was correlated significantly with N-acetyl-rich HS puncta, but less significantly with N-sulfo-rich HS puncta, which are reactive to NAH46 and HepSS-1 monoclonal antibodies, in the presence of sFRP2 (Figure 12F-G). These GFP-Wnt3a puncta were dependent on HSPG, because they disappeared with expression of a membrane-bound form of HepIII (Figure 12A', H, I). I have already shown that N-sulfo-, but not N-acetyl-, HS puncta co-localize with Wnt signalosomes, where components involved in transduction of Wnt/β-catenin signaling are assembled across the cell membrane (Mii *et al*, 2017). Therefore, the co-localization pattern of GFP-Wnt3a with N-acetyl-rich HS puncta supports the idea that sFRP2 increases cell-surface binding of Wnt3a independently of Wnt/β-catenin signaling.

To further examine the involvement of exosomes in Wnt secretion from *Xenopus* embryonic cells, I injected GFP-Wnt3a and sFRP2 mRNAs into different blastomeres at the four-cell stage (Figure 12A) and examined secreted Wnt3a on exosomes 24 h after injection (Figure 12J). To recover secreted Wnt3a in blastocoel and the extracellular space in embryos, I dissociated *Xenopus* embryos into individual cells in modified phosphate buffer with EDTA, then incubated for 5 h, followed by ultracentrifugation 100,000 x *g*. Even in this situation, sFRP2 increased the amount of Wnt3a protein recovered in the P100 fraction. Taken together, these results show that the interaction of sFRP2 with Wnt3a is specific not only in cell culture, but also in embryos.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GFP-Wnt3a
+
mRuby
sFRP2~4
A'
GFP-Wnt3a
+
mRuby
sFRP2
+
Lyn-mBFP
HEPIII
mRuby
GFP-Wnt3a
Merge
Magnified
Control
sFRP2
sFRP3
sFRP4
B
B'
B''
C
C'
C''
C'''
D
D'
D''
E
E'
E''
J
P100 from embryos
Control
sFRP2
86
70
55
GFP-Wn3a
HS
Merge
Magnified
F
F'
F''
F'''
N-acetylated (NAH46)
G
G'
G''
G'''
N-sulfated (HepSS-1)
mRuby
GFP-Wnt3a
Lyn-mBFP
NAH46
Merge
Magnified
Control
HEPIII
H-1
H-2
H-3
H-4
H-5
H-6
I-1
I-2
I-3
I-4
I-5
I-6
mRuby
GFP-Wnt3a
mBFP
Merged
Magnified
Control
sFRP1
K-1
K-2
K-4
L-1
L-2
L-3
L-4
L-5

Figure 12 sFRP2 specifically increases Wnt3a attachment on cell surfaces in vivo

Schematic figures of mRNA microinjection into fertilized *Xenopus* eggs (**A, A'**). GFP-Wnt3a mRNA and various mRNAs including sFRP mRNAs without (**A**) or with (**A'**) heparinase III (HEPIII) mRNA were injected into different blastomeres at the four-cell stage of *Xenopus* embryos. In most experiments, except that shown in **J**, injected embryos were fixed with MEMFA at stage 11.5. GFP-Wnt3a-expressing cells are marked by membrane-bound Ruby (mRuby) expression (red), while neighboring cells expressing sFRP or controls are identified by Lyn-mBFP expression (blue). **B-E.** Effect of sFRP proteins on the distribution of GFP-Wnt3a on surfaces of neighboring cells expressing sFRP2. Images of mRuby (**B-E**) and GFP-Wnt3a (**B'-E'**), as well as their merged images (**B''-C''**) are indicated. A magnified image of the area surrounded by a white line in **c''** is also shown (**c'''**). Injection with sFRP2 (**C**), but not with sFRP3 (**D**) or sFRP4 (**E**), increases GFP-Wnt3a accumulation on the cell surface and internalization into the cell (white arrowheads), compared to controls (**B**). **F, G.** Correlation in the spatial pattern of GFP-Wnt3a and HSPG at the surface of sFRP2-expressing cells. Immunostaining of fixed embryos injected with GFP-Wnt3a and sFRP2, with antibodies specific for N-acetyl-rich (NAH46; **F**) or N-sulfo-rich (HepSS-1; **G**) HS chains. Images of GFP-Wnt3a (**F, G**) and HS chains (**F', G'**), as well as their merged images (**F", G"**), are indicated. A magnified image of the areas surrounded by white lines in **F"** and **G"** is also shown in **F'''** and **G'''**. White arrowheads in **F'''** indicate co-localization of GFP-Wnt3a puncta and N-acetyl-rich HS puncta. **H, I.** Effect of heparinase III (Hep III) on GFP-Wnt3a accumulation at the surface of sFRP2-expressing cells. Accumulation of GFP-Wnt3a on sFRP2-expressing cells was examined with (**I**) or without (**H**) expression of a membrane-tethered form of HepIII. Images of mRuby (**H-1, I-1**), GFP-Wnt3a (**H-2, H-2**), Lyn-mBFP (**H-3, I-3**), as well as merged images of mRuby and GFP-Wnt3a (**H-5, I-5**), are indicated. A magnified image of the areas surrounded by white lines in **H-5** and **I-5** is also shown in **H-6** and **I-6**. To confirm activity of HEPIII to remove the HS rich region, embryos were fixed and stained with NAH46 at stage 11.5 (**H-4, I-4**). The distribution of GFP-Wnt3a in sFRP2- expressing cells **(H-2**) was disrupted by expression of HEPIII (**I-2**). **J.** Effect of sFRP2 in the exosome-mediated Wnt3a secretion in *Xenopus* embryos. Recovery of GFP-Wnt3a in the P100 fraction is shown. Equal number of embryos injected with GFP-Wnt3a with or without sFRP2 were dissociated at stage 11.5 and incubated another 5 h at 17ºC, followed by fractionation by ultracentrifugation at 100,000 x *g*. The

