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# Mammalian Wnt3a is Released on Lipoprotein Particles

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**Little is known about the release and intercellular transport of Wnt proteins from mammalian cells. Lipoproteins may act as carriers for the intercellular movement and gradient formation of the lipid-linked morphogens Wingless and Hedgehog in *Drosophila*. To investigate whether such a mechanism can occur in mammals, we have studied Wnt release in cultured mammalian cells. Wnt3a associated with lipoproteins in the culture medium and not with extracellular vesicles or exosomes. Although Wnt3a was associated with both high-density lipoproteins (HDL) and low-density lipoproteins, only HDL allowed Wnt3a release from mouse fibroblasts. Remarkably, Wnt3a lacking its palmitate moiety was released in a lipoprotein-independent manner, demonstrating the dual role of palmitoylation in membrane and lipoprotein binding. We additionally found that Wnt3a can be released from enterocyte cell lines on endogenously expressed lipoproteins. We further discuss the physiological implications of our findings.**

**Key words: lipid modification, lipoproteins, lipoprotein receptors, signaling, Wnt3a**

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Wnt proteins constitute a family of signaling molecules that control growth and patterning during animal tissue development (1) as well as the maintenance and the regeneration of adult tissues (2,3). Disturbances in Wnt signaling result in both degenerative diseases and cancer (4). In mice, about 20 different Wnt proteins have been identified serving different functions (for more information, visit the Wnt homepage: http://www.stanford.edu/~rnusse/wntwindow.html). Wnt proteins act as morphogens. They are expressed locally, released into the extracellular space where they establish a concentration gradient eliciting distinct, concentration-dependent responses in the neighboring cells. However, Wnt proteins tightly bind to membranes, most likely through covalent lipid modifications. Several family members have been shown to be modified by a palmitate moiety at a conserved cysteine residue and by a palmitoelate moiety at a conserved serine residue (5–9). Whereas the first lipid modification is important in Wnt signaling activity (5,8,10), the second modification is involved in proper intracellular sorting (6,7,9,11). Another important morphogen is Hedgehog, which is also lipid modified (12). How molecules with strong membrane affinity can spread in an aqueous environment and how their concentration gradient is formed remain elusive (13). Several mechanisms have been proposed to explain how the lipid-modified morphogens Hedgehog and Wnt move through tissues. One of the simplest models is the formation of micelle-like multimers (14) in which the hydrophobic groups of Wnt or Hedgehog are arranged in such a way that the protein complex becomes soluble in a polar medium. Another possibility is that cells pass on Wnt proteins through cell–cell contact sites or through long cellular extensions called cytonemes (15,16). Alternatively, Wnt proteins may assemble onto membrane vesicles that are released from cells, such as exosomes (17,18), surfactant-like particles (19) or nodal vesicular parcels (20).

Recently, lipoprotein particles were proposed to act as vehicles for the intercellular movement of lipid-modified proteins (21). Lipoprotein particles are large, globular complexes composed of a central core of hydrophobic lipids that are associated with apolipoproteins and surrounded by a monolayer of membrane phospholipids. Lipoproteins allow intercellular transport of water-insoluble lipids and fat throughout the circulation of multicellular organisms. In theory, the lipid modification of Wnt can anchor the protein in the exoplasmic leaflet of cell membranes as well as in the outer phospholipid layer of lipoproteins. *Drosophila* Wingless/Wnt was found to copurify with lipoproteins from tissue homogenates and with lipoprotein particles in the developing wing epithelium. Furthermore, reduction of lipoprotein levels narrowed the range of Wingless signaling (21). However, direct evidence that lipoproteins function as intercellular carriers for lipid-modified morphogens is lacking. Furthermore, it is unclear whether this mechanism could operate in organisms other than *Drosophila*. For the transport of lipid-modified proteins between cells, they first have to be extracted from





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the membrane of the producing cells and then they have to be transported and bind to the receiving cells. In this study, we have investigated whether lipoproteins are involved in the release of Wnt3a from mammalian cells.

Studying the role of lipoproteins as protein carriers is hampered by the complexity of multicellular organisms, especially in mammals (13). More than 10 different apolipoproteins are expressed, which form or associate with different classes of lipoproteins. Furthermore, the formation of lipoproteins depends on the developmental stage and is tissue specific. Additionally, a number of lipoprotein receptors are expressed that bind more than one kind of lipoprotein and can act redundantly. The circulation and the extracellular space contain significant amounts of lipoproteins, whereas the action of lipid-modified proteins is restricted in space. Another experimental complexity is that interference with lipoprotein metabolism in living animals gives rise to disturbances in nutrient supply, complicating the interpretation of phenotypes. For these reasons, we studied the release of Wnt from cultured mammalian cells.

## Results

### *Active Wnt3a cofractionates with lipoproteins*

Mouse fibroblast L-cells stably transfected with Wnt3a (L-Wnt3a) release active Wnt3a in the medium (5). To 2 determine if the protein is either present in the medium as a soluble protein or associated with exosomes or with lipoprotein particles, we fractionated conditioned medium by differential centrifugation and analyzed all fractions by western blotting. To mark exosomes, we additionally transfected L-Wnt3a cells with CD63-green fluorescent protein (GFP). CD63 is a tetraspanin that localizes to internal vesicles of multivesicular bodies and is released on exosomes (22,23). Exovesicles were pelleted by ultracentrifugation, and in contrast to CD63, which was pelleted completely, Wnt3a remained in the supernatant, as did lipoproteins (Figure 1A). To distinguish between freely soluble Wnt3a and Wnt3a with lipoproteins, the supernatant was subjected to isopycnic density centrifugation. During this procedure, lipoproteins float to the top of a self-forming density gradient, whereas soluble proteins migrate to the bottom fractions of the gradient. Wnt3a (Figure 1B) as well as Wnt3a-myc (Figure 3A) cofractionated with lipoproteins in the top low-density fraction, while soluble proteins, such as immunoglobulin Gs (IgGs), are present in bottom, higher density fractions (Figure 1B). This suggests that Wnt3a was associated with lipoproteins in the medium. Because of its lipid modifications, Wnt proteins are hydrophobic, which could target them to cellular membranes. Site-directed and naturally occurring mutations of the palmitate-modified cysteine (C77 of mouse Wnt3a) gave rise to normally released but inactive Wnt, indicating that the lipid moiety is important for signaling (5,6,8). Removal of the palmitate moiety by site-directed mutagenesis resulted in efficient release of Wnt3a-C77S-myc from L-cells (Figure 1B). Wnt3a-C77S-myc was present as a soluble protein in the medium because it migrated to the bottom fractions together with soluble serum proteins but no longer cofractionated with lipoproteins on isopycnic density gradients (Figure 1B). Taken together, these results suggest that Wnt associates with lipoprotein particles in the medium, depending on the presence of the palmitic anchor. Most likely, one lipid modification is not sufficient for binding of Wnt to membranous structures, as it is known for small guanosine triphosphatases (GTPases) (24).

