Wnt-inducible Lrp6-APEX2 interacting proteins identify ESCRT machinery and Trk-fused gene as components of the Wnt signaling pathway

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The canonical Wnt pathway serves as a hub connecting diverse cellular processes, including β-catenin signaling, differentiation, growth, protein stability, macropinocytosis, and nutrient acquisition in lysosomes. We have proposed that sequestration of β-catenin destruction complex components in multivesicular bodies (MVBs) is required for sustained canonical Wnt signaling. In this study, we investigated the events that follow activation of the canonical Wnt receptor Lrp6 using an APEX2-mediated proximity labeling approach. The Wnt co-receptor Lrp6 was fused to APEX2 and used to biotinylate targets that are recruited near the receptor during Wnt signaling at different time periods. Lrp6 proximity targets were identified by mass spectrometry, and revealed that many endosomal proteins interacted with Lrp6 within 5 min of Wnt3a treatment. Interestingly, we found that Trk-fused gene (TFG), previously known to regulate the cell secretory pathway and to be rearranged in thyroid and lung cancers, was strongly enriched in the proximity of Lrp6. TFG depletion with siRNA, or knock-out with CRISPR/Cas9, significantly reduced Wnt/β-catenin signaling in cell culture. In vivo, studies in the Xenopus system showed that TFG is required for endogenous Wnt-dependent embryonic patterning. The results suggest that the multivesicular endosomal machinery and the novel player TFG have important roles in Wnt signaling.

The Wnt/β-catenin pathway is an evolutionarily conserved signaling cascade that plays a fundamental role in animal embryonic development, adult stem cell homeostasis, and is dysregulated in a number of human diseases, including cancer1,2. In the absence of Wnt stimulation, the scaffold proteins Axin and Adenomatous Polyposis Coli (APC) form a ‘destruction complex’ with Casein Kinase1 (CK1) and Glycogen Synthase Kinase 3β (GSK-3β) which sequentially phosphorylate β-catenin and mark it for ubiquitination and proteasome-mediated degradation3. Canonical Wnt signal transduction is initiated when a Wnt ligand binds to the seven-pass transmembrane receptor Frizzled (Fzd) and the Low-density lipoprotein receptor-related protein 5/6 (Lrp5/6). The activated co-receptors recruit a number of effectors, including CK1 isoforms4, the adaptor Dishevelled (Dvl), Axin1 and GSK-3β, to the cytosolic side of the plasma membrane forming a complex known as the signalosome5. Signaling requires specific protein–protein interactions and the phosphorylation of several components, including the cytoplasmic tail of Lrp66–9. Subsequently, activated Wnt receptor clusters50 are internalized into multivesicular bodies by Endocytic Sorting Components Required for Transport (ESCRT)-driven microautophagy of GSK-3β and other negative Wnt regulators such as Axin, sequestering them away from their cytosolic targets51,52. As a result, β-catenin protein is stabilized and translocates into the nucleus, where it binds to T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) DNA-binding proteins to regulate the transcription of Wnt-target genes.

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Figure 1. The Lrp6-APEX2 fusion protein receptor retains signal transducing activity, proper subcellular localization, and biotinylates cellular proteins. (A) Schematic diagram of the Lrp6–APEX2 construct. The human Lrp6 contains an N-terminal VSV-G and a linker Flag-tag followed by the APEX2 enzyme. The construct also contains an internal ribosomal entry site (IRES) followed by a GFP-tagged Puromycin N-acetyltransferase (PAC) used for selection of permanently transfected cells. (B) Diagram depicting the biotinylation of Lrp6–APEX2 proximal targets. During the Wnt signal, in the presence of Biotin-phenol (BP) and H$_2$O$_2$, proteins recruited to the receptor are biotinylated by the APEX2 peroxidase, including known Wnt targets (Dvl, Axin, APC, etc.) and novel targets (X, Y). (C–E) Control embryos from oocyte-host transfer experiments show normal development (100%, n = 24), as compared to oocytes depleted with a phosphorothioate antisense DNA oligo targeting Xenopus Lrp6, which develop as ventralized embryos (75%, n = 22). Co-injection of 300 pg of Lrp6–APEX2 mRNA into oocytes completely rescued axis formation (60%, n = 17), indicating that the fusion protein is fully active. Scale bar represents 500 μm. (F–H) Lrp6-APEX2 is trafficked to the plasma membrane in stably transfected HEK293T cells. Lrp6 was detected through its Flag-tag and Phalloidin, which stains cortical F-Actin, confirmed cell surface localization of Lrp6. Scale bar represents 10 μm. (I–L) Lrp6-APEX2 changes subcellular localization by Wnt, Dkk1 and Bighead treatments. In presence of control conditioned medium (CM), Lrp6 is located mostly at the plasma membrane (79%). Treatment with Wnt3a CM for 30 min increases the number of intracellular vesicles containing Lrp6 (53%). Treatment with Dkk1 protein (200 ng/ml) or Bighead CM induced relocation of Lrp6 to the juxtanuclear bay area where lysosomes are located (63% and 53%, respectively). Quantification was obtained counting cells showing the observed immunostaining over total nuclei, in 10 different micrographs. Scale bars represent 10 μm. (M) Western blot stained with Streptavidin-IRDye 800 (in green) showing controls indicating that Lrp6–APEX2 biotinylates proximal proteins only in the presence of both BP and H$_2$O$_2$ (compare lane 5 with 2–4). Negative control cells not expressing the APEX2 peroxidase had no biotinylation even in presence of BP/H$_2$O$_2$ (lane 1). Note the presence of three Streptavidin-positive bands in all lanes, which correspond to endogenous biotinylated carboxylases. Gapdh served as a loading control.