P100 pellet was dissolved and subjected to Western blotting**. K, L.** Effect of sFRP1 proteins on the distribution of GFP-Wnt3a on surfaces of neighboring cells.

3.5 Wnt3a forms heterodimers with sFRP2, but not with other sFRPs

Wnt forms protein complexes with carrier proteins in the extracellular space. In vertebrates, several sFRP proteins act as carriers of Wnt in cell cultures and embryos. Since sFRP1 and sFRP2, but not sFRP3 or sFRP4, specifically increase cell-surface attachment of Wnt3a, I speculated that the former two and the latter two sFRPs may differ in their interactions with Wnt3a in the CS. To test this possibility, I directly examined whether sFRPs form complexes with Wnt3a in the CS. I investigated this using a non-invasive approach, i.e., analytical ultracentrifugation with a fluorescence detection system (AUC-FDS) (Nelson *et al*, 2016; Schuck *et al*, 2002; Zhao *et al*, 2014), which allowed us to directly measure the size of molecules of interest in CS without artificial manipulation using detergent. By AUC-FDS analysis, our previously showed that sFRP2 forms heterodimers with secreted GFP-Wnt3a in the CS of GFP-Wnt3a-expressing cells co-cultured with sFRP2-expressing cells (Takada *et al*, 2018). Using this method, I tested interactions of Wnt3a with other sFRPs. Since the secretion level of sFRP1 was quite low, for unknown reasons, I examined whether GFP-Wnt3a can form protein complexes with sFRP2, sFRP3 or sFRP4. In contrast to sFRP2, GFP-Wnt3a did not form heterodimers with sFRP3 or sFRP4 in the CS under the same conditions in which GFP-Wnt3a/sFRP2 heterodimers were detectable (Figure 13A-D,). Thus, only sFRP2 can form stable heterodimers with GFP-Wnt3a under conditions in which I examined the effect of sFRP proteins on Wnt3a in cell culture. Based on these results, I speculated that the formation of stable Wnt3a/sFRP2 heterodimers is involved in efficient attachment of Wnt3a to the cell surface, followed by increased endocytosis and exosome-mediated re-secretion of Wnt3a.