Mammals have five major classes of plasma lipoproteins that can be distinguished on the basis of their physical density using fractionation techniques. To investigate on which class of lipoproteins Wnt3a is present, we separated conditioned medium from L-Wnt3a cells by discontinuous density centrifugation (25). Wnt3a was found in the low-density lipoproteins (LDL) as well as in the high-density lipoproteins (HDL) density fractions (Figure 1C). The physical interaction of Wnt3a with lipoproteins in the medium was directly assessed by coimmunoprecipitation. L-Wnt3a cells were incubated in the presence of human or bovine serum, and Wnt3a was precipitated with anti-Wnt3a antibodies. To detect lipoproteins, we used antibodies that recognize human but not bovine apolipoprotein AI and B100, the major protein constituents of HDL and LDL, respectively (Figure 1D).

To determine whether Wnt3a is active under these con- 3 ditions, we subjected conditioned medium of L-Wnt3a cells to isopycnic density fractionation and divided the gradient in 3 or 12 fractions, exchanged the KBr solution to normal medium and performed a luciferase-based activity assay using the superTopflash system (STF, 26). In this system, luciferase is expressed as a reporter gene, responding to Wnt signaling activity. When fractions were incubated with L-cells stably expressing STF for 24 h, only the lipoprotein-containing fraction induced luciferase activity (Figure 1E).

### *Wnt is released on HDL*

From the preceding data, we concluded that Wnt3a in the medium is present on both HDL and LDL particles. We wondered how Wnt3a is transferred from cells to lipoprotein particles in the medium. Secreted proteins having hydrophobic properties might generally stick to membranous serum components like lipoproteins after they are released from cells. Alternatively, Wnt3a may be selectively delivered to lipoproteins. When L-Wnt3a cells were incubated with increasing amounts of delipidated fetal calf serum (DL-FCS), Wnt3a was not found in the medium, showing that the presence of lipoproteins is required for its release (Figure 2A). In contrast, mutant Wnt3a lacking its palmitic anchor was released into the medium also in the absence of lipoproteins (data not shown). The addition of increasing amounts of LDL did not lead to release of