During the 30 years since its discovery, the Wnt pathway has been the subject of intense studies, but its molecular mechanisms have not yet been fully elucidated, in particular the role of membrane trafficking in receptor activation. To dissect the dynamics of Wnt signaling, we decided to use a protein interaction approach to identify additional regulators of the pathway. In recent years, proximity-dependent biotin labeling approaches have emerged as a valuable tool to define functional protein complexes or organelle-specific proteomes. Two classes of enzymes can be used in proximity labeling (PL) experiments: the promiscuous E. coli biotin ligase BirA R118G (BirA*) and its variants TurboID and miniTurboID, or the engineered soybean Ascorbate Peroxidase 2 (APEX)1. These enzymes attach biotin-derivatives covalently to nearby proteins in vivo, while still in their native cellular environment. Biotinylated targets can then be extracted, purified by streptavidin-bead pull-down, and identified by mass spectrometry (MS). The APEX2 peroxidase technology offers several advantages: faster kinetics, robust biotinylation activity, and the possibility to activate the labeling at specific moments by adding H$_2$O$_2$ to living cells. APEX2 requires the use of biotin-phenol (also known as biotin-tyramide), which in combination with H$_2$O$_2$ is converted to a highly reactive, short-lived (<1 ms) radical with a labeling radius of 20 nm. The high spatial and temporal specificity provided by APEX2 has allowed the study of the proteome of organelles or subcellular compartments difficult to isolate, such as the mitochondrial intermembrane space and endoplasmic reticulum (ER) membrane, and to study G-protein-coupled receptor (GPCR) signaling in great detail.

Here, we describe the results obtained with a Human Embryonic Kidney 293T (HEK293T) cell line stably expressing a chimeric Lrp6–APEX2 receptor. Our proteomic data indicate that in presence of Wnt3a, the Lrp6-interactome was enriched in components of the ESCRT machinery that forms MVBS, providing independent support to our previous findings that endocytosis is at the core of Wnt pathway intracellular regulation. We also discovered that Tropomyosin-receptor kinase fused gene (Trk-fused gene, TFG), a protein involved in several pathologies including cancer, was a highly enriched target of Wnt-activated Lrp6–APEX2 and localized to endosomal vesicles. Cell culture knock-out assays and in vivo experiments using Xenopus laevis embryos revealed an important role for TFG in Wnt/β-catenin signaling and antero-posterior (AP) patterning.

Results

Lrp6–APEX2 receptor fusion is active in Wnt signaling. To study the protein complexes that are recruited in proximity of the receptor complex during Wnt signaling, we appended APEX2 to the cytoplasmic tail of the Lrp6 receptor (Fig. 1A,B). The chimeric receptor maintained functionality, since mRNA overexpression could rescue the ventralized phenotype caused by maternal depletion of Xenopus Lrp6 in frog embryos (Fig. 1C–E), and induced axis duplication in a ligand dependent manner when overexpressed in a ventral blastomere (Fig. 1A–G). Interestingly, Lrp6–APEX2 also restored the response to Wnt ligands by Lrp5/6 double knockout HEK293T cells, corroborating its functionality (Fig. 1H). Next, we established a HEK293T stable clonal line expressing the Lrp6–APEX2 receptor component. Our proteomic data indicate that in presence of Wnt3a, the Lrp6-interactome was enriched in components of the ESCRT machinery that forms MVBS, providing independent support to our previous findings that endocytosis is at the core of Wnt pathway intracellular regulation. We also discovered that Tropomyosin-receptor kinase fused gene (Trk-fused gene, TFG), a protein involved in several pathologies including cancer, was a highly enriched target of Wnt-activated Lrp6–APEX2 and localized to endosomal vesicles. Cell culture knock-out assays and in vivo experiments using Xenopus laevis embryos revealed an important role for TFG in Wnt/β-catenin signaling and antero-posterior (AP) patterning.
A

-30 min  Wnt3a treatment  -1 min  0 min

Biotin-phenol  Wnt3a CM  H₂O₂  Cell lysis and protein extraction

B

Mock  5  20

Wnt3a (min)

HGS/HRS  STAM1  STAM2  TSG101  VPS28  VPS37C  VPS37B  CHMP4B

C

Mock  5  20

Wnt3a (min)