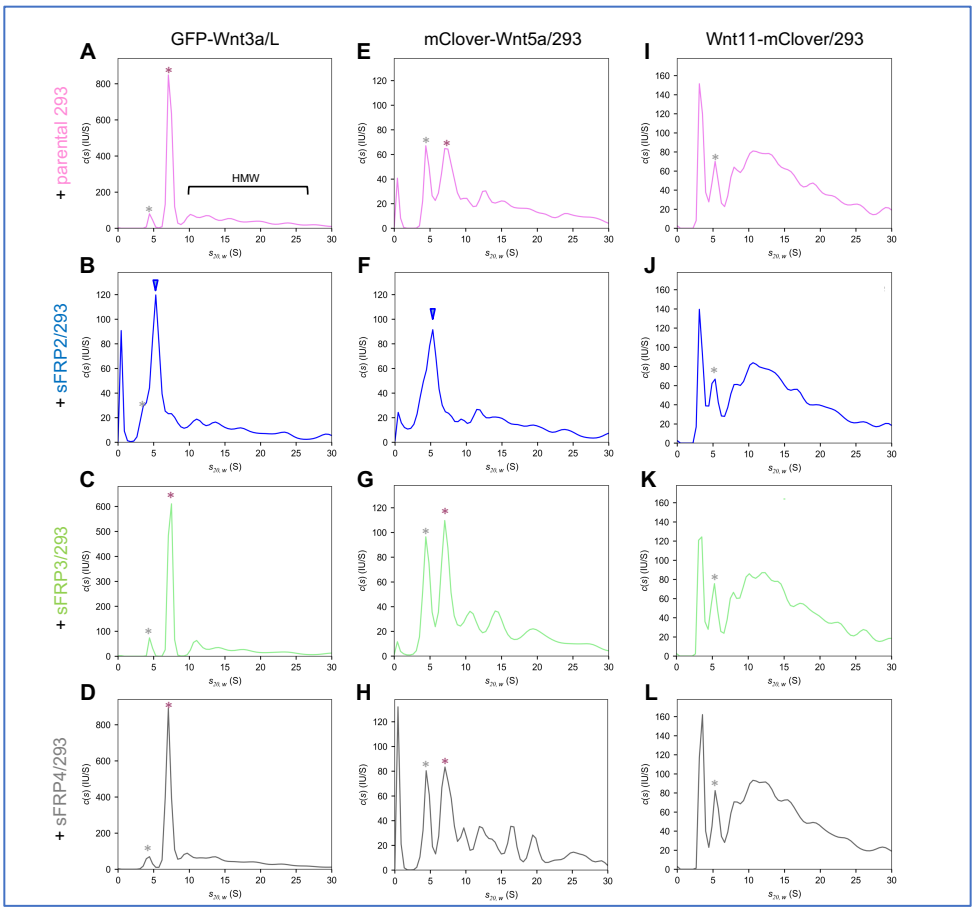

Figure 13 Analytical ultracentrifugation analyses of green fluorescent-tagged Wnt proteins with various sFRPs

AUC-FDS analysis of the CS of parental (pink; **A, E, I**), sFRP2- (blue; **B, F**, **G**), sFRP3- (green; **C, G, K**), or sFRP4-(gray; **D, H, L**) expressing HEK293 cells, co-cultured with GFP-Wnt3a/L (**A-D**), mClover-Wnt5a/HEK293 (**E-H)** or Wnt11-mClover/HEK293 (**I-L**). Peaks indicated by red asterisks corresponds those of the Wnt/afamin complex (7.0S peak, ~150 kD) while a bracket indicates high-molecular-weight (HMW) complexes. Since a small peak at ~4.2 S is detectable even in the CS of normal L cells, this fluorescence appears to be derived from serum components, probably albumin, associated with bilirubin (gray asterisks)**.** In addition, a small peak at 3S in the CS of Wnt11-mClover/HEK293 cells (**I-L**) appears due to degradation of Wnt11-mClover. As with GFP-Wnt3a, which forms a 1:1 complex with afamin, as previously reported (**A**), mClover-Wnt5a (**E**) also show peaks of the same size (S value), suggesting that mClover-Wnt5a form a 1:1 complex with afamin. In the presence of sFRP2, these peaks disappear and a new peak (indicated

by blue arrowheads) appears at 5.4S, the size of which corresponds to Wnt/sFRP2 1:1 complex, in the CS of GFP-Wnt3a (**B**) or mClover-Wnt5a (**F**), but not Wnt11-mClover.

3.6 Wnt specificity in the effect of sFRP2 on exosome-mediated re-secretion

I next examined whether sFRPs promote exosome-mediated re-secretion of other Wnt proteins, utilizing Re-secretion assay #1 or #2 (Figure 14A). Exosome-depleted CS (input S100 fraction) of Wnt5a-expressing cells mixed with that of sFRP2- or sFRP3-expressing cells were treated with MDCK or L cells. Wnt5a in lysate of secondary cells and re-secretion on exosomes was also promoted only by sFRP2 (Figure 14B, C). However, exosome-mediated re-secretion of Wnt11 and Wnt11 in lysate of secondary cells did not increase in the presence of sFRP2 (Figure 15). Consistently, AUC-FDS analysis indicated that mClover-tagged Wnt5a was able to form heterodimers with sFRP2, but not with sFRP3 or sFRP4 (Figure 13E-H), as in the case of Wnt3a. But mClover-tagged Wnt11 did not form protein complexes with any of the three sFRPs (Figure 13I-L). Thus, the ability to form heterodimers with sFRP is highly correlated with promotion of exosome-mediated re-secretion of Wnt proteins.