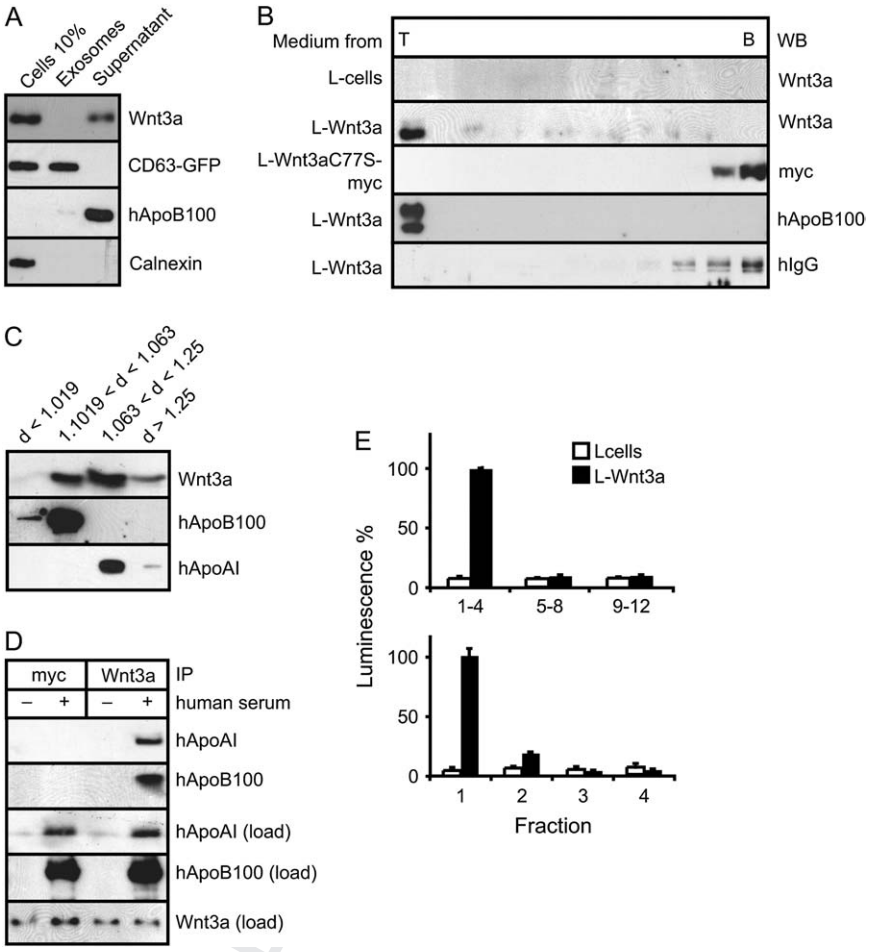


**Figure 1: Association of Wnt3a with lipoprotein particles.A)**L-cells stably expressing Wnt3a (L-Wnt3a) were transiently transfected with CD63-GFP and incubated with culture medium containing 10% FCS and 0.5% human serum for 3 days. Cells were scraped and pooled with the 10 000 × ***g***pellet from the medium (cells). The supernatant was centrifuged for 1 h at 120 000 × ***g***and separated into pellet (exosomes) and supernatant (S120). B) Twelve fractions were taken from top (T) to bottom (B) of KBr isopycnic density gradients of the S120 from media of L-cells stably or transiently (mock) expressing Wnt3a or mutant Wnt3a-C77S-myc (L-Wnt3a-myc) and analyzed by western blotting for the migration of Wnt proteins and compared them with lipoprotein-associated proteins (h-apoB100) or soluble proteins (hIgGs). C) S120 from media of L-Wnt3a cells were analyzed by discontinuous KBr density fractionation to separate different classes of lipoproteins and analyzed for the presence of Wnt3a and apolipoproteins (h-apoAI and h-apoB100) by western blotting. D) L-Wnt3a cells were incubated with culture medium containing either 10% FCS (–) or 5% FCS and 5% human serum (+) for 3 days. Wnt3a from the medium was adsorbed to protein A–Sepharose with rabbit antisera against Wnt3a or myc as a control (IP). Immunoprecipitates were subjected to SDS–PAGE and western blotting. Coprecipitated human lipoproteins were detected using antisera against h-apoA1 and h-apoB100, respectively. The relative amounts of lipoproteins and Wnt3a in the media prior immunoprecipitation (load) were determined on a fraction of the media by western blotting. Coprecipitation was specific because immunoprecipitation with a control antibody (GAPDH) did not pull down either h-apoAI or h-apoB100. E) To measure which fraction of the medium contained active Wnt3a, conditioned medium of L-cells or L-Wnt3a cells was subjected to a KBr gradient (as in Figure 1B) and the fractions were exchanged to normal medium. L-cells stably expressing the superTopFlash system (L-STF) were incubated with fractions overnight, and luciferase activity was determined.

Wnt3a from cells either. However, Wnt3a release into the medium was only restored by the addition of HDL (Figure 2A). To confirm that lipoproteins are required for the release of functional Wnt3a, conditioned medium from L-Wnt3a cells was produced with and without 10% FCS. Only medium containing lipoproteins exhibited activity, whereas medium without lipoproteins only showed minor activity (Figure 2B). To exclude that structures or particles that generally have the capability to sequester hydrophobic molecules lead to the extraction of Wnt3a from plasma membranes, we tested whether small unilamellar vesicles (SUVs) were capable to solubilize Wnt3a. However, release of Wnt3a after incubation with vesicles was not observed (Figure 2C).

### *Release of Wnt3a is facilitated by the SR-BI/II receptor*

Our results argue against an unspecific release of Wnt3a on lipoproteins or other serum components but indicate the specific use of HDL particles. This prompted us to

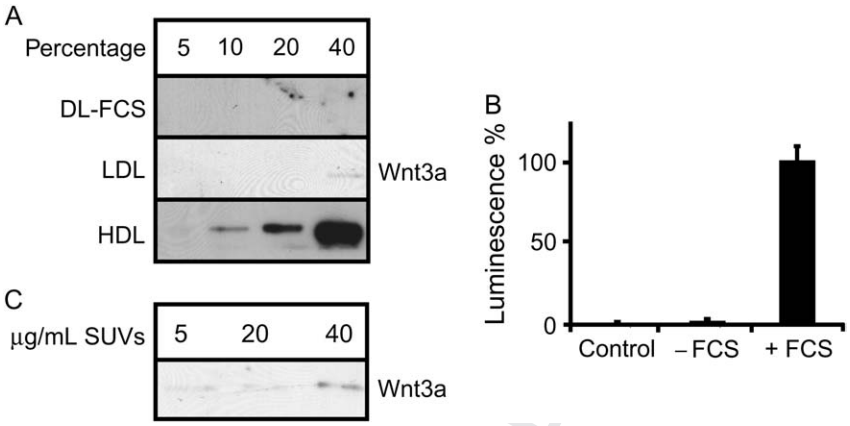


**Figure 2: Release of Wnt depends on HDL particles.**A) L-Wnt3a cells were incubated for 3 days with culture medium containing the indicated concentrations of DL-FCS or 10% DL-FCS plus increasing concentration of purified LDL or HDL particles. The soluble fraction of the medium was subjected to KBr isopycnic density centrifugation. The two top fractions were pooled and analyzed by western blotting against Wnt3a. B) Conditioned medium of L-cells grown with 10% FCS (control) or L-Wnt3a cells grown with or without 10% FCS was incubated overnight on L-STF cells, and luciferase activity was measured to determine Wnt activity in the medium. To exclude that cells grown without FCS are less viable or produce less Wnt3a, we determined the ability to activate the STF system in a co-culture assay after the collection of conditioned medium. No differences between both conditions were observed (data not shown). C) L-Wnt3a cells were incubated for 3 days with culture medium containing the indicated concentrations of small unilamellar phosphatidylcholine/cholesterol/phosphatidylserine vesicles. The soluble fraction of the medium was subjected to KBr isopycnic density centrifugation, and the two top fractions were pooled and analyzed by western blotting against Wnt3a.

investigate their role in Wnt3a release in more detail. Cells have two principal mechanisms to exchange lipids with HDL utilizing distinct cell surface proteins and HDLs as key components: (i) the scavenger receptor class B type I or II (SR-BI/II), which is a receptor that binds lipid-rich HDL with high affinity and mediates both selective lipid uptake from and release to HDL and (ii) the ATP-binding cassette transporter A1 (ABCA1), which mediates cholesterol efflux to lipid-poor pre-HDL and to lipid-free apoAI (27,28). To test whether SR-BI or other lipoprotein receptors are involved in Wnt3a release, we transfected Wnt3a-myc into LDL receptor-deficient CHO-*ldl*A-7 cells (ldlA, 29), which express only low amounts of HDL binding/selective uptake activity (30). Whereas Wnt3a-myc was barely released from these cells into the medium, transfection of SR-BI or its isoform SR-BII resulted in a significant increase in Wnt3a-myc release (Figure 3A). Wnt3a-myc was not released when *ldl*A cells were cotransfected with the LDL receptor (Figure 3A), ABCA1 or ABCG1, another ABC transporter that facilitates the efflux of cholesterol to HDL (Figure 3B). This indicates that SR-BI/II facilitated Wnt3a-myc release onto HDL, most likely by binding and subsequently release to exogenous HDL. Mutant Wnt3a lacking the palmitic anchor was released normally from *ldl*A cells (data not shown), suggesting that the mutant protein cannot be anchored sufficiently to cellular membranes. To test whether non-lipidated apoAI allows the release of Wnt3a, we incubated L-Wnt3a cells with increasing concentrations of purified apoAI, but no release could be observed (Figure 3C). Moreover, in the presence of serum lipoproteins, the addition of lipid-free apoAI inhibited Wnt3a release from L-Wnt3a cells in a dose-dependent manner. Because lipid-free apolipoproteins can also bind to SR-BI and compete with binding of lipid-rich HDL (31,32), apoAI possibly blocked the release of Wnt3a by interfering with SR-BI function, explaining the inhibitory effect of apoAI on Wnt3a release. These results suggest a role for SR-BI/II in Wnt release. Therefore, we wanted to test whether SR-BI/II expression would be required for the release of Wnt3a from L-cells. Surprisingly, transient knockdown of SR-BI/II in L-Wnt cells leads to an increase of Wnt activity in conditioned medium, which was dependent on the presence of lipoproteins (Figure 3D).