AXIN1  DVL1  DVL2  DVL3  APC

D

| Protein ID | Gene Name  | Description                                      | GO Molecular Function                                    |
|------------|------------|--------------------------------------------------|----------------------------------------------------------|
| Q92734     | TFG        | Trk-Fused Gene                                   | COPII-mediated vesicle transport                         |
| Q14789     | GOLGB1     | Golgin subfamily B member 1                      | COPI-mediated anterograde transport                      |
| Q90C02     | TNKS1BP1   | Tankyrase-1-binding protein                      | cadherin binding                                         |
| Q7Z2K8     | GPRIN1     | G protein-regulated inducer of neurite outgrowth 1| neuron projection development                             |
| Q9Y6N7     | ROBO1      | Roundabout homolog 1                             | Netrin-1 signaling                                       |
| Q86X9      | LSR        | Lipolysis-stimulated lipoprotein receptor        | tricellular tight junction assembly                      |
| P468Z1     | MAP1B      | Microtubule-associated protein 1B                | microtubule binding                                     |
| Q5UQ88     | BAIAP2     | Brain-specific angiogenesis inhibitor 1-associated protein 2 | regulation of actin cytoskeleton organization |
| Q5UDY2     | TIP2       | Tight junction protein ZO-2                       | regulation of membrane permeability                      |
| Q14847     | LASP1      | UM and SH3 domain protein 1                      | actin filament binding                                  |
| Q99995     | AKAP9      | A-kinase anchor protein 9                         | positive regulation of microtubule polymerization        |
| Q04771     | NOTCH2     | Neurogenic locus notch homolog protein 2         | Notch signaling pathway                                 |
| O14964     | HGS        | Hepatocyte growth factor-regulated tyrosine kinase substrate | Clathrin-mediated endocytosis                           |
| O00151     | PDLIM1     | PDZ and LIM domain protein 1                     | actin cytoskeleton organization                          |
| P25054     | APC        | Adenomatous polyposis coli protein               | Degradation of β-catenin by the destruction complex     |
biotinylation depends on the availability of certain amino acid residues, like tyrosine, to detect some known Lrp6 interactors, like Frizzled. However, it should be noted that APEX-mediated interactions at 20 min were maximal, except for APC which was maximal at 5 min (Fig. 2C). We were surprised that the canonical Wnt pathway, such as APC, Dvl 1–3 and Axin and were biotinylated upon Wnt3a treatment, although in this case the most enriched protein in this list.

Although showing that APC is a major regulator of endocytosis, the Wnt antagonists Dkk1 or Bighead induced endocytosis of Lrp6-APEX2 into large vesicle clusters in the cytoplasm next to the nuclear bay region, where the lysosomal system is located (Fig. 1K,L). Next, we assessed the activity of the APEX2 enzyme. The western blot in Fig. 1M includes several control treatments showing that APEX2-mediated biotinylation only occurred in the presence of both biotin-phenol and H₂O₂. This activity could also be detected by Streptavidin immunofluorescence (Fig. S1J–O). Altogether, our data show that the chimeric receptor Lrp6-APEX2 can replace the wild type receptor and can be used for biotin-labeling studies.

Compared to the parental HEK293T cell line, Lrp6-APEX2 HEK293T cells showed elevated levels of phosphorylated Dvl2 (pDvl2) (Fig. S2A); however, this Dvl2 phosphorylation was blocked upon preincubation with the Porcupine inhibitor IWP-2, indicating a higher sensitivity to an unknown endogenous Wnt present in HEK293T cells (Fig. S2B). In the presence of IWP-2, high levels of phospho-Dvl2, active (i.e., non-phosphorylated) β-catenin and phospho-Lrp6 (pLrp6) were only observed when cells were exposed to Wnt3a conditioned medium and, interestingly, this response was increased by R-Spondin 1 and 2 (Fig. S2C). Thus, our Lrp6-APEX2 cells are entirely dependent on exogenous Wnt extracellular ligands, providing a new and sensitive system to explore the interactions of the Lrp6 receptor during Wnt signaling.

Wnt induces Lrp6-APEX2 biotinylation of components of the ESCRT machinery. Lrp6-APEX2 HEK293T cells (preincubated with Porcupine inhibitor to eliminate endogenous Wnts) were treated for 30 min with biotin-phenol in T225 flasks (Fig. 2A). Next, duplicate samples were treated with either control or Wnt3a conditioned medium containing biotin-phenol (fortified by the addition of 50 ng/ml R-Spo1 and 2 which were found to strongly increase Wnt3a responsiveness; Fig. S2D). Two time points were analyzed, 5 and 20 min. The labeling reaction was triggered by a 1-min incubation with H₂O₂, followed by cell lysis and pull-down of biotinylated proteins (Fig. 2A). Each treatment included biological duplicates. Biotinylation and β-catenin stabilization were confirmed by western blot in each sample (Fig. S2E). Affinity-purified samples were then processed for tandem mass spectrometry (MS/MS) of tryptic peptides as described in “Methods”. All original datasets generated in this study are publicly available at the MassIVE public repository resource (accession number MSV00084335).