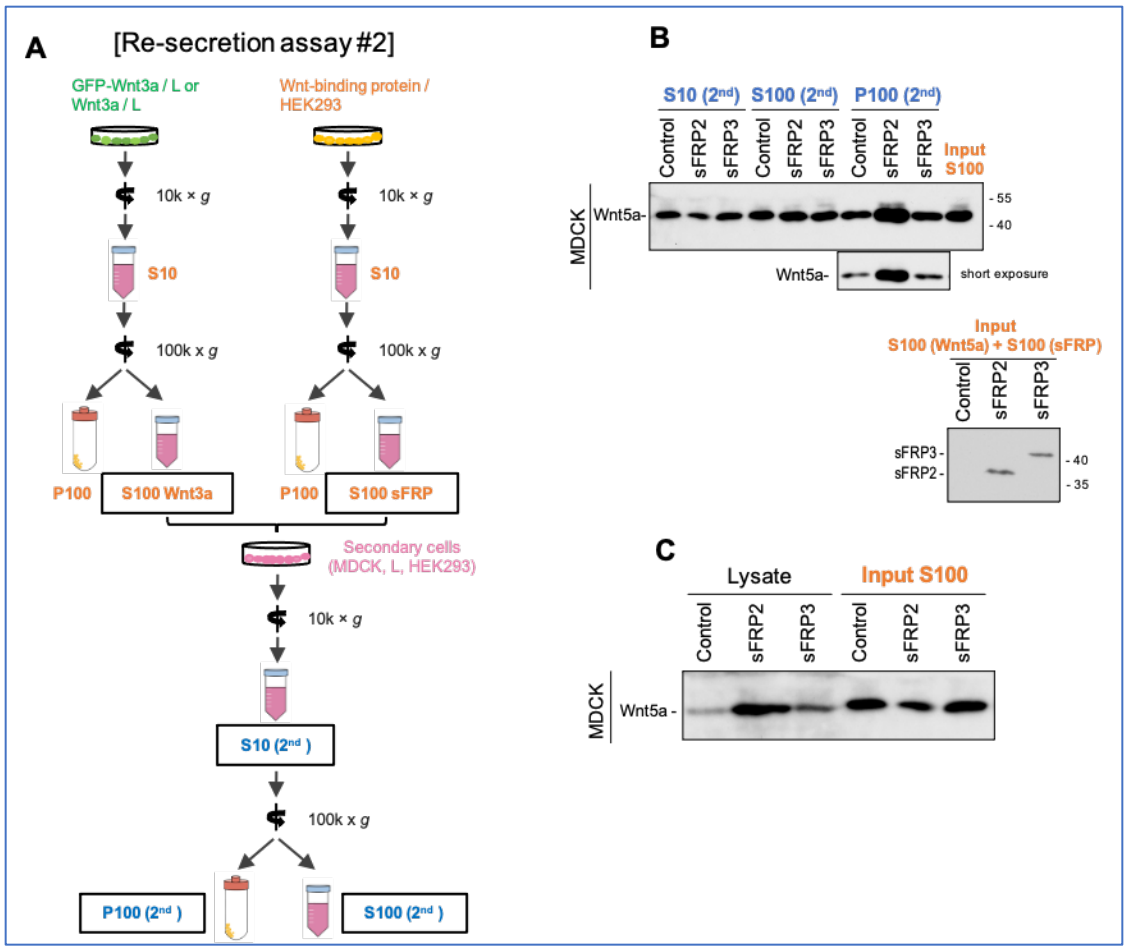

Figure 14 Re-secretion of exosomal Wnt5a is increased specifically by sFRP2

Exosome-mediated re-secretion of Wnt5a was examined according to the procedure shown in **A** (Re-secretion assay #2) for (**B).** Western blot analysis of S10 (2nd), S100 (2nd), and P100 (2nd) for examining the specificity of sFRP to Wnt5a. S100 mixture from separately cultured CSs of non-tagged Wnt5a/L cells and sFRP2-, sFRP3-, expressing or control HEK293 were used to treat MDCK cells in (**A**). **C.** Amounts of Wnt5a in the lysate of MDCK cells treated with mixture of exosome-depleted CS (S100) of non-tagged Wnt5a/L with those of sFRP2- or sFRP3-expressing, as well as control, HEK293 cells. Cell lysates were prepared 2 h after treatment of CSs.