### *Release of Wnt through endogenous lipoproteins*

In our cell culture system, Wnt3a release was dependent on exogenous lipoproteins, similar to the *Drosophila* model. However, in mammals, some cell types that express Wnt proteins also express and secrete (apo)lipoproteins (33). Therefore, we wanted to test whether Wnt proteins could be secreted together with endogenous lipoprotein particles. The human enterocyte cell line Caco-2 represent polarized epithelial cells from the intestine and express Wnt proteins (34,35) as well as lipoproteins, mostly the LDL-related chylomicrons (36). PD-7 cells, a subclone of Caco-2, produce only low amounts of mature lipoproteins compared with other Caco-2 clones (37) and are therefore a good model to distinguish between endogenously produced and exogenously added lipoproteins. Secreted lipoproteins from PD7 cells fractionated in the high-density bottom fraction of the density gradient (Figure 4A) and were absent from the top fraction, indicating that they are poorly lipidated. Although exogenous lipoproteins were present, Wnt3a-myc secreted from PD7 cells cofractionated with apoB100 in the high-density fractions of the gradient, also when cells were grown on Transwell™ inserts (data not shown). Thus, Wnt3a

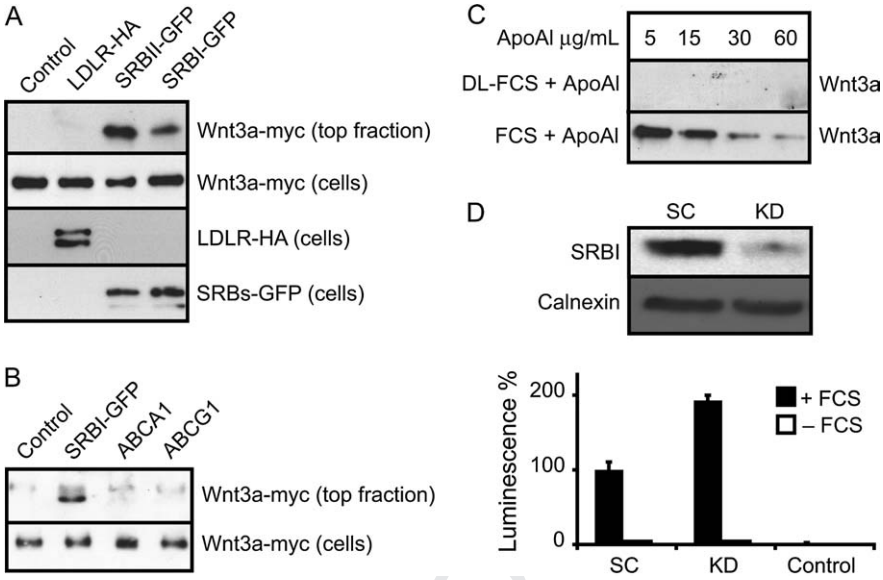


**Figure 3: Role of SR-BI/II in Wnt3a release.** A) To investigate if HDL receptors are involved in Wnt3a release, we made use of a cell line defective in lipoprotein receptors *ldl*A (66). *ldl*A cells transiently (mock) expressing HA-tagged LDL-R and GFP-tagged SR-BI or SR-BII were cotransfected with Wnt3a-myc and incubated in culture medium with 20% FCS for 3 days. The relative amount of Wnt3a in the lipoprotein fraction of the media was analyzed by western blotting (top fraction). The cellular expression levels of the lipoprotein receptors and Wnt3a were determined (cells). B) Cells can bind HDL through different cell surface receptors. To test of other receptors than SR-BI/II are involved in Wnt3a release, *ldl*A cells transiently (mock) expressing GFP-tagged SR-BI, ABCA1-V5 or ABCG1-GFP were cotransfected with Wnt3a-myc and incubated in culture medium with 10% FCS for 3 days. Media and cells were analyzed as above. C) SR-BI/II can bind to lipid-poor apoAI or lipidated HDL. To study which particle can facilitate Wnt3a release, L-Wnt3a cells were incubated with 10% DL-FCS (panel 1) or 10% FCS (panel 2) and increasing amounts of purified apoAI for 3 days. The soluble fraction of the medium was subjected to isopycnic density centrifugation, and the first two fractions were pooled and analyzed by western blotting for the presence of Wnt3a. D) L-Wnt3a cells were transfected with scrambled (SC) siRNA (Qiagen) or siRNA-targeting SR-BI/II (KD). Expression of SR-BI was determined by western blotting and compared with the expression of calnexin as a loading control. Conditioned media containing 20% FCS (+FCS) of SC, KD cells were collected after 2–3 days and medium was changed to serum-free medium (−FCS) and incubated for another 24 h. Conditioned media or normal medium (Control) was incubated overnight on L-STF cells, and luciferase activity was measured to determine Wnt activity in the medium.

could be released as a soluble protein or be associated to the poorly lipidated apoB100. To test the interaction between Wnt3a-myc and lipoproteins under these conditions, we tested whether apoB100 can be coprecipitated with Wnt3a-myc. We observed that apoB100 is not pulled down in the absence of Wnt3-myc or with a control antibody but specifically coprecipitated with anti-myc antibody, indicating a direct interaction between Wnt3a-myc and apoB100 (Figure 4B). In contrast, the parental Caco-2 cells secrete lipoproteins that are fully lipidated and fractionate in the top fraction of a density gradient. In Caco-2 cells stably expressing Wnt3a-myc (Caco-2-Wnt3a), Wnt3a-myc cofractionates with lipoproteins and is absent from the high-density bottom fraction (Figure 4C). These experiments show that Wnt3a-myc associated with endogenously produced lipoproteins, which may have taken place in the secretory pathway or at the cell surface.

