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WLS Retrograde Transport to the Endoplasmic Reticulum during Wnt Secretion

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SUMMARY

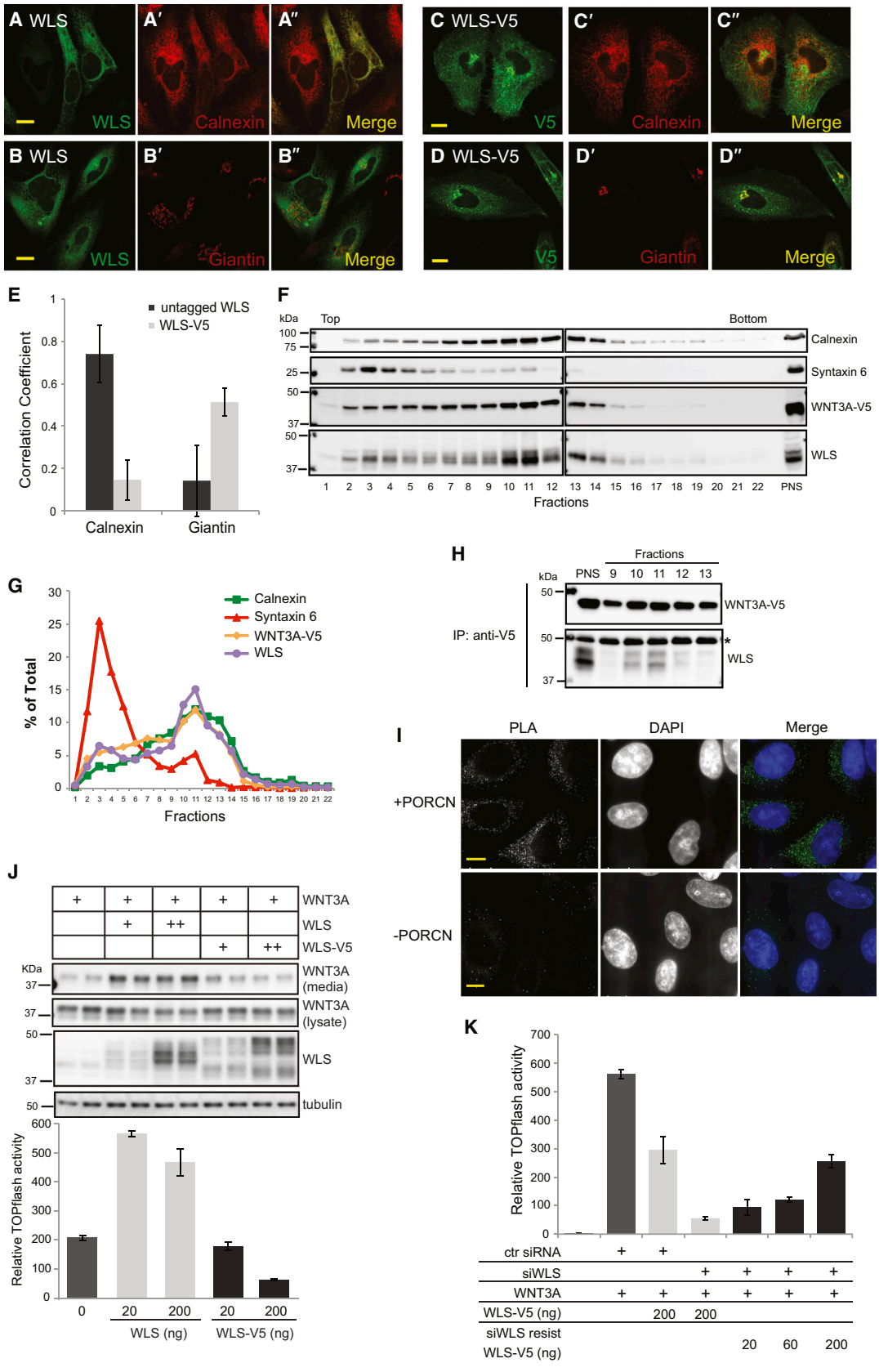
Wnts are transported to the cell surface by the integral membrane protein WLS (also known as Wntless, Evi, and GPR177). Previous studies of WLS trafficking have emphasized WLS movement from the Golgi to the plasma membrane (PM) and then back to the Golgi via retromer-mediated endocytic recycling. We find that endogenous WLS binds Wnts in the endoplasmic reticulum (ER), cycles to the PM, and then returns to the ER through the Golgi. We identify an ER-targeting sequence at the carboxyl terminus of native WLS that is critical for ER retrograde recycling and contributes to Wnt secretory function. Golgi-to-ER recycling of WLS requires the COPI regulator ARF as well as ERGIC2, an ER-Golgi intermediate compartment protein that is also required for the retrograde trafficking of the KDEL receptor and certain toxins. ERGIC2 is required for efficient Wnt secretion. ER retrieval is an integral part of the WLS transport cycle.

INTRODUCTION

Wnt proteins are an evolutionarily conserved family of secreted signaling molecules that play key roles in embryonic development and adult tissue homeostasis. Wnts travel from sending cells to nearby receiving cells to engage a panoply of cell surface receptors, leading to stabilization of β-catenin as well as activation of diverse additional signaling pathways. Wnts and their downstream signaling cascades are dysregulated in a broad spectrum of human diseases, and therefore Wnt expression, secretion, and signaling are the subject of extensive studies (Clevers and Nusse, 2012). While the 19 human Wnts regulate distinct pathways via interaction with multiple cell surface receptors, they appear to share a common biosynthetic and secretion pathway (Harterink and Korswagen, 2012; Najdi et al., 2012; Proffitt and Virshup, 2012). Although most of the elements required for Wnt secretion have been identified, significant gaps remain in our understanding of how newly synthesized Wnts are transported to the cell surface.

All Wnts have signal peptides that direct newly synthesized protein to the lumen of the ER, where they are palmitoleated (Rios-Esteves and Resh, 2013). The lipid-modified Wnts are then transported to the cell surface by the carrier protein WLS. WLS is a core component of the Wnt secretion pathway. Initially identified in genetic screens, *WLS* encodes a multipass transmembrane protein that is evolutionarily conserved in metazoans from cnidarians to *C. elegans* to human (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006; Guder et al., 2006). In mice, germline knockout of *Wls* is lethal early in development due to impairment of body axis formation (Fu et al., 2009). On the other hand, tissue-specific knockouts of *Wls* support its critical role for Wnt signaling in diverse processes including retinal angiogenesis, hair follicle formation, and bone mass regulation (Augustin et al., 2013; Cornett et al., 2013; Huang et al., 2012; Jiang et al., 2013; Stefater et al., 2011; Zhong et al., 2012). In human cells, we demonstrated that WLS binds to and is required for the activity of virtually all Wnts (Najdi et al., 2012). Wnt must be palmitoleated by the ER-resident membrane-bound *O*-acyltransferase Porcupine (PORCN) in order to bind to WLS. Mutation of the Wnt acylation site, knockdown of PORCN, or inhibition of PORCN enzymatic activity with small molecules all prevent Wnt-WLS interaction. As secretory vesicles reach the cell surface, Wnts are released from WLS in a pH-dependent manner (Coombs et al., 2010). Unloaded WLS is recycled from the cell surface via clathrin-mediated endocytosis, followed by retromer- and sorting nexin 3-dependent retrieval from early endosomes to the *trans*-Golgi network (Belenkaya et al., 2008; Franch-Marro et al., 2008; Harterink et al., 2011; Port et al., 2008; Zhang et al., 2011).

A major gap in our understanding of Wnt secretion remains. Addition of the palmitoleate group renders Wnts prone to aggregation unless bound to a carrier protein, and it is unclear how palmitoleated Wnts are transported from the ER to the Golgi. Protein transport from the ER to the Golgi complex relies on a number of protein sorting events in the early secretory pathway. The cycling between ER and Golgi is a dynamic and highly regulated process, with the vesicular-tubular cluster called the



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ER-Golgi intermediate compartment (ERGIC) mediating the trafficking. Specific coat protein complexes (COP) are involved in this bidirectional trafficking. In general, COPII mediates the anterograde transport from the ER to the Golgi, whereas COPI generates the retrograde trafficking from the Golgi back to the ER (Lorente-Rodríguez and Barlowe, 2011). Recently, p24-family proteins have been implicated in Wnt ER exit, although they lack specificity for the Wnt pathway (Buechling et al., 2011; Port et al., 2011). Although WLS is an ideal candidate to mediate Wnt trafficking from the ER, its role in ER exit has been discounted because most studies of WLS have found the protein to be localized to the Golgi and to recycle between the Golgi and plasma membrane (PM) via endocytosis and retrograde transport (Belenkaya et al., 2008). However, an additional Wnt carrier protein that accommodates palmitoleated Wnt in the ER has not been identified, and would necessitate an extra handoff.

Here we reexamine the question of WLS localization and function using methods that visualize the localization of the native protein. We find that in contrast to the carboxy-terminal epitope-tagged WLS used in prior published studies, there is a substantial pool of native WLS resident in the ER that interacts with both PORCN and palmitoleated Wnts. The retrograde trafficking of WLS from Golgi to ER requires an unusual ER-targeting sequence at the carboxyl terminus of WLS. This dynamic targeting signal is conserved from *Drosophila* to humans. By surface biotin labeling, we show that WLS can travel from the cell surface to the ER through the Golgi. Consistent with a key role of the carboxyl terminus in the localization of WLS, a primate-specific splice variant (WLSv2), which differs only in the carboxyl terminus, does not recycle to the ER. Confirming the importance of Golgi-to-ER shuttling of WLS in the Wnt secretory pathway, we find that ERGIC2, a protein essential for retrograde traffic of various toxins, is crucial for both WLS ER localization and optimal Wnt secretion. The ERGIC2-dependent retrograde shuttling of WLS from Golgi to ER closes the loop on the core Wnt secretory machinery. WLS is thus an integral membrane carrier protein that cycles from ER to PM and back to the ER.