Principal Component Analysis (PCA) showed that each replicate clustered according to the type of treatment, validating the reproducibility of the assay (Fig. S2F). Analysis of the peptide data generated a list of over 4,000 putative biotinylated proteins (all of which are listed in Tab 1 of Supplementary Table S1). Of these, over 2,000 could be accurately quantified using MS1-based label free quantitation (Supplementary Table S1, Tab 2), as explained in “Methods”. Of those 2,000-plus proteins, 217 and 237 proteins were significantly enriched (p-value < 0.05) in the 20 and 5 min Wnt3a samples, respectively, compared to no biotin controls (Supplementary Table S1, Tab 3). Amongst the most highly enriched proteins was the Trk-Fused Gene (TFG). The full list of identified proteins (including their spectral counts) that interact with Lrp6-APEX2 after 5 or 20 min of Wnt3a treatment is shown in Supplementary Table S1 and represents a rich resource to search for downstream interactions of the Lrp6 receptor during Wnt signaling.

Gene Ontology analyses using the Panther database revealed that the top hit induced by Wnt3a conditioned medium was the ESCRT machinery required for MVB formation. Heatmaps derived from the normalized list of proteins showed a clear involvement of the ESCRT machinery, with a marked enrichment of Hrs, Stam1/2, Chmp4, Vps28, Vps35b, Vps35c and Tsg101 among others, which was strongest after 5 min of Wnt3a treatment (Fig. 2B). Confirming that the Lrp6-APEX2 interactions were Wnt-induced, known targets of the canonical Wnt pathway, such as APC, Dvl 1–3 and Axin and were biotinylated upon Wnt3a treatment, although in this case the interactions at 20 min were maximal, except for APC which was maximal at 5 min (Fig. 2C). We were surprised not to detect some known Lrp6 interactors, like Frizzled. However, it should be noted that APEX-mediated biotinylation depends on the availability of certain amino acid residues, like tyrosine, on the target protein and factors like steric hindrance and accessibility may affect efficient labeling. It is possible that insufficient biotinylation precluded Frizzled detection on our proteomic analysis.

Proteins involved in actin cytoskeleton remodeling were also prominent among Wnt-inducible interactions. Since canonical Wnt signaling triggers macropinocytosis through the actin contractile machinery, this might be indicative of a role of Lrp6 in plasma membrane macropinocytosis. Figure 2D shows a partial list including...
the 15-most differentially enriched biotinylated proteins upon a 20 min Wnt3a treatment, which included TFG, Tankyrase-1 binding protein, Hrs/Hgs and APC.

We also carried out proteomic analysis of a single sample treated for 1 h with Wnt3a conditioned medium (CM) or CM from control cells not expressing Wnt3a, designated Mock CM (Supplementary Table S1, Tab 4). This longer time point (which did not include the use of R-spondin 1 and 2) revealed that the top three Lrp6-APEX2 interactors were α-Spectrin, β-Spectrin and their ancestor α-Actinin (from which spectrins evolved) 36. These proteins form a meshwork at the plasma membrane (together with protein band 4.1) that serves to link actin filaments to the plasma membrane36. The continuous involvement of the actin cytoskeletal machinery is consistent with a sustained role for nutrient acquisition via macropinocytosis in canonical Wnt signaling, which depends on Gsk3 inhibition16,37,38. We also performed MS analysis on whole protein extracts derived from the 1 h Wnt3a treatment, before any streptavidin-based enrichment, confirming the stabilization of β-catenin by Wnt (Supplementary Table S1, Tab 5).

We next analyzed more deeply this global proteomic data using the Metascape online software. Wnt3a-induced biotinylated proteins were ranked for enrichment over Mock control CM samples. The lists of the top 150 proteins were analyzed for Gene Ontology groups at 5 and 20 min and 1 h, as shown in Fig. S3A–C. After 5 min endocytosis was the top hit, followed by small GTPase-mediated signal transduction. Interestingly, Kras

Figure 3. TFG co-localizes with the ESCRT-0 protein Hrs/Hgs and is required for Wnt signaling. (A–F) Immunostaining on HeLa cells for endogenous Hrs and TFG. Weak co-localization was observed in absence of Wnt signaling (arrow in panel C). However, 5 min of Wnt3a treatment was sufficient to strikingly increase the number of endocytic vesicles containing both Hrs and TFG (arrows). Scale bar 10 µm. (G) Wnt signaling (5 min) increased the number of Hrs positive vesicles quantified as fluorescence per cell; this supports the rapid formation of MVBs in Wnt signaling. (H) Wnt3a increased co-localization of TFG puncta with the Hrs MVB marker by Pearson’s correlation coefficient. (I) TFG knock-down by siRNA reduced Wnt signaling, as assessed by β-catenin Activated Reporter (BAR/Renilla) Luciferase assay in HEK293T cells.
TFG localizes to endocytic vesicles and is required for Wnt signaling. Unexpectedly, one of the most highly enriched proteins upon 20 min of Wnt3a treatment was TFG (Fig. 2D), a protein containing multiple domains (Fig. S4A). N-terminal in-frame fusions of TFG to other genes are found in thyroid and lung cancers, indicating that TFG is required for Wnt signaling in vivo. In line with this, TFG loss of function in one half of the embryo (marked by co-injection of nuclear LacZ mRNA but not human TFG mRNA (Fig. 5H–J). Co-injection of human Flag-tagged TFG mRNA completely rescued the anteriorizing effect of Wnt8 DNA rescued eye and cement gland development (Fig. 5M and Fig. S6E–G), indicating that TFG is required for endogenous Wnt signaling and endocytosis. Importantly, it independently confirms our earlier proposals that the ESCRT machinery and endocytosis participate in Wnt signaling.

TFG is required for Wnt signaling in *Xenopus embryos*. To address the developmental role of TFG in vivo, we turned to *Xenopus* embryos as a model of choice. RT-PCR data showed that TFG was a maternal gene and its expression remained uniform until late stages of development (Fig. S4H). Unlike Chordin, a prototypical Spemann organizer gene, TFG did not show dorsal-ventral polarity (Fig. S4I). In situ hybridization showed that TFG was expressed in the animal pole of early cleavage embryos, and was later strongly expressed in the cement gland, as reported by others (Fig. S4J–N), in addition to staining in the notochord as shown here (Fig. S4O).

To perform loss of function studies in vivo, we designed a morpholinio antisense oligo which targeted both *Xenopus laevis* TFG homeologs (Fig. 5A) and efficiently blocked the translation of microinjected *Xenopus* flag-tagged TFG mRNA but not human TFG mRNA differing in the target sequence (Fig. S6A). Embryos microinjected with TFG morpholino displayed a strong anteriorization, with an enlargement of the cement gland and head (Fig. 5B,C, and Fig. S6B). Head enlargement was confirmed by in situ hybridization using the anterior marker Otx2 (Fig. S6C,D). Co-injection of human Flag-tagged TFG mRNA completely rescued the anteriorizing effect, demonstrating that the phenotype of the TFG morpholino oligo was specific (Fig. 5D). Overexpression of human or *Xenopus* TFG mRNAs were without phenotypic effects on their own. However, human TFG lacking the MO target sequences was able to rescue the large head structures caused by TFG knockdown (Fig. 5D). The phenotype of TFG knockdown was very similar to that caused by the overexpression of known Wnt antagonists, such as Dkk1 (Fig. 5E), indicating that TFG is required for Wnt signaling in vivo. In line with this, TFG loss of function in one half of the embryo (marked by co-injection of nuclear LacZ mRNA) caused a marked decrease in the expression of the Wnt target gene *Engrailed-2* (Fig. 5E,F), while expression of the hindbrain marker Krox20 was still present although shifted posteriorly. The pan-neural marker Sox2 was expanded by TFG morpholino, an effect also observed upon Wnt inhibition, and was rescued by co-injection with human TFG mRNA (Fig. 5H–J).

Next, we injected xWnt8 DNA into *Xenopus* embryos that, as expected, caused the inhibition of anterior structures such as eyes (Fig. 5K,L) and cement gland (Fig. S6E,F). However, injection of TFG-MO together with xWnt8 DNA rescued eye and cement gland development (Fig. 5M and Fig. S6E–G), indicating that TFG is required for Wnt activity in vivo. To show that TFG-MO is required for endogenous late Wnt signaling we used knock-down of the Wnt antagonist Bighead, which is known to result in an increase of Wnt activity. We found that while Bighead-MO caused loss of anterior structures in *Xenopus* embryos, this effect could be rescued by co-injection...
Figure 4. CRISPR-Cas9 mediated TFG knock-out inhibits Wnt-dependent β-catenin stabilization and Wnt-induced reporter activity. (A) Schematic diagram of Cas9 cleaving the TFG genomic DNA target sequence. The target sequence (which is on the reverse strand) is shown in 5′–3′ orientation (left–right); the PAM (protospacer adjacent motif) sequence is in bold. (B) Western blot of HEK293T cells confirming complete elimination of TFG protein in Cas9 knock-out cells. β-actin was used as loading control. (C–F) Immunostaining for β-catenin on WT or TFG KO cells. Cells were treated with control conditioned medium or with Wnt3a conditioned medium for 3 h before immunostaining. Note that TFG knock-out decreases Wnt3a-induced β-catenin accumulation; DAPI was used for nuclear counter-staining. Scale bars represent 20 µm. (G) Cas9-mediated TFG knock-out reduces response to Wnt3a by 50% in HEK293T BAR/Renilla β-catenin reporter cells. Cells were treated with Wnt3a or control medium for 16 h before luciferase analysis. (H) Luciferase assay of HEK293T BAR reporter cells treated with the GSK3 inhibitor CHIR99021 at 5 µM concentration for 16 h before being processed for luciferase assay. Note that TFG KO causes an 80% reduction of β-catenin luciferase activity in response to the GSK3 inhibition. Error bars represent standard deviation from triplicate experiments. Statistical significance was calculated with a paired 2-tailed t-Student test. *P < 0.05; **P < 0.01.
of TFG-MO (Fig. S5–P), indicating that TFG functions downstream of Lrp6. Taken together, the results indicate that the Lrp6-APEX2 interacting protein TFG is required for Wnt/β-catenin signaling in Xenopus development.

**Discussion**

In the present study, we used biotin-dependent proximity labeling to study the early molecular interactions following Wnt/β-catenin activation. Proteomic analyses generated a searchable list of Lrp6 interactors that is provided in Supplementary Table S1, and can be mined by the research community to identify additional Wnt regulators. Importantly, our data showed that ESCRT proteins such as Hrs/Hgs, Stan1/2, CHMP4, Vps28, Vps37, and Tsg101 were recruited close to the Lrp6 Wnt receptor soon after Wnt stimulation (5 min) and were still significantly enriched 20 min later. In agreement with this, our immunostaining on cultured cells showed that 5 min of Wnt3a treatment was sufficient to significantly increase the number of Hrs-containing endocytic vesicles. Thus, the results presented here strongly support the view that multivesicular endosome formation is a key downstream step in Wnt signaling11,12, as indicated in the model shown in Fig. 6. Our proteomic data also indicate a dynamic enrichment of different proteins to Lrp6, over time, as one would expect from a signaling cascade. According to our data, the earliest event would be the recruitment of endosome-associated protein, triggering the endocytosis of the activated Wnt receptor. This is immediately followed by the recruitment of Wnt signalosome proteins (including Axin, Dishevelled etc.) whose enrichment begins at 5 min, but is maximized at later time point (20 min in our analysis). Perhaps the assembly of the Wnt signalosome is stabilized upon endocytosis of the receptor complex. Of note, APC was enriched at 5 min, but strongly down-regulated at 20 min. APC has been shown to inhibit clathrin-mediated endocytosis of Lrp6, a process that prevent inadvertent activation of Wnt pathway38. It is possible that, upon Wnt stimulation, APC is removed from Lrp6, which is then endocytosed into MVBs together with other signalosome components.

The connection between Wnt and endocytosis is well known45,50, and several factors have been found to modulate Wnt through endosomal mechanisms. For example, the protein arginine methyltransferase 1 (PRMT1), which interacts with Lrp651, was shown to be indispensable for the sequestration of GSK3 into MVBs by microautophagy15,35 in order to sustain canonical Wnt signaling. In recent work, a proteomic proximity approach similar to the one reported here for Lrp6-APEX2, made use of a Fzd9b-APEX2 fusion to discover that Epidermal Growth Factor Receptor (EGFR)-mediated phosphorylation of Fzd9b was required for receptor internalization and signal transduction in response to Wnt9b37. In that study, the proteomic data showed that endosome-associated Rabs and the Clathrin-endocytic pathway interacted with Fzd9b upon Wnt9a signaling, supporting the view that the endosomal machinery is an early component of the Wnt/β-catenin pathway. Importantly, in this study we found that Kras, which is a major regulator of macropinocytosis53, interacts with Lrp6-APEX2 after Wnt treatment. A central role for endocytosis in Wnt signaling is consistent with the observations reported here, which revealed striking interactions with the ESCRT machinery that generates MVBs after Wnt treatment (Fig. 2B). In addition, Wnt3a treatment induced interactions between Lrp6 and components of the actin cytoskeletal machinery, which was particularly prominent at early (5 min) and later time points (1 h). This finding was expected, as endocytosis requires remodelling of the cytoskeleton and plasma membrane. While the exact molecular links between Wnt, endosomes and actin cytoskeleton are still unclear, the data presented here support our recent findings that Wnt is essential for macropinocytosis, an actin-driven endocytic process with important consequences on cell metabolism that requires Gsk3 inhibition30,37,38.

The best validation of the proximity biotinylation approach was the identification of an unexpected player in the Wnt pathway. Among the most enriched Lrp6 interactors we found TFG, a protein that was first identified in papillary thyroid carcinomas as a result of an in-frame oncogenic rearrangement between its N-terminal region and the tyrosine kinase domain of Neurotrophic tyrosine kinase receptor type 1 (NTRK1, also designated as Trk)39. The ability to self-oligomerize, conferred by the TFG N-terminal coiled-coil domain, leads to constitutive activation of the tyrosine kinase domain responsible for the transforming activity of this chimeric oncogenic protein40. Similarly, translocations between the TFG and anaplastic lymphoma kinase (ALK) genes result in a fusion oncprotein in some lung cancers41. The physiological function of wild type TFG has been previously investigated. Studies in C. elegans and human cultured cells reported that TFG localizes to the ER exit sites adjacent to the ER-Golgi intermediate compartment known as the ERGIC compartment, a cluster of membranes that secretory cargos traverse on their route to the Golgi apparatus55. TFG hexamers were shown to co-localize and interact with the coat protein complex II (COPII) components Sec16 and Sec23, promoting the trafficking and secretion of cargo proteins, including synaptobrevin55,56. The importance of TFG in membrane traffic is also highlighted by the presence of several pathogenic mutations that impair TFG activity in ER function and are linked to different neurological disorders, including a hereditary motor and sensory neuropathy (HMSN-P)57.

Our findings revealed an additional function for TFG, a protein that localized to the same endosomal vesicles as Hrs. Wnt3a treatment, which increases the number of late endosomes/MVBs55,56, stimulated the co-localization of TFG and HRS. Interactions between TFG, Hrs and other components of the ESCRT machinery such as Tsg101 have been reported on BioGRID (https://thebiogrid.org/), a repository for protein–protein interactions that curates data derived from high-throughput datasets. Moreover, β-catenin reporter assays in cell culture showed that TFG is required for Wnt signaling, as confirmed both by siRNA mediated knock-down and CRISPR/Cas9 knock-out of TFG. Our Cas9 genome editing experiments generated a cell line expressing only the first 90 amino acids of TFG. The resulting short protein lacks the coiled-coil domain, which spans from amino acids 97 to 12449. This detail is of importance, as the coiled-coil domain is involved in TFG oligomerization and is required for its function and transforming activity40, suggesting that our truncated TFG acts potentially as a null mutant that resulted in a 50% inhibition of canonical Wnt signaling. We do not know why the effect of TFG loss of function is only partial on stimulation by Wnt ligands, but we speculate that structurally related proteins may partially compensate for TFG absence.
**TFG.L**

CACGGAGCCCACAATGAAATGGACAACTGGACTTTAAGCG

**TFG.S**

GTCAGAGCCCACAATGAAATGGACAACTGGACTTTAAGCG

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**B**

Ctrl

**C**

TFG-MO

**D**

TFG-MO + hTFG

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**E**

Dkk1

**F**

En2/Krox20

**G**

En2/Krox20

---

**H**

Sox2

**I**

Sox2

**J**

Sox2

---

**K**

Rx2a/MyoD

**L**

Rx2a/MyoD

**M**

Rx2a/MyoD

---

**N**

Ctrl

**O**

BH-MO

**P**

(BH+TFG)-MO

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**TFG.MO and TFG.S MO +22**
The requirement for TFG during Wnt signaling is strongly supported by experiments in *Xenopus laevis* embryos. In vivo loss of function experiments using a TFG morpholino showed that TFG was required for correct antero-posterior (A-P) patterning and head development in frog embryos. In *Xenopus*, A-P patterning is regulated by an activity gradient of Wnt signaling which is lower in the rostral (head) region of the embryo. Numerous secreted Wnt inhibitors, including Dkk1, Frzb and Bighead, are required to maintain low levels of Wnt and allow head development. Embryos injected with TFG morpholino developed larger heads and cement glands, mimicking the effects of Dkk1 overexpression and pointing to a reduction of the endogenous Wnt signal. In agreement with this view, TFG knock-down also abolished the posteriorizing activity exerted by Wnt8 DNA together with 72 ng of TFG-MO showed rescue of Rx2a development (84%, n = 32), indicating that TFG is required for Wnt8 signaling. (N–P) Embryos injected with Bighead morpholino (BH-MO) showed reduced head and cement gland development (71%, n = 21), compared to normal head development in Bighead MO embryos (90%). Scale bars represent 500 μm.

**Methods**

**Cloning and plasmids.** pCS2 was used as a backbone for cloning recombinant DNA. First, the basic pCS2 plasmid was made compatible with the Gateway recombination system, by cloning a ccdb/chloramphenicol cassette (Invitrogen, 11828029) into the pCS2 StuI restriction site. Flag-tagged cytosolic APEX2 (Addgene, 49386) was then amplified by polymerase chain reaction (PCR) and sub-cloned in frame with the 3xFlag epitope tag at the C-terminus of the Gateway cassette (Invitrogen, 11828029) into the pCS2 StuI restriction site. VSV-G-tagged human Lrp6 was obtained from Addgene (27282), amplified by PCR using primers containing Gateway sequences, and sub-cloned through a Gateway reaction following manufacturer’s instructions. Finally, an IRES sequence followed by a GFP-tagged Puromycin N-Acetyltransferase (PAC) gene, obtained from Addgene plasmid 45567, was amplified by PCR and sub-cloned into XbaI/SnaBl sites, downstream of the Lrp6-APEX2 open reading frame (ORF), to allow for selection of stable transfectants with the antibiotic puromycin. A human TFG clone was obtained from Dharmacon, sequence verified, and sub-cloned into pCS2 vector containing a 3xFlag epitope tag at the C-terminus. *Xenopus laevis* TFG was PCR-amplified from cDNA obtained from frog eggs, Gateway-cloned into pCS2-3xFlag and pCS2-Myc-Strep-tact. All cloned sequences were verified by DNA Sanger sequencing (Genewiz). Primer sequences are shown on Table S1. The plasmids used in this study will be made available on the Addgene plasmid repository. All animal experiments procedures were approved by the UCLA institutional Animal Research Committee.

**Stable line transfection.** The pCS2 Lrp6-APEX2-IRES-GFP-PAC plasmid was transfected into Human Embryonic Kidney 293 T (HEK293T) cells using Lipofectamine2000 (Thermo Fisher Sci.). 48 h after transfection puromycin was added to the culture medium, at a final concentration of 4 μg/ml. Stable transfected cells were continuously expanded and selected with antibiotic for at least two weeks, after which monoclonal lines were derived by limiting dilution. Two independent clones were obtained, which maintained stable and uniform expression of the Lrp6-APEX2 and GFP-PAC chimeras after many cell passages.

**Mass spectrometry analyses.** Affinity-purified biotinylated protein samples were reduced and alkylated using 5 mM Tris (2-carboxyethyl) phosphine and 10 mM iodoacetamide, respectively, and then proteolyzed by the sequential addition of trypsin and lys-C proteases at 37 °C as described. The digestion reactions were stopped by the addition of 5% formic acid, desalted using Pierce C18 tips (Thermo Fisher Sci.), and then dried and resuspended in 5% formic acid. Peptide digests were fractionated online using a 25 cm long, 75 μm inner...
diameter fused silica capillary packed in-house with bulk C18 reversed phase resin (1.9 µm, 100A pores, Dr. Maisch GmbH). A 140-min water-acetonitrile gradient was delivered to a maximum of 80% buffer B using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Sci.) at a flow rate of 300 nl/minute (Buffer A: water with 3% DMSO and 0.1% formic acid and Buffer B: acetonitrile with 3% DMSO and 0.1% formic acid). Peptides were ionized using a distal 2.2 kV spray voltage and a capillary temperature of 275 °C and analyzed by tandem mass spectrometry (MS/MS) using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Sci.). Data was acquired by a Data-Dependent Acquisition (DDA) method comprised of a full MS1 scan at 120,000 resolution followed by sequential MS2 scans (Resolution = 15,000) to utilize the remainder of the 3 s cycle time. Data analysis was performed using two discrete bioinformatic pipelines. The first analysis used the Integrated Proteomics pipeline 2 (Integrated Proteomics Applications, San Diego, CA) to generate peptide and protein lists that were quantified using spectra counting. In this case, MS2 spectra were searched using the ProLuCID algorithm against the EMBL Human reference proteome (UP000005640 9606) followed by filtering of peptide-to-spectrum matches (PSMs) by DTASelect using a decoy database-estimated false discovery rate of < 1%. In the second approach, an in-house Galaxy-based pipeline was used to provide label-free MS1 scan intensity-based quantification for the samples. MS2 spectra were searched against the EMBL Human reference proteome (UP000005640 9606) using the MSGF+ search engine. False detection rates for evaluating the peptide-spectrum-match (PSMs) were determined using Percolator while protein identification confidence was estimated via the stand-alone implementation of FIDO such that analytes had respective score cut-off q-values at or below 0.01 at both PSM and protein level. The Integrated peak areas of extracted ion chromatograms across multiple runs were calculated for each peptide using the Skyline software platform. In order to calculate the fold change and determine the protein abundance changes, the MSStats R-package was used to normalize across runs using quantile normalization, summarize peptide-level intensities into a protein-level abundance, and perform statistical testing to compare protein abundance across conditions. The raw dataset generated by mass spectrometry has been deposited in the online mass spectrometry interactive virtual environment (MassIVE) repository, with accession number MSV000084335. Additional methods, including proximity labeling, embryo and cell culture procedures are available in Supplementary Information.

Figure 6. Model of how Lrp6-APEX2 may interact with the ESCRT machinery and TFG in multivesicular body formation during Wnt-induced endocytosis. The endocytosed Wnt receptor complex on the surface of multivesicular endosomes becomes clustered in clathrin-containing invaginations that give rise to the intraluminal vesicles (ILV's) of late endosomes. MVBs are the obligatory pathway through which plasma membrane proteins reach the lysosome. We found that most ESCRT GO components are brought into proximity of Lrp6-APEX2 5 min after treatment with Wnt3a conditioned medium. The top interactor with Lrp6-APEX2 after 20 min of Wnt3a was TFG, which we show here is required for Wnt signaling. TFG protein co-localizes with the ESCRT-0 protein Hrs/Hgs after Wnt stimulation. We did not detect interactions with intraluminal vesicle components such as CD63; a possible explanation is that the acidic medium or lysosomal proteases might interfere with APEX2 activity.
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Author contributions
G.C. and E.M.D.R. designed research; G.C., Y.J.A., A.D., N.T.M., and L.V.A. performed research; G.C., Y.J.A., A.D., N.T.M., L.V.A., E.S., J.A.W. and E.M.D.R. analyzed data; G.C., Y.J.A., J.A.W. and E.M.D.R. wrote the paper.

Competing interests
The authors declare no competing interests.

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