## Discussion

Although many components of the Wnt signaling cascade have been identified and characterized, only little is known about the release of Wnt into the extracellular space and its trafficking to neighboring cells, particularly in mammals. Several mechanisms have been proposed for the transport of lipid-modified proteins through the aqueous environment of cells and tissues. Recently, it was suggested that Wingless, a Wnt homologue in *Drosophila*, is released and transported through lipoproteins (21). In this study, we observed that Wnt3a was released from mammalian cells by lipoproteins present in the culture medium and that the protein was active under these conditions. The release of Wnt3a occurred specifically to HDL particles, and ectopical expression of SR-BI/II facilitated SR-BI/II Wnt release; however, knockdown of SR-BI/II had the same effect. Furthermore, Wnt3a was released on newly synthesized lipoprotein particles from enterocyte cell lines.

### *Lipid modifications are required for the association to lipoproteins*

Members of the Wnt family have been shown to be lipid modified by palmitic acid (16:0) at a conserved cysteine residue (C77 in Wnt3a) (5) and by palmitoleic acid (16:1) at a conserved serine residue (S209 in Wnt3a) (6). Whether all members of this family carry a lipid anchor is not clear;

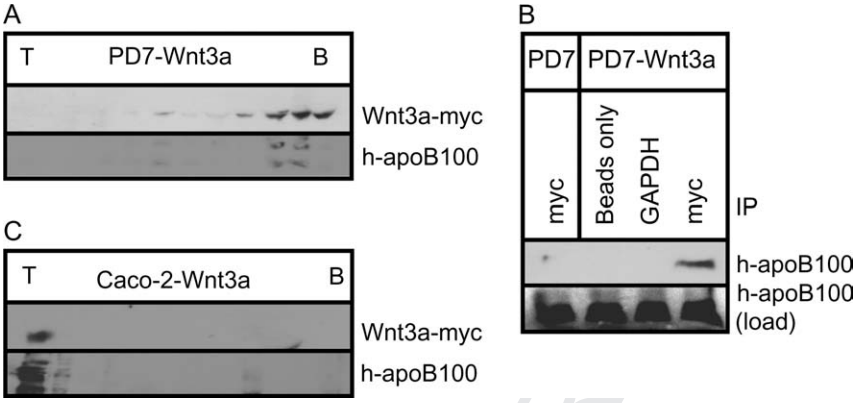


**Figure 4: Wnt3a can be secreted through endogenous lipoprotein particles.** A) PD7 cells stably expressing Wnt3a-myc (PD7-Wnt3a) were incubated with medium containing 10% FCS for 3 days. The conditioned medium was subjected to a KBr isopycnic gradient, and fractions were analyzed by western blotting for Wnt3a-myc and newly synthesized h-apoB100. Both proteins cofractionate in the bottom fraction of the gradient. B) To examine if both proteins associate, conditioned medium of PD7 cells or PD7-Wnt3a cells was produced as above and probed by coimmunoprecipitation for interaction of Wnt3a with lipid-poor apolipoproteins. Conditioned medium of PD7 cells or PD7-Wnt3a cells was divided into three parts, and Wnt3a-myc was adsorbed to protein G–Sepharose without antibody or mouse anti-GAPDH antibody as controls (IP) or with mouse anti-myc antibody. Immunoprecipitates were subjected to SDS–PAGE and western blotting against h-apoB100. The relative amount of h-apoB100 in media prior to immunoprecipitation (load) was determined on a fraction of the media by western blotting. C) Compared with PD7 cells, wild-type Caco-2 cells secrete more lipoproteins into the culture medium (37). Caco-2 cells stably expressing Wnt3a-myc (Caco2-Wnt3a) were incubated with medium containing 10% FCS for 3 days. The conditioned medium was subjected to a KBr isopycnic gradient, and fractions were analyzed by western blotting for Wnt3a-myc and h-apoB100.

however, the critical residues for lipid modifications are highly conserved between family members and species. Nonetheless, only a number of Wnt proteins have been characterized in respect to the lipid anchor so far (5–9). As proteins with different lipid anchors associated with lipoproteins in *Drosophila*, we expect the association of Wnt proteins with lipoproteins also to occur with Wnt proteins other than Wnt3a. However, very recently, it was shown that WntD, a *Drosophila* Wnt protein that does not have a mammalian homologue, is not lipid modified (38). Removal of the palmitate moiety allows normal release of Wnt to the cell culture medium but impairs its function, whereas removal of the palmitoleate moiety impedes transport of Wnt from the endoplasmic reticulum (ER) to the plasma membrane (5–11), indicating several distinct roles of the lipid modifications. Despite the normal release from cells of mutant WntC77S, which lacks the palmitate moiety, into the medium, we find that mutant WntC77S was present as a soluble protein, in contrast to wild-type Wnt3a, which was associated with lipoproteins. This indicates that the palmitoleate moiety on Ser209 by itself is not able to anchor Wnt3a to lipoproteins. Probably, a single lipid modification is not sufficient to efficiently target proteins to membranous structures as is the case for cytosolic small GTPases (24). However, it cannot be excluded that the removal of the lipid anchor leads to a structural rearrangement of Wnt, which impairs signaling activity and lipoprotein binding.