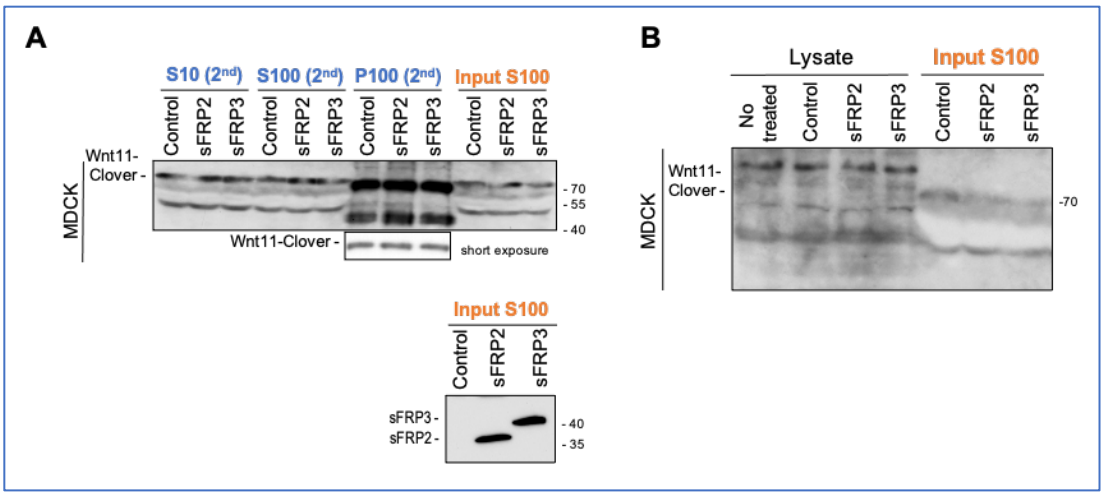

Figure 15 Re-secretion of exosomal Wnt11 is not increased specifically by sFRP2

A. Exosome-mediated re-secretion of Wnt11 was examined according to the procedure shown in Figure 4A (Re-secretion assay #1). Western blot analysis of S10 (2nd), S100 (2nd), and P100 (2nd) for examining the specificity of sFRP to Wnt11 proteins. Exosome-depleted CS of Wnt11-mClover/HEK293 cells co-cultured with sFRP2-, sFRP3-expressing or control HEK293 cells were used to treat MDCK cells in. **B.** Amounts of Wnt11 in the lysate of MDCK cells treated with mixture of exosome-depleted CS (S100) of Wnt11-mClover/HEK293 cells with those of sFRP2- or sFRP3-expressing, as well as control, HEK293 cells. Cell lysates were prepared 2 h after treatment of CSs.

3.7 Wnt heterodimerization increases re-secretion via exosomes

If heterodimer formation and subsequent dissociation are crucial for endocytosis and exosome-mediated re-secretion of Wnt ligands, a mutant form of Wnt3a that cannot form heterodimers with sFRP2 should be defective in sFRP2-mediated enhancement of these processes. Previously, our group found that a mutant form of Wnt3a, Wnt3a-C77A, in which Cys77 is substituted for Ala, aggregates, forming barely dissociable protein complexes (Takada *et al*, 2018; Zhang *et al*, 2012). Using AUC-FDS analysis, our group found that Wnt3a-C77A forms heterodimers poorly with sFRP2 (Figure 16A, B). In addition, the amount of Wnt3a in the cell lysate was apparently decreased in MDCK cells co-cultured with CS of Wnt3a (C77A)-expressing L cells (Figure 16C). Furthermore, I found that membrane association of GFP-Wnt3a-C77A (Figure 16D-G) and the effect of sFRP2 in increase of exosomal Wnt3a-C77A re-secretion is reduced, compared to control

Wnt3a (Figure 16H). Thus, exosome-mediated re-secretion with sFRP2 seems to be crucial for sFRP2-mediated endocytosis and exosome-mediated re-secretion of Wnt3a (Figure 17).