RESULTS

Tagging of the WLS Carboxyl Terminus Affects Its Subcellular Localization

The current model for Wnt secretion holds that Wnts are palmitoleated in the ER and then transported to the Golgi, where they bind to WLS. WLS then carries Wnts to the PM, releases the Wnts, and is recycled via endocytosis, sorting nexins and the retromer complex back to the *trans*-Golgi network (TGN). This model is based on the finding that WLS is localized to the PM, Golgi, and cytoplasmic vesicles and is not seen in the ER by indirect immunofluorescence microscopy (Bartscherer et al., 2006; Belenkaya et al., 2008; Harterink et al., 2011; Yang et al., 2008). However, low-abundance ER proteins are difficult to visualize by immunofluorescence microscopy due to the large area of the ER. In our studies of Wnt secretion, we generated monoclonal antibody YJ5 that recognizes the first predicted extracellular loop of human WLS and showed that this antibody specifically recognizes both overexpressed and endogenous WLS by immunoblotting in multiple cell lines (Coombs et al., 2010). Using YJ5, we confirmed previous reports that WLS with either a V5 or GFP tag at the carboxyl terminus localizes predominantly to the Golgi (Figures 1C–1E; Figure S1A available online) (Yang et al., 2008). However, when we removed the carboxy-terminal V5 epitope tag by the simple expedient of reintroducing the native stop codon, we found that under identical transfection conditions the subcellular localization changed. Transiently expressed untagged WLS colocalized prominently with the ER chaperone protein Calnexin, but not with the Golgi marker Giantin, indicating it is in the same compartment in which Wnt is synthesized and palmitoleated (Figures 1A, 1B, and 1E; Figures S1B and S1C). The ER localization of untagged WLS was verified in multiple cell types including HeLa (cervical epithelial cancer), U2OS (osteosarcoma), and HUH7 (hepatocellular carcinoma) (Figure S2A and data not shown). To confirm that these results were not antibody specific, the studies were verified using a rabbit polyclonal antibody raised against the carboxyl terminus of WLS (Figures S3A and S3B). These results indicate that a significant fraction of untagged WLS is present in

Figure 1. Native WLS Is in the ER Rather Than the Golgi

(A–D) HeLa cells transiently expressing untagged (A and B) or carboxy-terminal V5-tagged WLS (C and D) were stained with either YJ5 or anti-V5 antibody. Calnexin was used as an ER marker, and Giantin was used as a Golgi marker. The images were taken using a Zeiss LSM 710 confocal microscope. Scale bars represent 20 μm.

(E) The colocalization of WLS with Calnexin and Giantin was quantified by calculating Pearson's correlation coefficient value using the Coloc 2 plugin of ImageJ. Error bars represent SD.

(F) Endogenous WLS is enriched in the ER membrane fractions. The subcellular distribution of Calnexin (ER marker), Syntaxin 6 (TGN and endosome marker), and endogenous WLS was analyzed with WNT3A-transfected HeLa cell postnuclear supernatant (PNS) using a continuous 10%–30% iodixanol gradient.

(G) Quantitation of Calnexin, Syntaxin 6, WNT3A-V5, and WLS from immunoblots in (F).

(H) Interaction of WNT3A and WLS takes place in the ER. ER fractions from (F) were used for immunoprecipitation (IP) with an anti-V5 antibody. PNS was included for IP as a positive control. Asterisk indicates IgG.

(I) WLS is interacting with PORCN by PLA. Twenty nanograms of untagged WLS with (upper panel) or without (lower panel) 20 ng of HA-PORCN expression plasmids was transfected into HeLa cells seeded in an eight-well chambered coverglass. PLA was performed at 48 hr posttransfection as per the manufacturer's instructions. The images were taken using a Nikon N-SIM confocal microscope. Scale bars represent 10 μm.

(J) Carboxy-terminal tags impair WLS function. Untagged WLS or WLS-V5 expression constructs (+, 20 ng; ++, 200 ng) were transfected together with 100 ng of pPGK-mWnt3A into STF cells in 12-well plates. Culture medium and lysates were collected 48 hr posttransfection. Twenty microliters of medium and 25 μg of lysates were assessed by SDS-PAGE and immunoblot. Bottom: luciferase activity was measured and normalized to LDH activity. Error bars represent SD.

(K) Carboxy-terminally tagged WLS has limited activity in the absence of endogenous WLS. WLS-V5 or siRNA-resistant WLS-V5 expression constructs were transfected into STF cells together with 100 ng of pPGK-mWnt3A in 12-well plates in the presence of 100 nM WLS siRNA as indicated. Luciferase activity was measured 48 hr posttransfection. Error bars represent SD.

See also Figure S1.

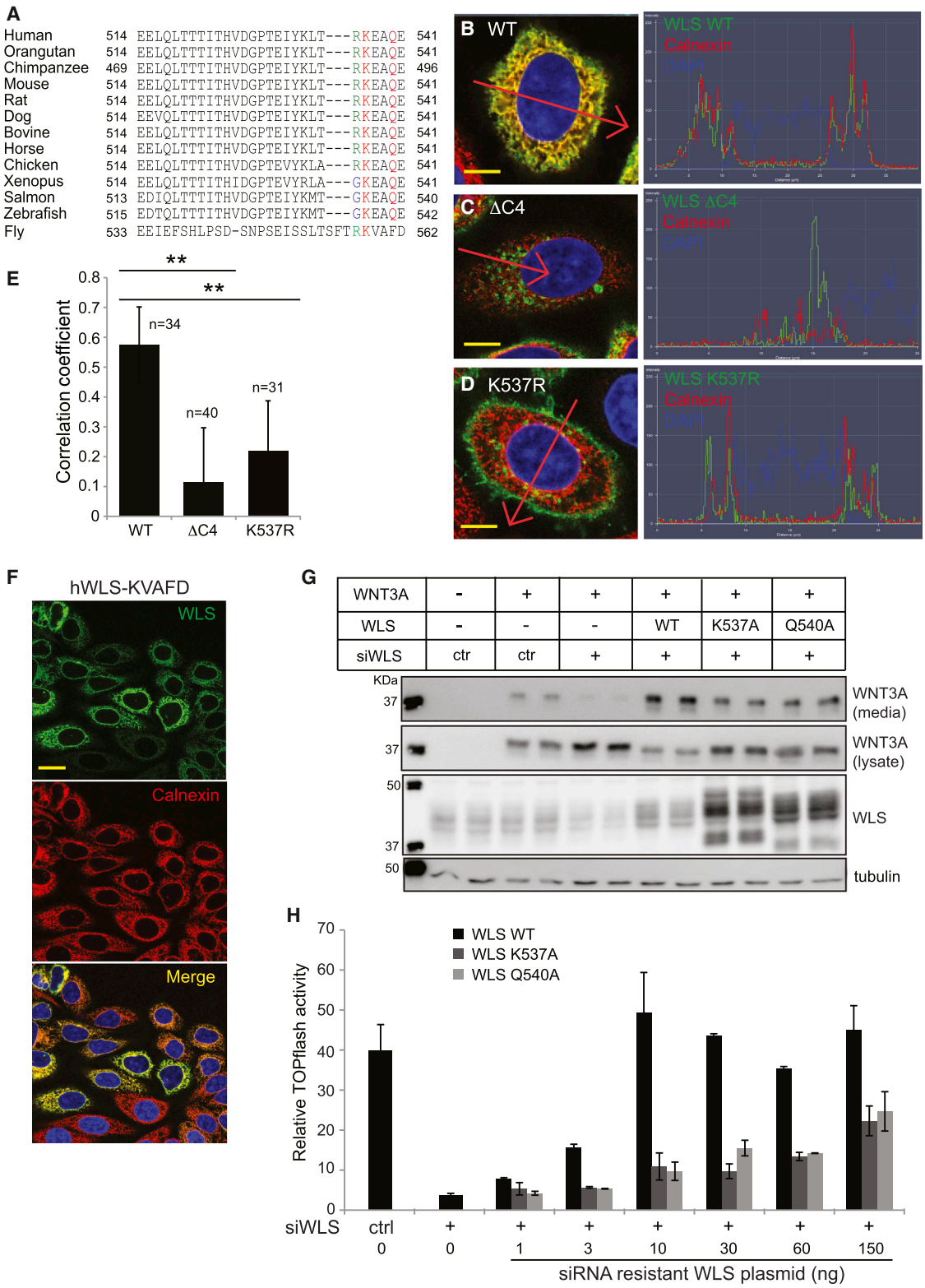

Figure 2. WLS Carboxy-Terminal Sequences Are Important for Subcellular Localization and Function

(A) WLS carboxy-terminal sequences are highly conserved in vertebrates.

(B–D) WLS carboxy-terminal mutants have altered subcellular localization. Wild-type WLS or the indicated mutants were transiently expressed in HeLa cells and visualized with YJ5 and anti-Calnexin antibodies using a Zeiss LSM 710 confocal microscope. Scale bars represent 10 μm. The fluorescence intensity of the staining along the indicated lines was quantified with Zeiss Zen 2009 software (right panel).