### *Lipoproteins and lipoprotein receptors*

In theory, all classes of lipoproteins have the capability to carry lipid-modified proteins in the phospholipid monolayer surrounding the fat core. A possible role for lipoprotein receptors is to bring lipoprotein particles in close vicinity to the exoplasmic leaflet of cell membranes, which contain Wnt. Then, Wnt can be transferred to the lipoprotein particle, either by passive diffusion or by an active, but yet unidentified, protein machinery. In our experiments, Wnt is released from cells only to HDL but not to LDL. However, once associated to lipoproteins, Wnt could freely exchange between lipoprotein particles, similar to other proteins, like the small apolipoproteins apoE and apoC (39–41). From *ldl*A cells, the release of Wnt was facilitated by the SR-BI/II receptor, and several other lipoprotein receptors tested did not show similar effects. In contrast, knockdown of SR-BI/II in L-Wnt cells resulted in an increase of Wnt release. The SR-BI/II knockout mouse does not show phenotypes related to impaired Wnt signaling (42), which would be in line with our results in L-cells. Both cell lines, *ldl*A and L-cells, are not very well characterized in terms of lipoprotein receptor expression. For *ldl*A cells, it is known that they have a defect in LDL receptor expression (29,43) and express only low amounts of HDL binding/selective uptake activity (30); therefore, these cells probably adapted to a situation with impaired lipid uptake from lipoproteins. It is difficult to predict, however, whether upon knockdown of SR-BI/II in L-cells, other lipoprotein receptors would be upregulated and also facilitate Wnt release. Furthermore, it is known that membrane structure is dependent on SR-BI/II expression, as shown in Sf9 cells and for the formation and organization of microvilli in adrenal gland cells (44–46). Hereby, the lack of SR-BI/II leads to a decrease in membrane thickness, possibly because of a decrease in membrane

cholesterol (45). It is possible that extraction of Wnt from the plasma membrane is facilitated under these conditions. Zhai et al. showed that Drosophila Wnt1 is present in cholesterol-rich, detergent-resistant membranes (9), which indicates that Wnt is organized in specialized membrane domains. Therefore, interference with membrane lipid composition may result in different membrane anchoring.

We find that transfection of *ldl*A cells with SR-BII leads to more efficient Wnt release than that with SR-BI. Hereby, sorting in the endocytic system, which was found to be essential for Wnt release (47–51), could be responsible for this effect. SR-BII preferentially localized to endocytic compartments and the plasma membrane (52), suggesting a role in HDL recycling. Possibly, Wnt can also be released on recycling lipoprotein particles other than HDL (53,54). Whereas LDL particles and their associated proteins are taken up through the LDL receptor and targeted for degradation to the lysosome, HDL can be recycled by SR-BI/II or ABCA1 (52,55,56). Thus, if Wnt would have been released onto LDL, both proteins would have been targeted to lysosomal degradation.

Certainly, the cell culture system we used is poorly understood. Therefore, it would have to be tested whether other lipoprotein receptors are also involved in Wnt release, for example from the large family of LDL receptor-related proteins or other scavenger receptors. Furthermore, it is important to decipher the mechanism, how Wnt is transferred to lipoprotein particles. Possibly, a change in membrane environment is required, for example, during recycling. Finally, the release should be studied *in vivo*. Perhaps, the use of inducible reporter genes for Wnt signaling in combination with inducible, tissue-specific knockdowns of lipoproteins and their receptors will give more insight in Wnt transport *in vivo*.

### *Wnt release through endogenously expressed lipoproteins*

Different from the *Drosophila* model, where lipoproteins are synthesized in the fat body and Wnt is produced in other cell types, some mammalian cell types producing Wnt are also able to synthesize lipoproteins (34,35). We find first evidence that in these cells, Wnt is present on endogenously produced lipoproteins. In contrast to the fibroblast system, Wnt3a cofractionated together with poorly lipidated lipoproteins in conditioned medium from PD7 cells, instead of being released onto exogenous lipoproteins, possibly because of the lack of expression of receptors or because of the sidedness in these cells as known, for example for SR-BI/II that expresses apically (57), whereas lipoproteins are secreted basolaterally (58) together with Wnt3a (data not shown). Whether in these cells the association of Wnt3a with lipoprotein particles occurs in the secretory pathway or as a consequence of extraction from the plasma membrane through secreted lipoproteins has to be further investigated. Nevertheless, lipid modification of Wnt and lipidation of lipoproteins take place in the ER. Therefore, it is likely that association of both proteins occurs in the same compartment. This pathway would give cells the opportunity to flexibly react to changes in nutrition. In mammals, the lipid-modified hormone Ghrelin was found to be associated to very low-density lipoprotein and HDL particles in serum (59). Ghrelin is a small peptide hormone carrying an octanoate moiety (60) and is secreted from the stomach and the intestine upon starvation to induce food uptake. This is another example of regulating fat metabolism by the action of lipid-modified proteins.

### *Other lipid-modified proteins*

A rising number of extracellular proteins have been identified as containing a lipid anchor. For all of these proteins, it is unclear how they are transported through an aqueous environment. Lipoproteins have the potential to be a vehicle for a variety of lipid-modified proteins. In *Drosophila*, the lipid-modified morphogens Hedgehog and Wingless as well as GPI-anchored proteins were found to be associated with lipophorin (21), indicating that the incorporation of lipid-modified proteins into the phospholipid monolayer of a lipoprotein is rather unspecific. In contrast, our findings suggest that the transfer of the lipid-modified protein Wnt3a occurs specifically to HDL. The fact that not all lipoprotein receptors facilitated the release of Wnt implies that, in addition to recruiting the lipoprotein to the membrane, a specific interaction between either the lipid-modified protein and the lipoprotein or the lipoprotein receptor could be involved. Theoretically, lipid-modified proteins and lipoprotein receptors could also be laterally restricted to distinct membrane domains, depending on the physical properties of the lipid modification and the transmembrane domains of the receptor. Thus, the interaction between lipoproteins and lipid-modified proteins may be rather unspecific, although in the context of lateral organization of membranes combined with individual protein–protein interactions, specificity in uptake can be obtained. Whether specificity is only relevant for efficient release or also serves additional functions remains to be determined. As many different proteins are associated to lipoprotein particles (21,61–63), the role of lipoproteins and their associated proteins should be reconsidered.