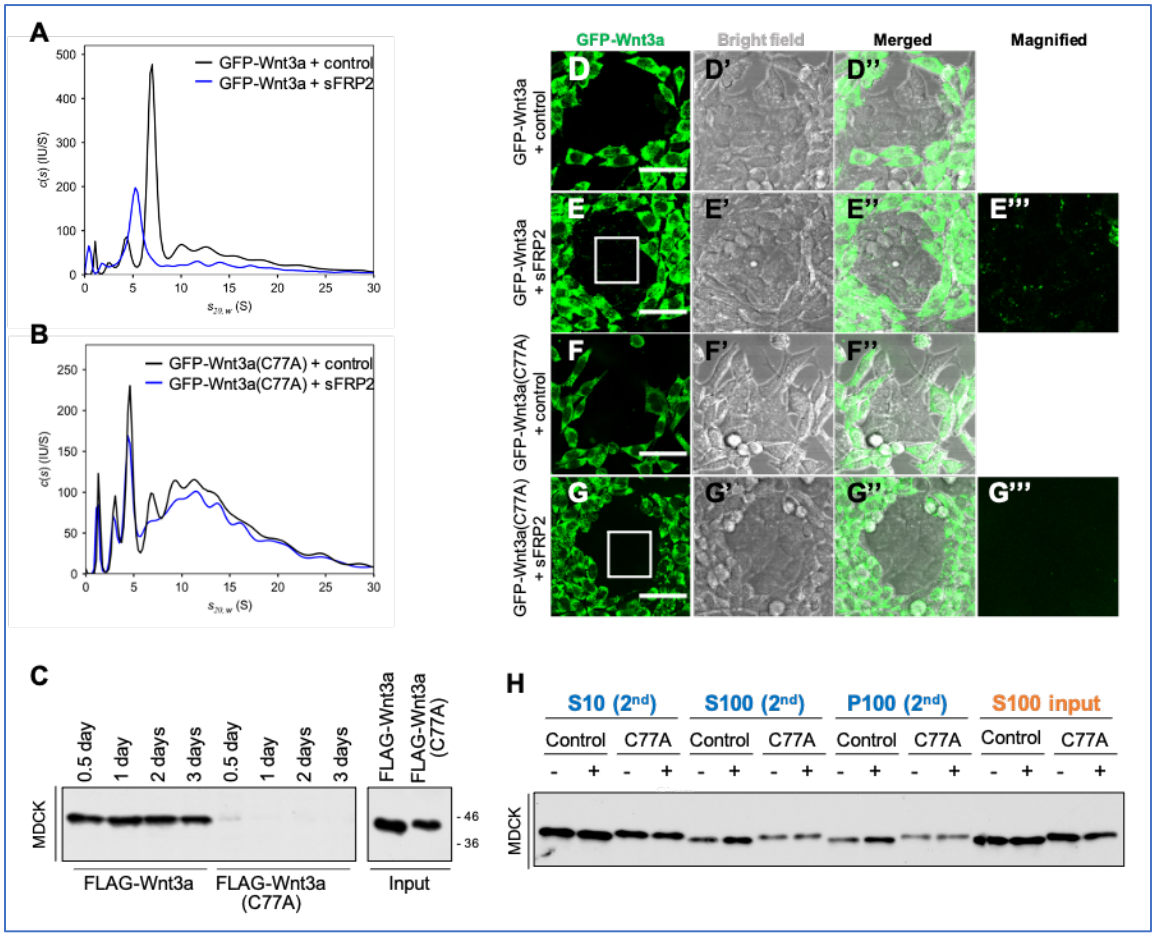

Figure 16 Wnt3a mutant defective in heterodimer formation with sFRP2 prevents re-secretion of Wnt3a on exosomes

A, B. Analysis with AUC-FDS of the CS from co-culture of GFP-Wnt3a (**A**) or GFP-Wnt3a (C77A) (**B**) expressing L cells with sFRP2-expressing or control HEK293 cells. In the presence of sFRP2, a shift in the peak position of control GFP-Wnt3a was observed, whereas peak positions of GFP-Wnt3a (C77A) remained largely unchanged. Thus, in contrast to control GFP-Wnt3a, heterodimers of GFP-Wnt3a (C77A) and sFRP2 were not formed. **C**.Western blot for detection of FLAG-Wnt3a in the lysate of MDCK cells treated with exosome-depleted CS of FLAG-Wnt3a or FLAG-Wnt3a (C77A) for indicated periods.

D-G. Distribution of GFP-Wnt3a (C77A) in sFRP2-expressing cells. To examine whether the Wnt3a (C77A) mutant is defective in distribution in the presence of sFRP2, GFP-Wnt3a- (**D, E**)

or GFP-Wnt3a (C77A; **F, G**) producing L cells were co-cultured with sFRP2-expressing cells (**E, G**), or control HEK293 cells (**D, F**) from 2 days before observation. All images were processed by maximum intensity projection. Images of GFP-Wnt3a (**D-G**), bright field (**D'-G'**), and their merged images (**D"-G"**) are shown. Magnified images of areas surrounded by white lines in **E** and **G**, are also shown in **E'''** and **G'''.**

H. Examination of exosome-mediated secretion of Wnt3a (C77A) following Re-secretion assay #1. Western blot for detection of Wnt3a recovered in S10, S100 and P100 fractions collected from CS of MDCK cells cultured with S100 of non-tagged Wnt3a/L or FLAG-Wnt3a (C77A)/L co-cultured with sFRP2-expressing or control HEK293 cells are shown.