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the ER and suggest that sequences at or near the WLS carboxyl terminus regulate its ER localization.

Endogenous WLS Is Predominantly Localized in the ER

To independently confirm that endogenous WLS concentrates in the ER, organelles were fractionated by equilibrium density gradient ultracentrifugation. The postnuclear supernatant extracted from exponentially growing HeLa cells was applied to 10%–30% continuous iodixanol gradients, and after overnight centrifugation, fractions were analyzed by SDS-PAGE and immunoblotting. As Figures 1F and 1G demonstrate, ER and Golgi membrane proteins were well separated. Similar to the observation with indirect immunofluorescence microscopy, endogenous WLS and ectopically expressed WNT3A were highly enriched in the ER fractions. The ER localization of WLS was not due to WNT3A overexpression, since identical WLS fractionation profiles were seen in nontransfected cells (Figure S4F). Smaller quantities of WLS and WNT3A were present in the Golgi fractions (Figure 1G, purple line), consistent with the role of WLS in transporting Wnt throughout the secretory pathway. Confirming that carboxy-terminal modification of WLS alters its localization, overexpressed WLS-GFP concentrated in the Golgi fractions, whereas untagged WLS was enriched in the ER fractions (Figures S1D–S1F). The function of WLS is to transport palmitoleated Wnts, and we confirmed that WLS in the ER fractions was indeed bound to WNT3A in a palmitoleation-dependent manner (Figure 1H; Figure S1G). Thus, ER-localized WLS can transport Wnts.

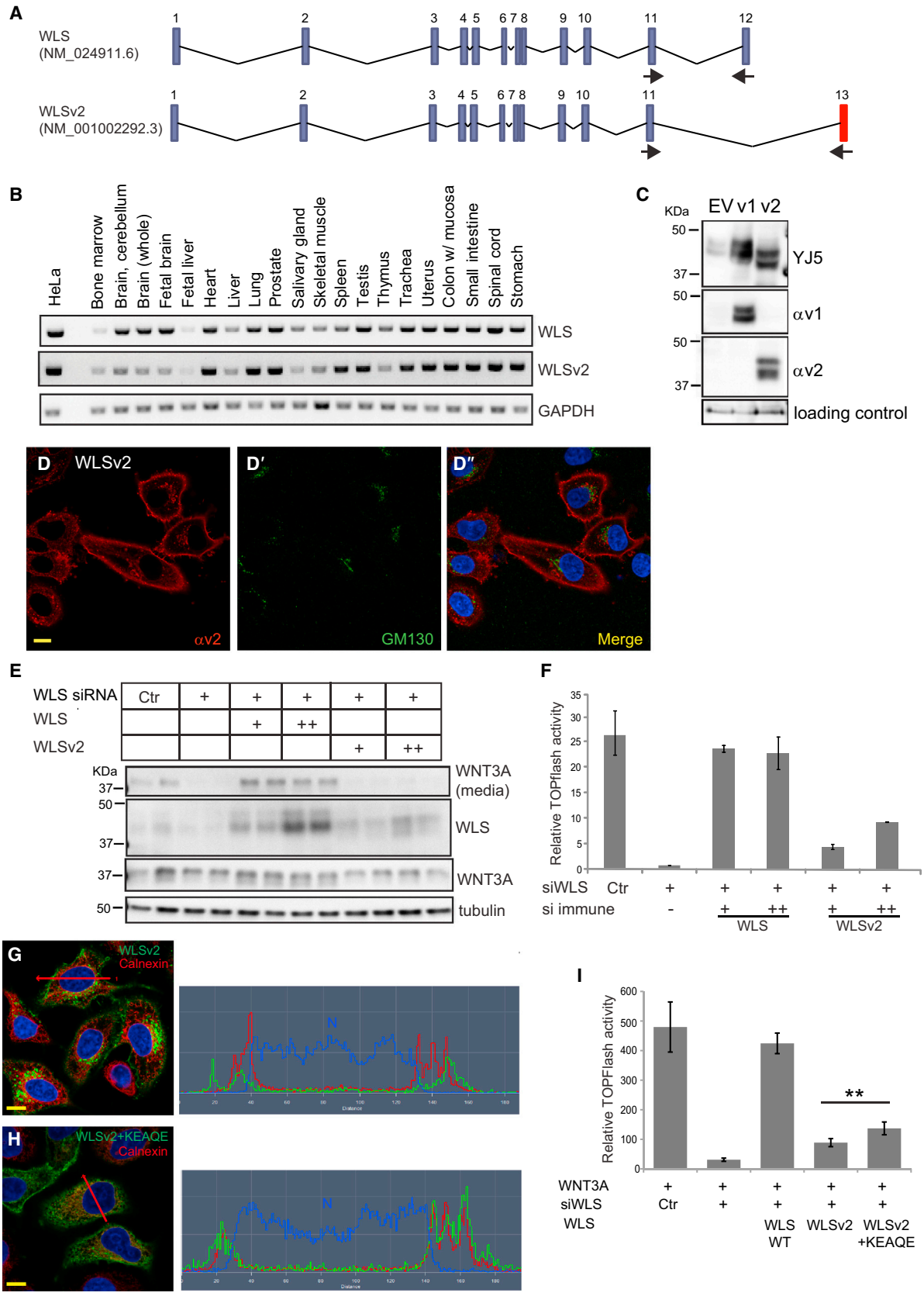
The colocalization and binding of WLS to WNT3A in the ER suggested that WLS is in close proximity to the site of Wnt palmitoleation. Indeed, endogenous WLS shows partial overlap with the Wnt acyltransferase PORCN in the ER (Figure S1H). In fact, WLS and PORCN appear to be in very close proximity, as we were also able to detect their interaction using a proximity ligation assay (PLA) (Figure 1I) (Söderberg et al., 2006). If WLS function were primarily required to transport Wnts out of the Golgi, one would expect Wnts to accumulate in the Golgi in the absence of WLS. However, our analysis of WLS-depleted cells did not reveal any Golgi accumulation but rather showed increased persistence of Wnts (Figures S1K and S1L) primarily in the ER (Figures S1I and S1J). Taken together, the data are consistent with the model that Wnts are palmitoleated by PORCN in the ER and then immediately bound to an ER pool of WLS for transport to the cell surface.

If the Golgi accumulation of carboxy-terminally tagged WLS is due to the blocking of a biologically important ER trafficking signal, then WLS with a V5 tag should be less active than wild-type WLS in its ability to shuttle active Wnts out of the cell. To test this, we expressed near-endogenous amounts of untagged or tagged WLS along with WNT3A in a HEK293 cell line that contains an integrated SuperTOPflash reporter (STF cells) (Xu et al., 2004). HEK293 cells have relatively low levels of endogenous WLS expression as compared to other cell lines (data not shown). Under these conditions, WLS was rate limiting for Wnt secretion and activity, since expression of untagged WLS stimulated Wnt secretion (Figure 1J, upper panel) and increased Wnt/β-catenin signaling (Figure 1J, lower panel). Transfection of low amounts of the WLS plasmid was sufficient to promote WNT3A secretion, as 20 ng of WLS expression plasmid gave near-maximal stimulation. Interestingly, increasing the amount of transfected wild-type WLS plasmid from 20 to 200 ng did not further increase WNT3A secretion (Figure 1J). The identical amount of plasmid expressing carboxy-terminal V5-tagged WLS suppressed Wnt/β-catenin signaling, presumably because it competes with endogenous WLS (Figure 1J, lower panel). However, when endogenous WLS was knocked down by siRNA, marked overexpression of WLS-V5 could rescue Wnt/β-catenin signaling (Figure 1K, black bars). This residual activity of carboxy-terminally tagged WLS may explain why overexpression of WLS-HA (hemagglutinin) under a strong promoter rescued a loss-of-function mutation in *wls* in *Drosophila* (Bänziger et al., 2006).

WLS Carboxy-Terminal Sequences Are Important for Its ER Localization and Function

The data suggest that WLS contains a carboxy-terminal ER-targeting signal that is hindered or blocked by the addition of an epitope tag. Integral membrane ER-resident proteins can be retrieved from the Golgi by means of a dilysine motif at the carboxyl terminus that facilitates their interaction with the COPI vesicles (Cosson and Letourneur, 1994; Nilsson et al., 1989). Sequence alignment of WLS revealed the highly conserved sequence 536-RKEAQE-COOH at the extreme carboxyl terminus (Figure 2A) with features suggestive of a dibasic motif. Illustrating the importance of this sequence, deletion of the last four residues, EAQE (at the extreme carboxyl terminus, designated ΔC4) resulted in distinct punctate localization of WLS (Figure 2C). Individual residues were then mutated to alanine to test their role in ER localization (Figures S2B–S2F). Mutants R536A, E538A, and E541A had ER localization similar to WNT3A and wild-type WLS. However, mutants K537A and Q540A each lost ER localization and instead colocalized with the Golgi marker Giantin (Figures S2H and S2I). To test whether the charge alone is important for the cellular localization, K537 was mutated to arginine. As is true for the dilysine ER-retrieval motif, arginine could not substitute for lysine at the −5 position of WLS, since the K537R mutant

(E) The correlation coefficient of the WLS and Calnexin staining was measured to assess the degree of their colocalization (see Experimental Procedures for detailed methods). Error bars represent SD. Statistical significance was calculated using a two-tailed unpaired t test. **$p < 0.001$.
(F) Human WLS with *Drosophila* carboxy-terminal sequence (hWLS-KVAFD) is colocalized with Calnexin. hWLS-KVAFD was transiently expressed in HeLa cells and visualized with YJ5 and anti-Calnexin antibodies using a Zeiss LSM 710 confocal microscope. Scale bar represents 20 μm.
(G) WLS localization mutants have impaired ability to support Wnt secretion. One hundred nanograms of the indicated WLS expression constructs resistant to the siRNA was transfected into STF cells along with 50 ng of pPGK-mWnt3A in 12-well plates in the presence of WLS siRNA as indicated. Culture medium and lysates were collected 48 hr posttransfection and analyzed as above.
(H) WLS localization mutants have impaired signaling activity. Where indicated, endogenous WLS was depleted with siRNA in STF cells, and signaling was rescued by expression of siRNA-immune wild-type or mutant WLS. All wells received 50 ng of pPGK-Wnt3A in a 24-well plate. Luciferase activity was measured 48 hr posttransfection and normalized to LDH activity. Error bars represent SD.
See also Figure S2.