## Materials and Methods

### *Materials*

Chemicals, if not indicated otherwise, were from Sigma and used in the highest purity available. Organic solvents were from Riedel de Haën, and cell culture media and reagents were from Invitrogen. Cell culture plastics were from Costar. Mouse anti-myc 9E10, rabbit anti-myc A14 and rabbit anti-hemagglutinin (HA) Y11 antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-calnexin H-70 was from Santa Cruz Biotechnology. Rabbit anti-mouse SR-BI NB400-104 was from Novus Biologicals. Rabbit anti-human apolipoprotein AI was from Calbiochem, and antisera against apolipoprotein B100 were from Calbiochem or from Santa Cruz

Biotechnology (C1.4). The mouse antibody against GFP JL8 was from Clontech. Rabbit anti-calnexin antiserum (Zhang et al., 1997) was a kind gift of Ineke Braakman (Utrecht University, The Netherlands). Rabbit anti-Wnt3a antiserum was a kind gift from Roel Nusse (Stanford University, Stanford, CA, USA). Mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Ambion (Applied Biosystems). Anti-human IgG (hIgG) was a kind gift of Jeanette Leusen (University Medical Center Utrecht, The Netherlands). Horseradish peroxidase-conjugated secondary goat antibodies were from Pierce. Myc-peptide was synthesized using an Applied Biosystems 431A peptide synthesizer.

### *Sera and lipoprotein preparation*

FCS (Hyclone) was delipidated (DL-FCS) by solvent extraction. Therefore, serum was mixed with an equal volume of a 2:1 mixture of diisopropyl ether: n-butanol, and stirred at room temperature for 30 min, and phases were separated by centrifugation at 5000 × ***g*** for 30 min. The aqueous phase was re-extracted with an equal volume of diisopropyl ether and subsequently dialyzed against PBS. This treatment resulted in the total loss of intact lipoprotein particles, as judged by the loss of proteins from the top fraction of a KBr gradient centrifugation that was analyzed by SDS–PAGE and Coomassie brilliant blue staining. The cholesterol content was reduced to less than 5% of untreated serum, as determined using the Amplex Red Cholesterol assay kit (Molecular Probes). Human serum was obtained from healthy fasting human donors, and HDL and LDL were prepared by discontinuous density centrifugation as previously described (25), dialyzed against PBS and stored under nitrogen at 4°C. The protein contents of the preparations were determined using the BCA assay (Pierce Chemical Co.). SUVs were generated by sonication on ice (64) of egg phosphatidylcholine: cholesterol:egg phosphatidylserine (50:50:1, mol/mol).

### *Plasmids*

pcDNA3-LDLR-HA was kindly provided by Jürgen Gent (Utrecht University, The Netherlands). The construction of pEGFP-SRBI and pEGFP-SRBII has been described (52). pcDNA3.1/V5-His-TOPO-ABCA1 and pEGFP-ABCG1 were from Gerd Schmitz (Regensburg, Germany). pEGFP-CD63 was from Gillian Griffiths (Oxford, UK). pcDNA3-Wnt3a was from Damien Coudreuse (Rockefeller University, New York, NY, USA). A myc tag was appended at the carboxy-terminus of Wnt3a by polymerase chain reaction (PCR) and ligated into pcDNA3 (pcDNA3-Wnt3a-myc). Cysteine 77 of Wnt3a was mutated into a serine by PCR mutagenesis, yielding pcDNA3-Wnt3a-C77S-myc. All synthetic complementary DNAs were verified by restriction analysis and dye termination sequencing.

### *Cell culture and transfection*

Mutant Chinese hamster ovary *ldl*A (clone 7) cells that are LDL receptor deficient and express very little SR-BI or HDL binding/selective uptake activity (30,65) were kindly provided by Monty Krieger (MIT Department of Biology, Cambridge, UK) and Jürgen Gent. *ldl*A and mouse L-cells [American Type Culture Collection (ATCC)] were grown in DMEM containing 4 mM L-glutamine, 4.5 g/L glucose and 10% FCS (culture medium) and were maintained at 37°C with 5% $CO_2$. Control L-cells were stably transfected with pcDNA3 and maintained in culture medium containing 0.4 mg/mL geneticin. L-Wnt3a cells (ATCC) were cultured in culture medium with 0.4 mg/mL geneticin. For Wnt activity measurements, L-Wnt3a were kindly provided by Daniele Tauriello (Utrecht University, The Netherlands), and cells were cultured in medium containing 50 μg/mL zeocin (Invitrogen). L-cells stably transfected with the superTOPflash system (L-STF) were kindly provided by Roel Nusse and cultured in medium containing 0.8 mg/mL geneticin.

For transient overexpression, cells were transfected with Lipofectamine 2000 (Invitrogen) and expression was induced by addition of 2 mM butyrate during all incubation times. For transient knockdown, cells were transfected with small interfering RNA (siRNA) oligonucleotides targeting SR-BI/II (sense: AGG UCA ACA UCA CCU UCA A; Qiagen) using oligofectamine (Invitrogen). Transfection was repeated after 2–3 days of incubation with siRNA. Protein expression was determined by western blot after 2–3 days.

### *Wnt conditioned medium*

Unless indicated otherwise, in experiments detecting human lipoproteins by western blotting, human sera were used. Cells on 3-cm dishes were transiently (mock) transfected using Lipofectamine 2000 (Invitrogen) and grown in Optimem (Invitrogen) with DL-FCS, FCS, purified HDL or LDL or lipid-free apoA1 at 37°C with 5% $CO_2$ for 3 days. Cell debris was removed by centrifugation at 10 000 × ***g***, and the supernatant was centrifuged for 1–3 h at 39 000 r.p.m. at 4°C in a SW41Ti rotor (Beckman). Lipoprotein particles were separated from soluble proteins by isopycnic density centrifugation. In short, KBr was added to the supernatant to a final concentration of 0.33 g/mL, and the sample was spun for 2 days at 37 000 r.p.m. at 4°C in a SW41Ti rotor. Twelve fractions were taken from the top. The proteins were precipitated with trichloroacetic acid. In some cases, the proteins from the supernatant were directly precipitated with trichloroacetic acid.