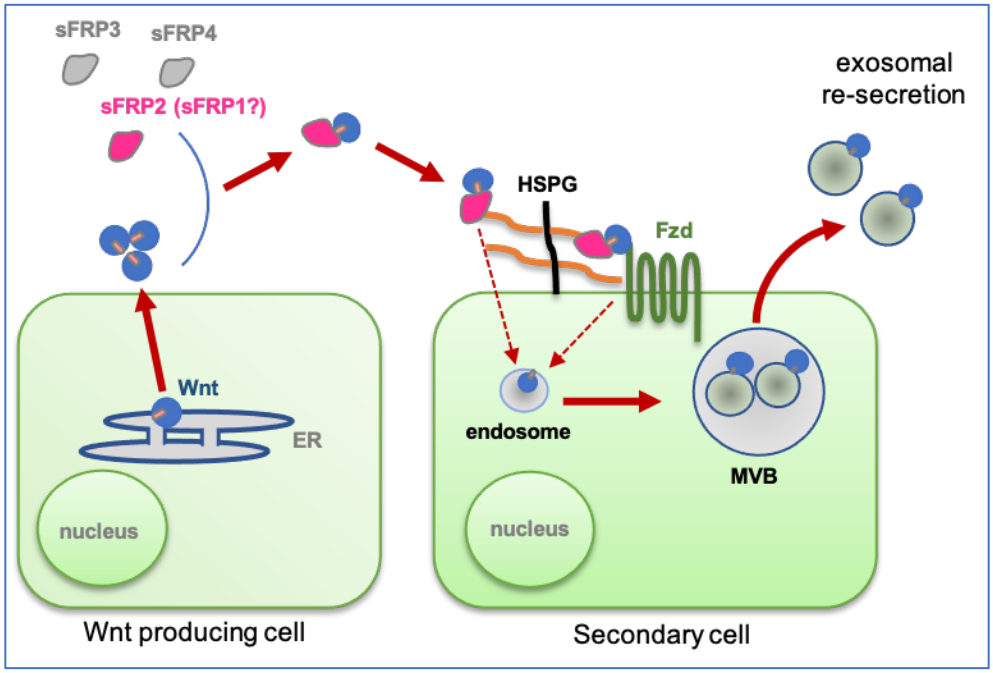

Figure 17 Schematic picture represents exosomal Wnt re-secretion model.

IV. Discussion

4.1 Re-secretion of Wnt3a on exosome is mediated by members of Frizzled-related proteins family

In this study, by establishment of a cultured cell assay system and Xenopus embryo system to recover Wnt re-secretion on exosome, I detected a significant increase in the level of Wnt3a ligands re-secreted on exosome in the presence of sFRP1 and sFRP2, but not sFRP3, sFRP4 or exFzd8. In addition, co-culture of GFP-Wnt3a expressing L cells with sFRP2 expressing HEK293 cells or microinjection of mRNA of GFP-Wnt3a and sFRP2 observed many GFP-positive puncta, clearly localized along cell boundaries of only sFRP2-expressing cells (Figure 9, 12). Interestingly, this localization in the cell boundaries of neighboring sFRP2-expressing cells and this effect of sFRP2 in the increase of re-secreted exosomal Wnt3a level were cancelled by expression of the membrane-bound form of heparinase, which cleaves HS chain in HSPG. Notably, this membrane-bound Wnt3a was co-localized with HSPG in the presence of sFRP2 in Xenopus embryos. Our results suggest that sFRP2 increases Wnt association with HSPG on cell membranes, resulting in increased Wnt internalization and exosome-mediated re-secretion.

On the other hand, sFRP proteins were initially thought to act as antagonists, primarily by sequestering Wnt (Leyns & Bouwmeester, 1997; Lin *et al*, 1997; Wang & Krinks, 1997). In addition to their antagonism of Wnt signaling, several sFRP proteins also act as carriers to transport Wnt ligands over long distances (Mii & Taira, 2011, 2009). Therefore, these conflicting results, including results in this study, suggest that sFRP may have multiple roles in Wnt signaling and behavior in tissues.

4.2 Wnt and sFRPs interaction

As discussed above, the conflicted results regarding sFRP2 multiple functions on Wnt signaling raise one of the important points to characterize the interaction between Wnt ligands and different members of sFRP family.

sFRPs are mediators of the Wnt signaling and proposed to antagonize Wnt activity by directly binding to Wnt ligands, then preventing Wnt-Fzd interaction. Wnt3a binds to four sFRPs (sFRP1, sFRP2, Fzb (sFRP3), sFRP4) with affinities in the nanomolar range (Wawrzak *et al*, 2007).

However, previous reports were conducted by using purified Wnt and sFRPs. During purification, the required addition of detergents (1% CHAPS) caused the dissociation of intact extracellular Wnt complexes, resulted in detection of only monomeric form of Wnts. Therefore, I believe that it is important to carefully consider the binding affinity between Wnt and sFRP under conditions that are more nearly physiological.

In this study, taking the advantage of non-invasive approach, AUC with a fluorescence detection system, which enables to examine protein complexes from cultured media without any requirement of detergent, it could be possible to measure the interaction of Wnt3a, Wnt5a and Wnt11 with three sFRPs (sFRP2, sFRP3 and sFRP4) secreted in culture supernatant. Unfortunately, I have not yet been able to obtain CS containing concentrations of sFRP1 high enough for AUC analysis. Using this method, the size of the Wnt complex can be measured in culture supernatant because Wnt proteins are labelled with a fluorescent tag (Takada *et al*, 2018). To our surprise, only sFRP2 and Wnt3a form stable heterodimers. Similarly, Wnt5a formed a stable heterodimer only with sFRP2, but Wnt11 could not form a complex with any of these three sFRPs. Since complex-forming ability is correlated with specificity of sFRP-induced cell surface accumulation and exosome-mediated re-secretion of Wnt proteins, I hypothesized that heterodimer formation under natural conditions is involved in the accumulation of Wnt on cell membranes, which seems to be followed by exosome-mediated re-secretion. In fact, a Wnt3a mutant with impaired heterocomplex-forming ability (Takada *et al*, 2018; Zhang *et al*, 2012) significantly reduced cell membrane accumulation and exosome-mediated re-secretion. I believe that these results strongly support our hypothesis. However, it is also necessary to investigate in more detail whether increasing binding of Wnt/sFRP2 heterodimers to HSPGs really contributes to increased Wnt endocytosis and exosomal re-secretion. At present, I cannot exclude the possibility that sFRP2 may also be involved in some other process of Wnt endocytosis, besides binding to HSPGs.

Interestingly, three members of the sFRP family proteins, sFRP1, sFRP2, and sFRP5 are more similar in amino acid sequence than sFRP3 and sFRP4. Thus, I assume that some structural characteristics common to the former group may be involved in binding Wnt3a and Wnt5a, which results in increased cell surface binding and exosomal re-secretion. In this study, I showed that sFRP1 increases cell surface accumulation and exosomal re-secretion of Wnt3a. Although AUC

analysis with sFRP1 has not yet succeeded, due to failure to establish a cell line that secretes high amounts of sFRP1, our results suggest that some structural characteristics common to the sFRP2-related subfamily may be crucial for binding to Wnt proteins

4.3 Involvements of membrane proteins in sFRP2-mediated exosomal re-secretion of Wnt

Interestingly, western blotting results showed that after treatment at 4°C of culture supernatant from co-culture of GFP-Wnt3a and sFRP2 expressing cells, where endocytosis is normally blocked, the level of Wnt3a in cell lysate was not decreased, but even slightly increased compared to those treated at 37 °C. These surprising results suggest the binding of Wnt ligands occurred before internalization even.

This study raises the question of how Wnt/sFRP2 heterodimers are incorporated into cells. Given that both cell surface binding and exosomal re-secretion of Wnts are dependent on HSPGs, it may be that Wnt/sFRP2 heterodimers are incorporated into cells with HSPGs. On the other hand, it also seems probable that Wnts, once trapped by HSPGs, are transported to other cell surface molecules that are incorporated into cells with Wnts. In terms of the latter possibility, this study shows that LRP5 and LRP6 of co-receptors (He *et al*, 2004; Kelly *et al*, 2004; Kathleen I. Pinson *et al*, 2000) are dispensable for the sFRP2-mediated increase of exosome-mediated re-secretion of Wnt ligands. This indicates that at least some molecular mechanism for signal transduction of Wnt ligands across the cell membrane is not required for Wnt endocytosis mediated by sFRP2. On the other hand, our results also indicate that some Fzd receptors are involved in this endocytosis, because loss of all Fzds results in reduction of exosome-mediated Wnt re-secretion. Since this reduction was not complete, it is probable that both Fzd-dependent and -independent mechanisms are involved in Wnt endocytosis enhanced by sFRP2. Thus, I speculate that some Wnts bound to HSPGs may be transferred to Fzd and incorporated into the cell, and that some may be taken up by a different mechanism, such as by cells with HSPGs. It will be interesting to understand how differences in endocytic mechanisms affect Wnt signaling and re-secretion.

4.4 Significance of sFRP2 in transport of extracellular Wnt ligands

Given that sFRP2 forms a stable complex with Wnt3a and Wnt5a, results of this study indicate that sFRP2 is directly involved as a carrier protein in extracellular transport of these Wnt proteins and indirectly promotes their re-secretion on exosomes. Which mode of Wnt transport is more efficient in dispersing Wnt proteins over long distances is still uncertain. Since it is assumed that each mode of Wnt transport has its own characteristics, the extent to which these two modes are used can affect the spatial pattern of Wnt activation in tissues. As far as I have investigated in cell culture, the amount of Wnt protein secreted on exosomes is much less than that which directly forms heterodimers with sFRP2 in the CS. Therefore, I speculate that carrier-protein-mediated transport may be the dominant means of transport of Wnt proteins and that exosome-mediated transport may serve a complementary function, at least in conditions like our cell culture system. On the other hand, it seems plausible that exosome-mediated re-secretion is involved in fine tuning the spatial pattern of Wnt signaling in tissues. In many developing tissues, sFRPs are expressed with Wnt and are likely to modify Wnt gradients. In cases in which sFRP2 acts as an antagonist to Wnt ligands, sFRP2-mediated exosomal re-secretion should change the impact of sFRP2 on Wnt signaling from negative to positive. As a result, the extent of this change should affect the spatial pattern of Wnt signaling gradients. Since the range of exosome-mediated Wnt transfer is still controversial, it is uncertain how long the exosomal resecretion affects Wnt gradient formation. Whether and how heterodimer-formation specificity of sFRP-mediated exosomal re-secretion is involved in regulation of Wnt signaling will have to be clarified in future studies.

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