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relocalized to the Golgi and plasma membrane as well as some cellular vesicles (Figure 2D) (Jackson et al., 1990). The visual impression of altered colocalization was confirmed by quantitation of colocalization of WLS with the ER marker Calnexin (Figure 2E). Interestingly, lysine at the −5 position is also conserved in the *Drosophila* WLS protein (Figure 2A). Human WLS with *Drosophila* sequences replacing the human carboxyl terminus (hWLS-VAFD) retains its ER localization (Figure 2F), indicating that the fly sequences can also act as a targeting signal. Thus, human and *Drosophila* WLS have an ER-targeting signal related to but distinct from the classic dilysine motif.

Confirming the functional importance of ER targeting of WLS, mutants defective in ER localization were impaired in their ability to support Wnt secretion in HEK293 cells as compared to the wild-type WLS (Figure 2G). Depletion of WLS by RNAi inhibited Wnt/β-catenin signaling, and this was fully rescued by transfection of as little as 10 ng of siRNA-immune WLS expression plasmid (Figure 2H). Notably, WLS K537A and Q540A, while expressed at comparable levels, were several-fold less active than wild-type WLS in rescuing the signaling defect caused by WLS knockdown. The findings that single amino acid changes can both shift localization from the ER to the Golgi and impair function confirm that ER localization is important for WLS function.

A Human WLS Splice Variant that Differs at the Carboxyl Terminus Has Alternative Functions

The data indicate that the conserved carboxyl terminus of WLS is critically important for its targeting to the ER compartment as well as its function. In fact, human WLS has an alternatively spliced transcript with a different carboxyl terminus (here called variant 2, or WLSv2; RefSeq accession number NP_001002292.3). A BLAST search revealed that, although variant 1 is evolutionarily conserved from *C. elegans* to human, variant 2 is primate specific. The two variants use alternative terminal exons, leading to distinct carboxy-terminal amino acid sequences and 3′ UTRs (Figure 3A). This allowed us to test whether a naturally occurring modification of the WLS carboxyl terminus alters its shuttling and function.

Using splice variant-specific PCR primers, we confirmed that variant 2 is widely expressed in human tissues (Figure 3B). We then generated splice variant-specific affinity-purified antibodies and confirmed their specificity in both immunoblots and immunofluorescence microscopy (Figures 3C and 3D; Figures S3A–S3C). Based on immunoreactivity after reciprocal knockdown of variant 1 and variant 2, both splice variants are endogenously expressed at the protein level, with variant 1 present at much higher levels than variant 2 (Figures S3D and S3E). WLS variant 2 was transiently overexpressed to determine what effect the alternative carboxyl terminus has on its localization. Notably, as assessed by both immunofluorescence and cell surface labeling, variant 2 was located at the plasma membrane and in cytosolic vesicles distinct from the ER and Golgi (Figure 3D; Figures S3F–S3H). Thus, splice variant 2 is not simply "not ER" but rather is specifically targeted differently from variant 1. Consistent with the model that ER localization is critical for WLS function in canonical Wnt signaling, WLS variant 2 when expressed at near-physiological levels could neither support efficient WNT3A secretion (Figure 3E) nor replace WLS variant 1 in stimulating Wnt/β-catenin signaling (Figure 3F). Finally, to test whether the variant 1 carboxyl terminus was sufficient for ER targeting, we added the sequence -KEAQE to the tail of WLSv2. WLSv2+KEAQE, unlike wild-type variant 2, has more overlap with Calnexin in the ER (Figures 3G and 3H; Figure S3I). This indicates that the KEAQE is sufficient to partially restore WLS ER localization. Similarly, WLSv2+KEAQE is significantly more active than WLSv2 in rescuing TOPflash activity after WLS knockdown (Figure 3I). These data support a critical role for the WLS carboxyl terminus in WLS function. However, the relatively weak ability of KEAQE to convert WLSv2 into an efficient component of the Wnt secretory pathway suggests that WLSv2 might have specific targeting functions of its own.

ER Localization of WLS Is Due to Retrograde Trafficking

WLS is known to traffic from the Golgi to the PM and recycle back to the Golgi via the retromer complex (Franch-Marro et al., 2008; Port et al., 2008). Our data suggest that in addition, WLS has a carboxy-terminal ER localization signal, and its modification, for example, by K537 mutation, leads to redistribution of WLS to the Golgi and beyond. To begin its retrograde transport from the cell surface, WLS is endocytosed via AP-2/clathrin-coated vesicles (Port et al., 2008). WLS endocytosis can be blocked by the WLS Y425A mutation (Gasnereau et al., 2011). We reasoned that a Y425A/K537A double mutant should differentiate the role of carboxy-terminal sequences between the following two routes during WLS trafficking. If the K537A mutant were defective in anterograde transport from the Golgi to the PM,

Figure 3. Alternative Splicing of Human WLS Causes Isoform Relocalization

(A) Cartoon representation of the WLS human exon splicing structure. The arrows indicate the primer positions for amplifying specific transcripts in (B).

(B) Both WLS variants are expressed in multiple human tissues. Semiquantitative RT-PCR was done using human total RNA Master Panel II (Clontech) from 20 different human tissues.

(C) Variant-specific WLS polyclonal antibody (αv1 for variant 1 and αv2 for variant 2) recognized overexpressed WLS variants. HeLa cells expressing either empty vector (EV), WLS variant 1 (v1), or WLS variant 2 (v2) were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies.

(D) WLSv2 has a distinct subcellular localization. Untagged WLSv2 (100 ng plasmid) was transiently expressed in HeLa cells, and subcellular localization was assessed with the αv2 antibody. GM130 is a Golgi marker. The images were taken with a Zeiss LSM 710 confocal microscope. Scale bar represents 10 μm.

(E and F) WLSv2 rescues WNT3A secretion less efficiently than variant 1. WLS or WLSv2 siRNA-immune expression constructs (+, 50 ng; ++, 200 ng) were transfected together with either control (Ctr) or WLS siRNA and 100 ng of pPGK-mWnt3A into STF cells in 12-well plates. Protein expression, Wnt secretion (E), and TOPflash activity (F) were assessed as above. Error bars represent SD.

(G and H) The WLS ER localization sequence KEAQE is sufficient to relocalize WLSv2 to the ER. The fluorescence intensity was measured along the indicated lines using Zeiss ZEN 2009 software (right: red, Calnexin; green, WLSv2). Scale bars represent 10 μm. N, nucleus.

(I) Addition of KEAQE to WLSv2 enhances its activity. The indicated WLS siRNA-immune expression constructs (10 ng) and pPGK-mWnt3A (100 ng) were transfected into STF cells treated with either control (Ctr) or WLS siRNA in 12-well plates. Luciferase activity was measured 48 hr after DNA transfection and normalized to LDH activity. Error bars represent SD. Statistical significance was calculated using a two-tailed unpaired t test. $^{**}p < 0.01$.

See also Figure S3.

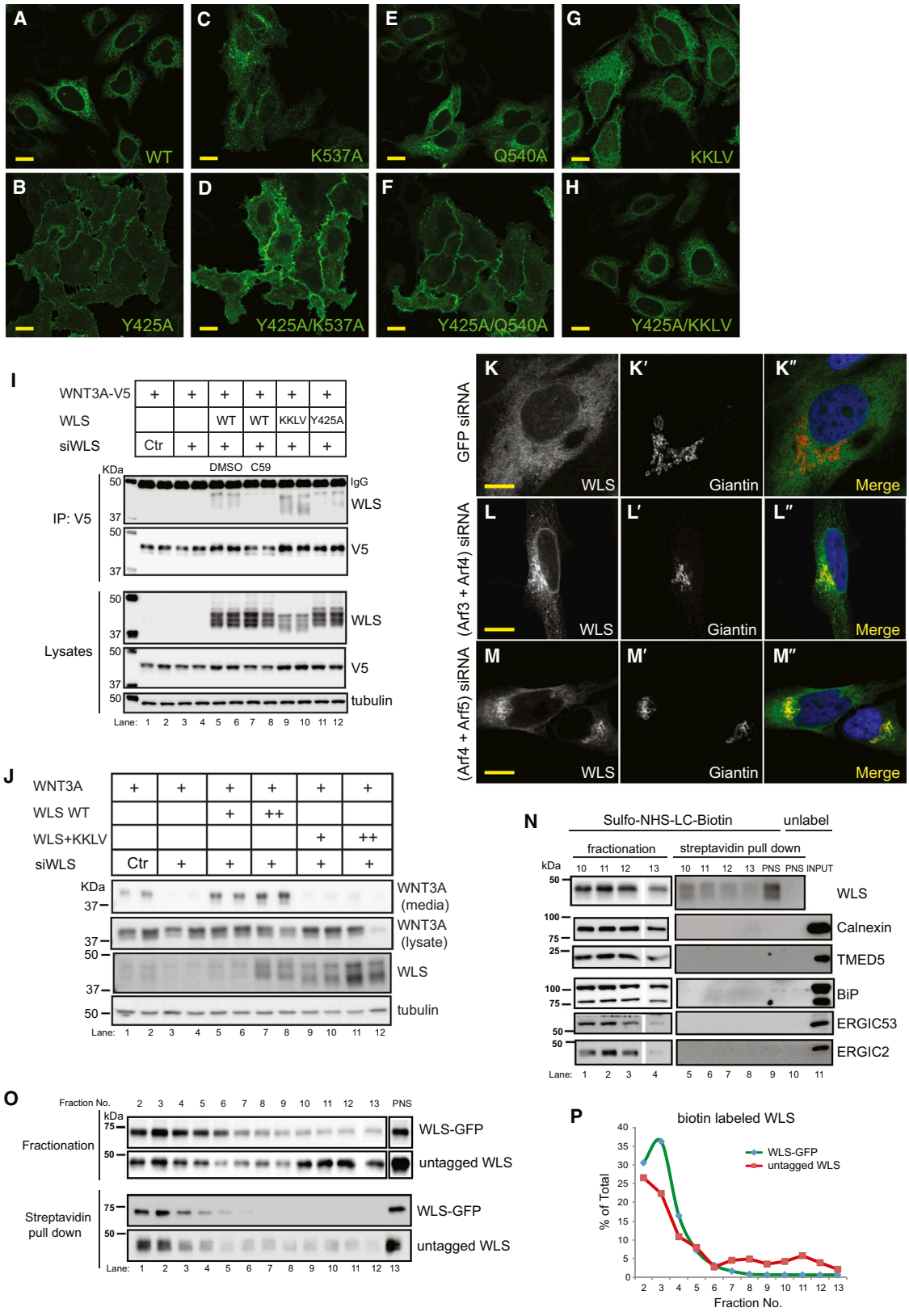

Figure 4. The WLS ER Localization Signal Functions Downstream of Endocytosis

(A–F) Endocytosis is upstream of ER localization. WLS ER localization mutants were transiently expressed in HeLa cells as single mutants or as double mutants in combination with the Y425A endocytosis mutant as indicated. Endocytosis mutants are not able to return to the ER. Scale bars represent 10 μm.

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the double mutant would show similar localization as the K537A. However, if the K537A mutant cannot function in retrograde traffic from the Golgi to the ER, the Y425A mutation would prevent it from ever reaching the Golgi. We first confirmed the finding of Gasnereau et al. (2011) that mutation of Y425 to alanine leads to the accumulation of wild-type untagged WLS at the plasma membrane (Figures 4A and 4B). We next tested the double mutants Y425A/K537A and Y425A/Q540A. In both cases, the double mutants accumulated predominantly at the PM but not in the Golgi (Figures 4C–4F). These results support the model that WLS cycles from the ER to the PM and back to the ER via the Golgi. The carboxy-terminal ER-targeting signal causes accumulation in the ER due to active recycling from the Golgi rather than accumulation of newly synthesized protein.

One additional prediction of the model is that if a strong dilysine ER retention signal were added to replace the dynamic ER-targeting motif on WLS, the strong signal would prevent the PM trafficking of WLS (and thus Wnts) and secretion of the Wnt proteins would be reduced. In order to test this, -RKEAQE was replaced with -KKLV, the last four residues in the p24 protein, and both the localization and activity in a Wnt/β-catenin signaling assay were tested. As expected, WLS-KKLV protein showed typical ER localization (Figure 4G). Confirming that ER-resident WLS can bind Wnts, WLS-KKLV bound to WNT3A in immunoprecipitation assays (Figure 4I, lanes 9 and 10). In contrast to the Golgi-localized mutants, additional mutation of Y425 did not cause WLS-KKLV to accumulate at the plasma membrane (Figure 4H), presumably because WLS-KKLV never left the ER. Consistent with this, WLS-KKLV was dominant negative in Wnt/β-catenin signaling and was not able to rescue WNT3A secretion defects due to WLS knockdown (Figure 4J; Figure S4A). Thus, either increasing or decreasing the strength of the WLS ER-retrieval sequence significantly impaired its ability to support Wnt signaling.

WLS Golgi-to-ER Retrieval Requires ARF and ERGIC2

We wished to better understand the retrograde trafficking of WLS from the Golgi to the ER. The best-characterized Golgi-to-ER retrograde trafficking route is dependent on the COPI coat, which consists of the small GTPase ARF1 and a cytosolic multimeric protein complex called coatomer (Girod et al., 1999). COPI is essential for the retrieval of ER-resident membrane proteins with the classic retrieval motif KKxx (Letourneur et al., 1994).

To test whether COPI is required for WLS retrieval, we perturbed the function of the ARF proteins involved in retrograde but not anterograde traffic between the ER and Golgi (Dascher and Balch, 1994). A combination of ARF isoform knockdowns was employed to achieve specific blockage of Golgi-to-ER transport. It has been reported that double knockdown of ARF3 and ARF4 or ARF4 and ARF5 increased the perinuclear localization of the KDEL receptor but had no or limited effects on anterograde or endosome-to-plasma membrane traffic (Volpicelli-Daley et al., 2005). Depletion of either ARF3 and ARF4 or ARF4 and ARF5 each shifted WLS from the ER to the Golgi (Figures 4K–4M), consistent with a role for ARF-mediated retrograde transport of WLS. Further supporting this model, overexpression of ARF1 Q71L, which blocks the Src-induced Golgi-to-ER relocalization of *N*-acetylgalactosaminyltransferase (GalNac-T) (Gill et al., 2010), markedly reduced WNT3A secretion (Figure S4B). ARF1 Q71L expression also shifted WLS localization from the ER to the Golgi (Figures S4C and S4D). These findings are consistent with a role for COPI vesicles in the retrograde trafficking of WLS from the Golgi to the ER.

Considering the fact that WLS can be recycled via endocytosis and retromer complex-mediated endosome-to-TGN retrograde transport, if the Golgi-to-ER targeting model is correct, a subset of WLS in the ER should be recycled from the cell surface. We tested this model using direct cell surface labeling, followed by detection of the labeled WLS in different cellular compartments. HeLa cells (with high endogenous WLS) were labeled with cell surface-impermeable biotin for 2 hr, and the postnuclear supernatant was fractionated by density gradient centrifugation as before (Figure S4F). The ER fractions (i.e., fraction numbers 10–13) were then subjected to streptavidin pull-down to precipitate surface biotin-labeled proteins. As illustrated in Figure 4N, among the many ER or ER/Golgi cycling proteins tested (including ER-resident proteins Calnexin and BiP, p24-family protein TMED5, and ER/Golgi intermediate compartment proteins ERGIC2 and ERGIC53), only WLS showed biotin labeling. Approximately 20% of the surface-biotinylated WLS was found in the ER. The cell surface protein epidermal growth factor

(G and H) Endocytosis is downstream of the strong ER localization signal KKLV. WLS-KKLV or the Y425A/KKLV double mutant was transiently expressed in HeLa cells. Localization was assessed by staining with YJ5 antibody. Scale bars represent 10 μm.

(I) WLS binds Wnt in the ER. WNT3A-V5 and either wild-type WLS, ER-localized WLS-KKLV, or endocytosis-defective WLS Y425A were coexpressed in STF cells. For lanes 5–8, cells were treated with either 0.1% DMSO or 10 nM PORCN inhibitor C59 for 20 hr. WNT3A was immunoprecipitated with anti-V5 antibody.

(J) WLS with a strong ER localization signal cannot rescue Wnt secretion. Wild-type or WLS-KKLV siRNA-immune plasmids (+, 50 ng; ++, 200 ng) were transfected into STF cells together with control (Ctr) or WLS siRNA and 100 ng of pPGK-mWnt3A in 12-well plates. Wnt secretion was assessed 48 hr later as above.

(K–M) Specific blocking of Golgi-to-ER retrograde transport by knocking down of ARF3/4 or ARF4/5 shifts the localization of WLS toward the Golgi. Each ARF siRNA (25 nM) was transfected into HeLa cells stably expressing WLS. Localization was assessed by staining with YJ5 and anti-Giantin antibodies. Scale bars represent 10 μm.

(N) Cell surface biotin-labeled WLS can be detected in the ER fractions. HeLa cells were labeled with Sulfo-NHS-LC-Biotin at 37°C for 2 hr, and the extracted PNS was centrifuged in a 10%–30% iodixanol density gradient. ER fractions (i.e., fraction numbers 10–13) were used for streptavidin pull-down to detect biotin-labeled proteins. Unlabeled cell PNS pull-down was used as a negative control (lane 10). INPUT is PNS without pull-down (lane 11). TMED5, integral ER/Golgi p24-family protein; BiP, lumenal ER-resident protein.

(O) Only untagged WLS, but not WLS-GFP, can recycle back to the ER. HeLa cells were transfected with either untagged WLS or carboxy-terminal GFP-tagged WLS (WLS-GFP). Forty-eight hours posttransfection, the cells were labeled with Sulfo-NHS-LC-Biotin at 37°C for 2 hr, and extracted PNS was spun in 10%–30% iodixanol density gradient ultracentrifugation. Fractions 2–13 were used for streptavidin pull-down to detect biotin-labeled proteins.

(P) Quantitation of surface biotin-labeled WLS protein (streptavidin pull-down blots) from (O). The data are represented as percentage of total labeled WLS.

See also Figure S4.

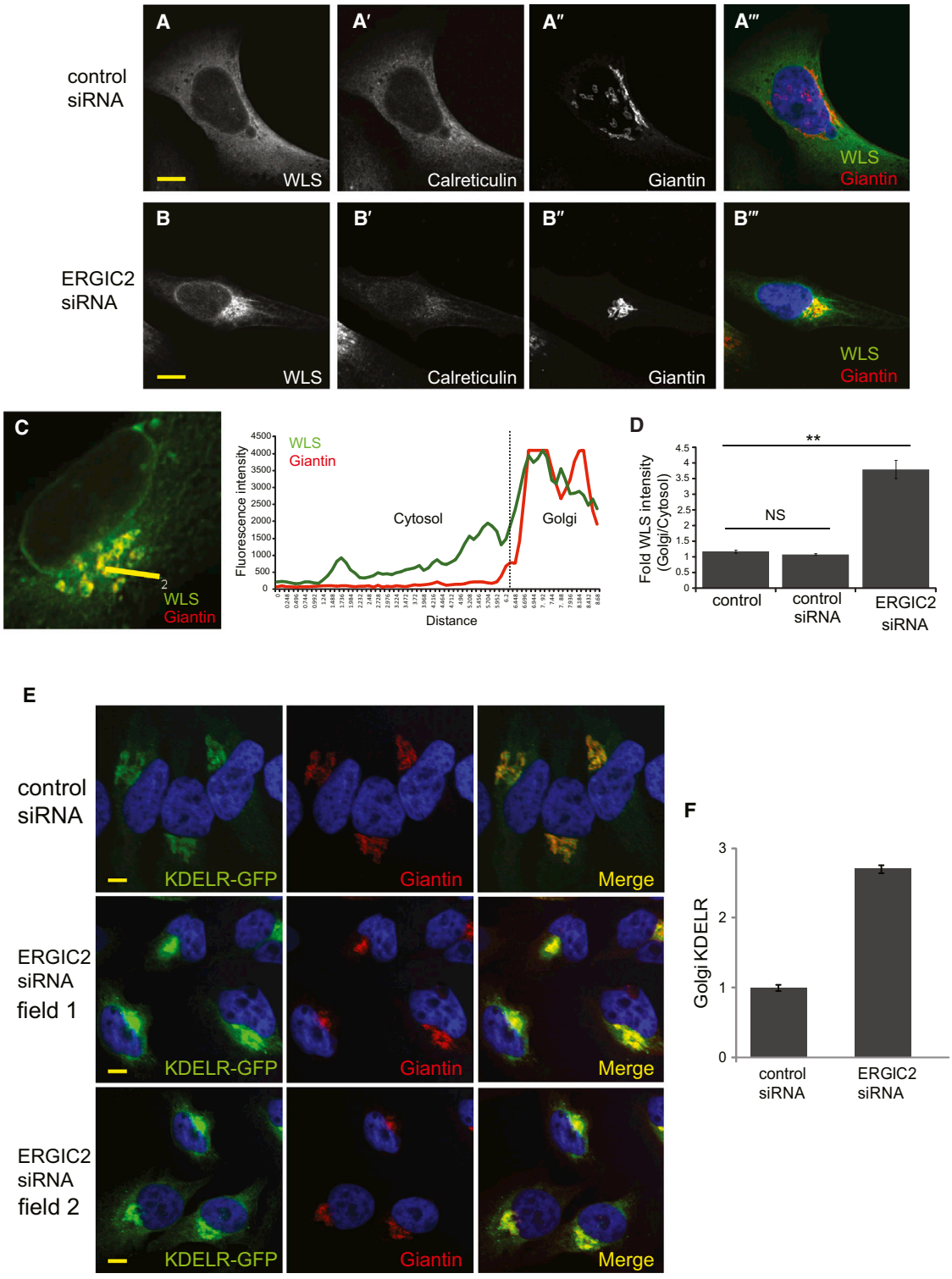

Figure 5. ERGIC2 Is Required for WLS Retrograde Localization to the ER

(A and B) HeLa cells with stable overexpression of WLS were transfected with control (A) or ERGIC2-specific (B) siRNA pool. ERGIC2 knockdown increases WLS colocalization with the Golgi marker Giantin. Scale bars represent 10 μm.

(C) Example of WLS localization in Golgi quantified in ERGIC2 knockdown cells by measuring staining intensity along the indicated line. Golgi-localized WLS was calculated as the ratio of WLS intensity in the Golgi over that in the cytosol (right panel).

(D) Thirty cells were quantified for each indicated condition. Golgi-localized WLS in an untransfected control is set to 1. WLS enrichment at the Golgi increased by almost 4-fold with ERGIC2 depletion. Statistical significance was calculated by two-tailed paired t test. **$p < 0.001$. NS, not significant. Error bars represent SEM.

(legend continued on next page)

receptor was labeled by biotin, but it only recycled to the early endosomes/Golgi fractions (Figure S4F, lower panel). The labeling was specific because proteins extracted from cells without biotin incubation were not pulled down by streptavidin (Figure 4N, lane 10). On the other hand, when WLS-GFP-transfected cells were labeled at the cell surface, biotin-labeled WLS-GFP was detected in the Golgi but not in the ER (Figures 4O and 4P), indicating that carboxy-terminal tagging reduces the ability of WLS to recycle back to the ER.

A limited number of proteins have been implicated in COPI-dependent Golgi-to-ER retrograde trafficking. Exotoxins including ricin, *Shiga* toxin, and *Pseudomonas* exotoxin exploit cellular trafficking pathways for retrograde transport from the cell surface to the ER (Sandvig et al., 2010). Genes required for individual steps in retrograde transport of these toxins were recently identified by a genome-wide RNAi screen (Moreau et al., 2011). In this screen, ERGIC2, a component of the ER-Golgi intermediate compartment, was identified as one of the few cellular proteins specifically required for the Golgi-to-ER retrograde trafficking of the exotoxins. We therefore tested whether ERGIC2 was involved in WLS ER localization. Knockdown of ERGIC2 caused relocalization of WLS from the ER to the Golgi (Figures 5A and 5B). Identical results were seen with two independent siRNAs against ERGIC2, but not with siRNA against a closely related protein, ERGIC3/Erv46p (Otte et al., 2001) (Figure S5). Quantification revealed that there was a 4-fold increase in Golgi-localized WLS after ERGIC2 knockdown as compared to nontargeting siRNA-treated cells (Figures 5C and 5D). Consistent with a role of ERGIC2 in general Golgi-to-ER retrograde transport of proteins, depletion of ERGIC2 also caused a significant increase of KDEL receptor (KDELR) localization in the Golgi (Figures 5E and 5F). The increased KDELR signal at the Golgi after ERGIC2 knockdown strongly suggests that Golgi-to-ER retrograde trafficking is slowed or blocked, while anterograde ER-to-Golgi transport remains functional.

ERGIC2 Is Required for Efficient Wnt Secretion and Signaling

If ERGIC2 is important in Wnt secretion, then depletion of ERGIC2 would be expected to inhibit Wnt signaling. Knockdown of ERGIC2 by four independent siRNAs (Figure 6A) reduced the expression of the endogenous Wnt/β-catenin target gene *AXIN2* by approximately 25% in HeLa cells transfected with a WNT3A expression plasmid (Figure 6B). Consistent with a role for ERGIC2 in Wnt secretion, ERGIC2 knockdown did not affect *AXIN2* expression induced by WNT3A-conditioned medium (Figure 6C). We confirmed that ERGIC2 is not required for general protein secretion, as the secretion of *Gaussia* luciferase was not affected by ERGIC2 knockdown (Figure S6). The defect in Wnt/β-catenin signaling caused by ERGIC2 knockdown may be due to a decrease in WLS recycling back to the ER to pick up Wnts. Consistent with this, total cellular WLS level was not affected by ERGIC2 knockdown (data not shown). Importantly, increasing the supply of cycling-competent wild-type WLS by overexpression rescued Wnt signaling activity after ERGIC2 knockdown, whereas expression of recycling-defective WLS K537A could not rescue signaling (Figure 6D).

The role of ERGIC2 in retrograde trafficking remains poorly understood, but its ortholog Erv41 is thought to participate in vesicles cycling between the *cis*-Golgi, the ER-Golgi intermediate compartment, and the ER in budding yeast (Otte et al., 2001). This suggests that a pool of ERGIC2 and WLS should be present in the same vesicles. Consistent with this, we find that WLS coimmunoprecipitates with GFP-ERGIC2 (Figure 6E). Finally, we tested whether WLS and ERGIC2 genetically interact in a Wnt-dependent developmental process in *Xenopus*. Knockdown of ERGIC2 by morpholino injection into two-cell embryos led to death after stage 10, when they failed to undergo gastrulation (Figure 6F). WLS overexpression rescued the initial stages of gastrulation, indicated by formation of the dorsal lip of the blastopore. Hence, WLS and ERGIC2 interact functionally, physically, and genetically.

DISCUSSION

While Wnts regulate diverse signaling pathways once outside the producing cells, secretion of all vertebrate Wnts uses several common core steps, including palmitoleation by PORCN in the ER and transport by WLS to the cell surface (Najdi et al., 2012; Proffitt and Virshup, 2012). Palmitoleation renders Wnts hydrophobic, and the carrier protein WLS interacts with the acyl group to bind Wnts during travel from the Golgi to the plasma membrane (Coombs et al., 2010). This left the unsolved problem of getting acylated Wnts from the ER to the Golgi. Here we show that WLS localizes in the ER in close proximity to PORCN where it can bind to newly modified Wnts, and that WLS carries Wnts from the ER to the PM. WLS then undergoes retrograde transport back through the Golgi, as has been shown previously. In addition, we find that WLS is transported from the Golgi back to the ER via the ER-Golgi intermediate compartment and that this retrograde transport is necessary for efficient Wnt secretion (Figure 7).

Prior studies concluded that WLS recycles from the PM to the Golgi, rather than to the ER. Our results differ, and we believe this is because we examined both endogenous and native WLS, rather than overexpressed epitope-tagged WLS. We verified our findings using immunofluorescence microscopy with two different antibodies (mouse and rabbit), proximity ligation assay, and subcellular fractionation using isopycnic density sedimentation. Previous identification of Golgi localization was because modification of the cytoplasmic carboxyl terminus by epitope tagging or point mutation prevents WLS Golgi-to-ER recycling. The WLS ER-targeting sequence KxxQx appears to be both necessary and sufficient, in the appropriate context, for ER targeting, since adding the KxxQx signal to WLS splice variant 2 partially restored its ER localization and improved activity. The

(E) ERGIC2 knockdown induces a marked accumulation of the KDEL receptor (KDELR) at the Golgi. HeLa cells with stable expression of KDELR-GFP were treated with the indicated siRNAs for 72 hr prior to fluorescence microscopy using constant image acquisition parameters. Scale bars represent 10 μm.
(F) KDELR-GFP accumulation at the Golgi was quantified in 30 cells for each indicated condition. The Golgi was identified by Giantin staining, and the integrated fluorescence intensity of KDELR-GFP was computed. Error bars represent SEM.
See also Figure S5.

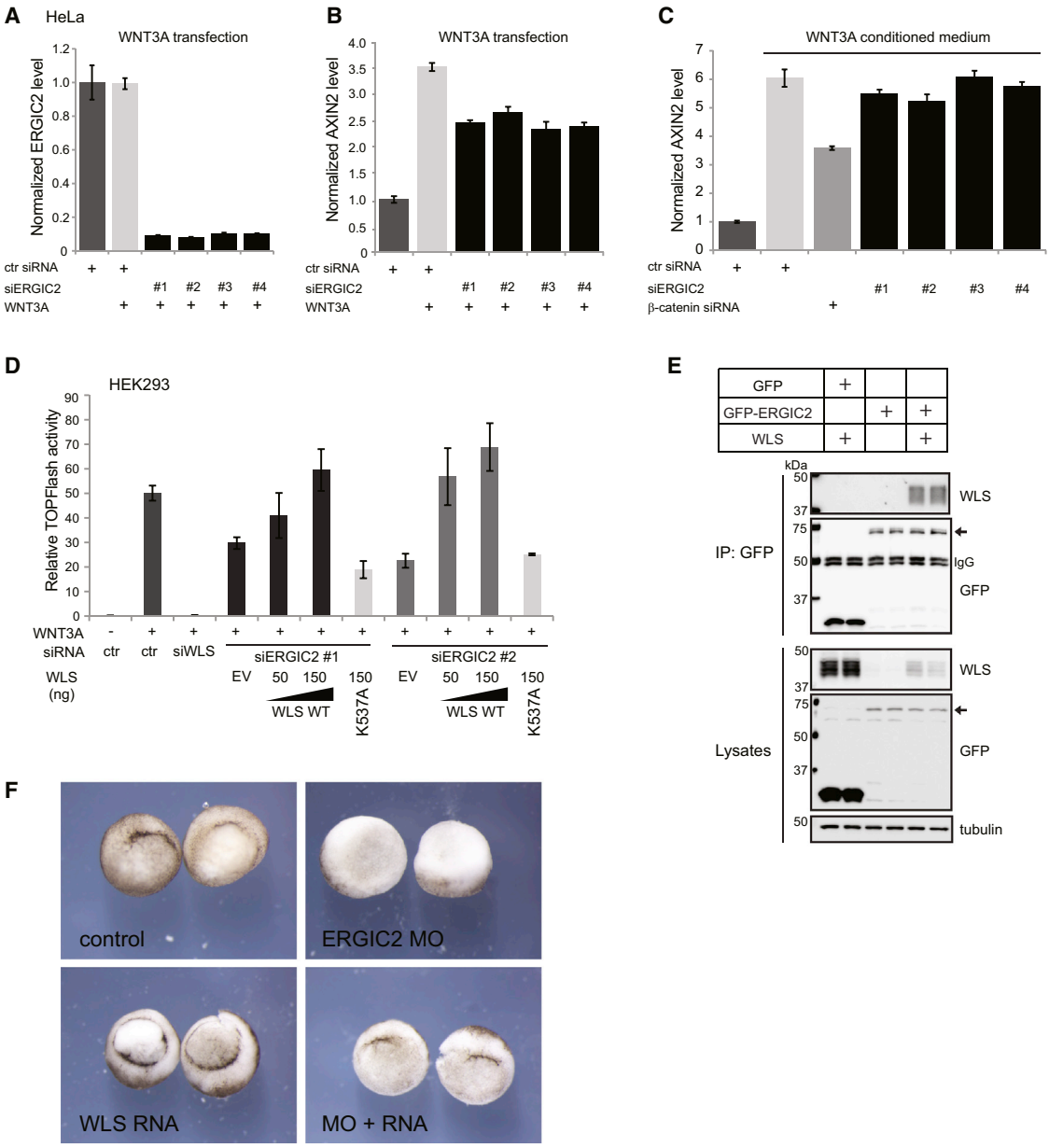

Figure 6. ERGIC2 Interacts with WLS and Is Required for Wnt Secretion

(A) Knockdown efficiency of four independent ERGIC2 siRNAs in HeLa cells. HeLa cells were transiently transfected with 50 ng of pPGK-mWnt3A and 50 nM siRNA in 12-well plates. Total RNA was extracted 48 hr posttransfection. *ERGIC2* mRNA abundance was determined by qPCR and normalized to *GAPDH*. Error bars represent SEM.

(B) Knockdown of ERGIC2 reduces expression of the Wnt target gene *AXIN2*. The same RNA samples as in (A) were examined by qPCR for *AXIN2* abundance. Error bars represent SEM.

(C) Knockdown of ERGIC2 does not affect *AXIN2* expression induced by WNT3A-conditioned medium. HeLa cells were treated with WNT3A-conditioned medium for 18 hr. β-catenin knockdown served as a positive control. Error bars represent SEM.

(D) Knockdown of ERGIC2 can be rescued by wild-type WLS but not mutant K537A overexpression. STF cells were transfected with 100 ng of pPGK-mWnt3A, the indicated amount of WLS plasmids (ng), and 50 nM siRNA in 12-well plates. Luciferase activity was measured 48 hr posttransfection as above. Error bars represent SD.

(E) Interaction of WLS with ERGIC2. STF cells were transfected with plasmids encoding GFP or GFP-ERGIC2, and wild-type WLS. Cells were lysed 50 hr posttransfection, and anti-GFP antibody was used for immunoprecipitation. The arrows indicate GFP-ERGIC2. Chicken polyclonal WLS antibody from GeneTex was used for the immunoblot.

(F) WLS rescues the effects of *ERGIC2* morpholino in *X. laevis* embryos. Control embryos undergoing gastrulation at stage 10 display a dorsal blastopore lip. Embryos injected with *ERGIC2* morpholino fail to initiate gastrulation at stage 10 and lack a dorsal blastopore lip (n = 70/80). *WLS* RNA overexpression results in premature blastopore closure by stage 10 (n = 72/80). The expression of *WLS* RNA is sufficient to rescue the *ERGIC2* MO blastopore lip phenotype (n = 70/80) compared to control embryos.

See also Figure S6.

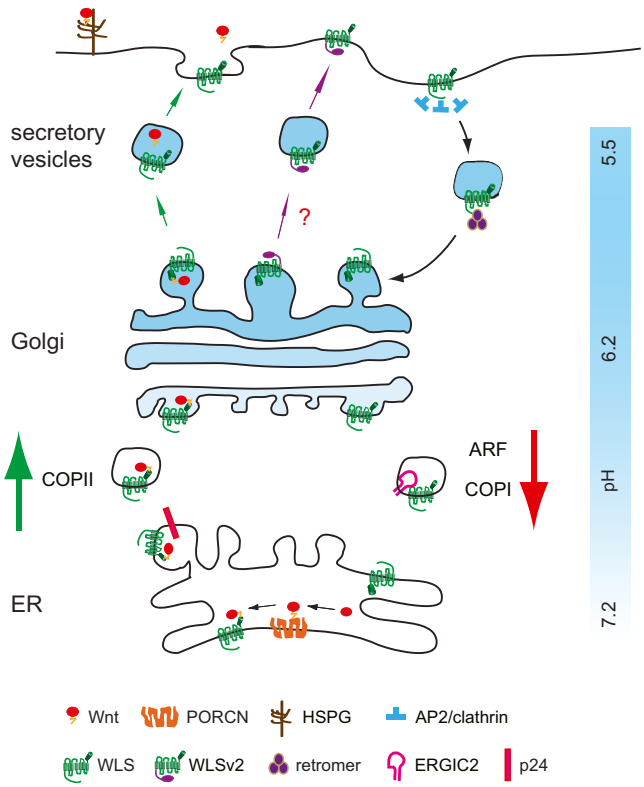

Figure 7. Revised Model of the Wnt Secretory Pathway Including WLS Recycling to the ER

WLS in the ER is derived from both newly synthesized and recycled protein. WLS binds newly palmitoleated Wnts and transports them on the secretory pathway to the plasma membrane, where acidification of vesicles induces their release from WLS (Coombs et al., 2010). WLS then recycles via clathrin-coated vesicles and the retromer complex to the Golgi. WLS retrograde movement from the Golgi to the ER requires ARF-regulated COPI vesicles and ERGIC2. The model is modified from Coombs et al. (2010) with permission.

data suggest that KxxQx either is a variant of a previously identified ER retention signal (e.g., a variation of KKxx) or interacts with novel retrieval components of the Golgi-to-ER retrograde pathway. The essential lysine at the −5 position (K537) is conserved even in *Drosophila* and *C. elegans*, and we found that the *Drosophila* sequence functions in the human WLS protein.

Retrograde transport into the ER is an area of active investigation in yeast and in mammals. The final step, Golgi-to-ER trafficking, requires the small GTPase ARF to assist in the formation of COPI-coated vesicles that bud from the Golgi and traffic to the ER. An RNAi screen identified ERGIC2 as a key component of the Golgi-to-ER pathway traffic used by exotoxins. Our data indicate that WLS uses the same pathway used by endogenous retrograde-transported proteins such as the KDEL receptor and also by exogenous toxins such as ricin, *Shiga* toxin, and *Pseudomonas* exotoxin (Moreau et al., 2011). While toxins and the secreted protein sulfatase modifying factor 1 can recycle from outside of the cell to the ER (Zito et al., 2007), to the best of our knowledge WLS is the only integral membrane protein with an ER-PM-ER life cycle. One speculation therefore is that WLS might be a receptor for toxins trafficking to the ER that do not exhibit a KDEL-like sequence.

Given the importance of the WLS ER signal in Wnt secretion, it is notable that primates have a unique splice variant of WLS that cannot recycle to the ER. We verified that this splice variant is widely expressed, and that it cycles to subcellular locations distinct from WLS variant 1. The physiologic role of this widely expressed splice variant is unclear. Wnts may exit cells by diverse mechanisms including secretion, exosomes, and transcytosis (Gallet et al., 2008; Gross et al., 2012; Korkut et al., 2009), and we speculate that variant 2 may be a primate-specific mechanism to facilitate this process. Establishing this, however, will require further studies using primate cells rather than existing animal models.

In conclusion, here we identify the basis for an early step in Wnt signaling, moving newly palmitoleated Wnts from the ER to the Golgi and then out of the cell. In the process, we find that WLS is a recycling cargo receptor that moves from the ER to the plasma membrane and back to the ER using key elements of the ER-Golgi intermediate compartment. Regulation of WLS localization at the ER may prove critical to its function.

EXPERIMENTAL PROCEDURES

For cell lines, constructs, antibodies, siRNA targeting sequences, and qPCR primer sequences, see Supplemental Experimental Procedures.

Luciferase Assay

Transfected cells were washed once with PBS and then lysed with 150 μl per well (for 12-well plates) of 1× Reporter Lysis Buffer (Promega) supplemented with complete protease inhibitor cocktail (Roche). Cell debris was removed by centrifugation at 11,000 × *g* for 10 min at 4°C. Twenty microliters of cleared lysates was mixed with 50 μl of luciferase assay reagent (Promega), and a 1 s integration time was used for measurement. Luminescence data were obtained using a Tecan Infinite M200 microplate reader and normalized to lactate dehydrogenase (LDH) as described previously (Coombs et al., 2010).

Immunoprecipitation

Transfected cells were lysed in HEPES lysis buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 0.6% IGEPAL CA-630, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitor cocktail). Cell debris was removed by centrifugation at 11,000 × *g* for 10 min at 4°C. One to two micrograms of the appropriate antibody was added to 400–500 μg of protein from total cell lysates and incubated at 4°C for 18 hr. Subsequently, 20 μl of protein A/G agarose suspension beads was added for an additional 2 hr to capture the immunocomplex. The pelleted immunoprecipitate was washed three times with lysis buffer, and the bound proteins were eluted with 2× Laemmli buffer, boiled at 95°C for 5 min, and then analyzed by SDS-PAGE and immunoblotting.

Indirect Immunofluorescence Microscopy

Cells were seeded on coverslips in a 12-well plate the day before transfection. When the cells reached 70%–80% confluence, they were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). For ERGIC2 knockdown experiments, reverse siRNA transfection was performed using HiPerFect (QIAGEN) and 25 nM siRNA with 40,000 HeLa WLS cells per well in 24-well plates with glass coverslips. Forty-eight to seventy-two hours post-transfection, medium was removed and coverslips were washed once with PBS prior to fixation with 4% paraformaldehyde at room temperature for 15 min. In some experiments, cells were fixed with methanol for 10 min at −20°C. Cells were permeabilized with 0.1% Triton X-100 at room temperature for 10 min, washed, and blocked with 2% BSA at room temperature for at least 30 min. Primary antibody incubation was typically performed overnight at 4°C. Secondary Alexa Fluor-conjugated antibodies were added after washing, for 30 min prior to repeated washing. Cells were mounted on glass slides using

either VECTASHIELD mounting medium with DAPI (Vector Laboratories) or FluorSave (Merck). The images were taken using either a Zeiss LSM 710 confocal microscope, Super Resolution Nikon N-SIM microscope, or Olympus IX81 inverted FluoView confocal microscope. For calculation of the correlation coefficient, scatter plots were generated in Microsoft Excel using fluorescence intensity of WLS and Calnexin along a randomly drawn line. The nuclear area was excluded for analysis due to its bias toward higher correlation. For Figure 1E, Pearson's correlation coefficient values (above threshold) were calculated using the ImageJ Coloc 2 function.

Subcellular Membrane Fractionation

To identify the subcellular membranes that contain WLS, gradient ultracentrifugation and fractionation were performed. HeLa cells grown to confluence on 15 cm dishes were resuspended in 1 ml of homogenization buffer (250 mM sucrose, 10 mM HEPES, 1 mM KCl, 20 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate [pH 7.5] with protease inhibitor cocktail). Cells were disrupted using a Dounce homogenizer with a tight-fitting pestle to obtain more than 90% broken cells. Nuclei and unbroken cells were pelleted by centrifugation at 3,000 × *g* for 10 min at 4°C. Postnuclear supernatant was overlaid on a preformed 10%–30% continuous iodixanol (OptiPrep density gradient medium; Sigma) gradient and was centrifuged at 50,000 × *g* for 15 hr using an SW41 rotor. The resulting gradient was collected as 500 μl fractions (total 22 fractions) that were analyzed by SDS-PAGE. For immunoprecipitation following fractionation, 200 μl of each fraction was used, topped up with 500 μl of HEPES lysis buffer. For streptavidin pull-down following fractionation, 400 μl of each fraction was used, topped up with 1 ml of HEPES lysis buffer. Fifty microliters of streptavidin agarose (Novagen) slurry was added for each fraction.

Proximity Ligation Assay

Untagged WLS (20 ng) and HA-PORCN plasmid (20 ng) were transfected as indicated into HeLa cells seeded in an eight-well chambered coverglass (Lab-Tek). Forty-eight hours posttransfection, the cells were fixed and permeabilized before the addition of primary antibodies (YJ5 and rabbit anti-HA antibody). PLA was performed as per the manufacturer's manual (Olink Bioscience) with anti-mouse PLA probe PLUS and anti-rabbit PLA probe MINUS. Following ligation and amplification, the nuclei were counterstained with DAPI solution. The results were visualized with a Nikon N-SIM inverted microscope.

Biotin Surface Protein Labeling

Sulfo-NHS-LC-Biotin (Thermo Scientific) was freshly dissolved in PBS to 1 mg/ml before use. HeLa cells were rinsed twice with PBS before adding the biotin solution. The cells were incubated at 37°C for 2 hr, followed by washing twice with PBS. The reaction was quenched by incubating with 100 mM glycine at room temperature for 20 min. After washing, the cells were scraped off the dish in cold homogenization buffer.

Xenopus Embryo Microinjection

Xenopus laevis oocytes were fertilized in vitro, chemically dejellied with 2.2% L-cysteine (pH 7.8–8.1), and maintained in 1/10 Marc's modified Ringer solution. For capped mRNA synthesis, *WLS* cDNA cloned in pCS2+ vector was linearized and transcribed with SP6 polymerase. *ERGIC2* antisense morpholino nucleotide (MO) 5′-GCCTCATTTTTGTAGTCTCCCCTGT-3′ was designed by and purchased from Gene Tools. Embryos were injected in both cells at the two-cell stage with either *ERGIC2* MO (50 ng), *WLS* RNA (2 ng), or both together and cultured until they reached stage 10. Animal husbandry and experimental procedures were carried out under the approval of the A*STAR Institutional Animal Care and Use Committee and with international accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.03.016.

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