### *Wnt activity measurements*

L-cells or L-cells stably transfected with Wnt3a were grown on 15-cm dishes. Protein expression was induced with 2 mM butyrate for 3–5 days when cells where 60% confluent. Conditioned medium (20 mL) was collected, and cell debris was removed by centrifugation. KBr gradients were performed and divided in 3 (pooled) or 12 fractions. Fractions were exchanged to normal medium using an Amicon column (Millipore) to normal medium without FCS. L-cells stably transfected with STF were grown on a 24-well and incubated overnight with a 1:1 dilution of a fraction, conditioned medium or normal medium in a final volume of 300 μL. Cells were lysed, and luciferase activity was measured using the BrightGlo system (Promega).

Cells transiently knocked down for SR-BI/II were incubated with Optimem containing 20% FCS during the incubations with siRNA. After 2–3 days, conditioned medium was collected and medium was exchanged to serum-free medium for 24 h. Cell debris was removed by centrifugation. Wnt activity was determined as above.

### *Immunoprecipitation*

Conditioned medium of cells was subjected to ultracentrifugation at 120 000 × ***g*** in a SW41Ti rotor (Beckman), and the supernatant was precleared during a 2-h incubation with 0.25 volumes of Sepharose CL4B beads. A fraction of each supernatant was used to determine relative amounts of apoA1 and apoB by western blotting. The remainder was incubated with anti-Wnt3a, anti-myc or anti-GAPDH adsorbed to protein A–Sepharose or protein G–Sepharose (GE Healthcare). Immunoprecipitates were washed at least 10 times with 5 volumes of PBS (without $Ca^{2+}$ and $Mg^{2+}$), 0.5%BSA and once with PBS (without $Ca^{2+}$ and $Mg^{2+}$). Proteins were eluted from beads using either 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 100 mM Tris–Cl (pH 8.3), 0.5% Nonidet P-40, 0.5% sodiumdeoxycholate and 0.1% sodium dodecyl sulfate or Laemmli sample buffer containing 5% β-mercaptoethanol, or proteins were eluted in a 1 mg/mL solution of myc peptide.

## Acknowledgments

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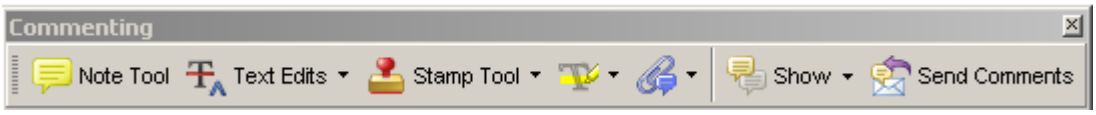


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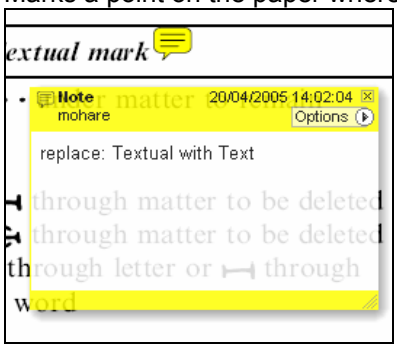


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2. Select Add Note and a yellow speech bubble symbol and text box will appear
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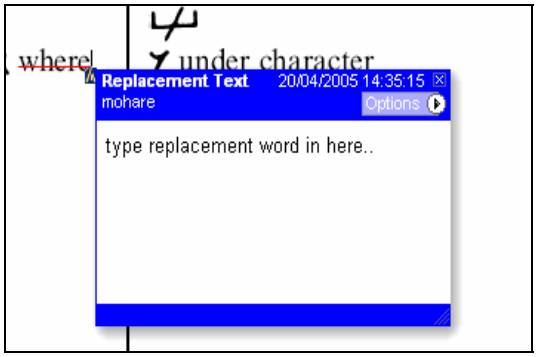


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3. Right click
4. Select Replace Text (Comment) option
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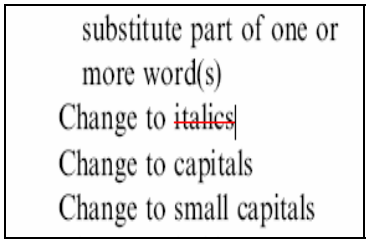


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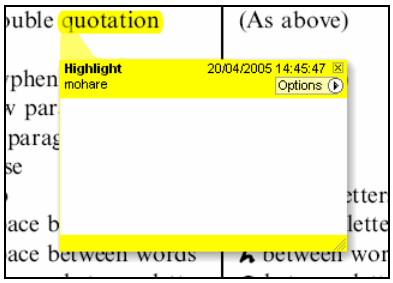


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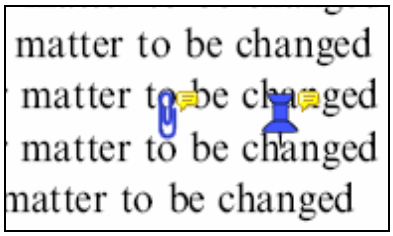


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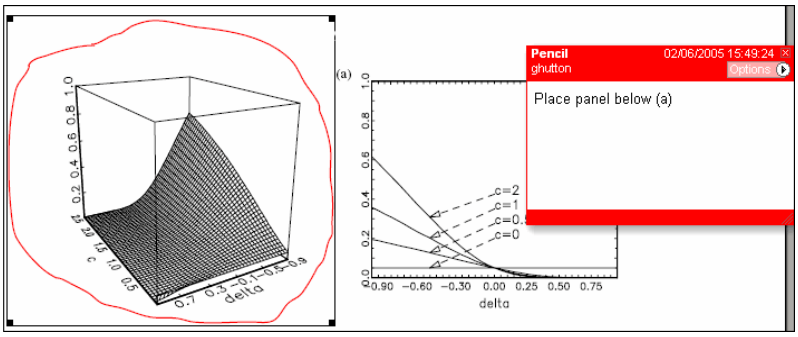
Creates freeform shapes with a pencil tool. Particularly with graphics within the proof it may be useful to use the Drawing Markups toolbar. These tools allow you to draw circles, lines and comment on these marks.




**How to use it:**

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
4. Once finished, move the cursor over the shape until an arrowhead appears and right click
5. Select Open Pop-Up Note and type in a details of required change
6. Click the X in the top right hand corner of the note box to close.

## Help

For further information on how to annotate proofs click on the Help button to activate a list of instructions: