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Presenilin Deficiency or Lysosomal Inhibition Enhances Wnt Signaling through Relocalization of GSK3 to the Late-Endosomal Compartment

Radek Dobrowolski,[1,3,4] Philipp Vick,[1,3] Diego Ploper,[1] Iwona Gumper,[2] Harriet Snitkin,[2] David D. Sabatini,[2] and Edward M. De Robertis[1,*]

[1]Howard Hughes Medical Institute and Department of Biological Chemistry, University of California, Los Angeles, CA 90095-1662, USA
[2]Department of Cell Biology, New York University School of Medicine, New York, NY 10016-6497, USA
[3]These authors contributed equally to this work
[4]Present address: Department of Biological Sciences, Rutgers University, Newark, NJ 07102, USA
*Correspondence: ederobertis@mednet.ucla.edu
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SUMMARY

Sustained canonical Wnt signaling requires the inhibition of glycogen synthase kinase 3 (GSK3) activity by sequestration of GSK3 inside multivesicular endosomes (MVEs). Here, we show that Wnt signaling is increased by the lysosomal inhibitor chloroquine, which causes accumulation of MVEs. A similar MVE expansion and increased Wnt responsiveness was found in cells deficient in presenilin, a protein associated with Alzheimer's disease. The Wnt-enhancing effects were entirely dependent on the functional endosomal sorting complex required for transport (ESCRT), which is needed for the formation of intraluminal vesicles in MVEs. We suggest that accumulation of late endosomal structures leads to enhanced canonical Wnt signaling through increased Wnt-receptor/GSK3 sequestration. The decrease in GSK3 cytosolic activity stabilized cytoplasmic GSK3 substrates such as β-catenin, the microtubule-associated protein Tau, and other proteins. These results underscore the importance of the endosomal pathway in canonical Wnt signaling and reveal a mechanism for regulation of Wnt signaling by presenilin deficiency.

INTRODUCTION

Canonical Wnt signaling is essential for embryonic development, stem cell and tissue homeostasis, and regeneration in adults (MacDonald et al., 2009; Angers and Moon, 2009). Aberrant Wnt signaling has been associated with human diseases such as cancer, bone disorders, and neurodegeneration (Clevers and Nusse, 2012; Boonen et al., 2009). In the absence of Wnt ligands, the adaptor protein and transcription cofactor β-catenin is phosphorylated by GSK3 in a destruction complex consisting of the tumor suppressor adenomatous polyposis coli (APC), Axin, casein kinase 1 (CK1), and the E3-polyubiquitin ligase βTrCP (Cadigan and Peifer, 2009; Li et al., 2012). Phosphorylations by GSK3 target β-catenin and other proteins for polyubiquitinylation and degradation in the proteasome (Kim et al., 2009; Taelman et al., 2010; Clevers and Nusse, 2012). Binding of Wnt ligands to their receptors Frizzled (Fz) and LDL-receptor related protein 5/6 (LRP5/6) triggers recruitment of Dishevelled (Dvl), Axin, and GSK3 to the plasma membrane (Bilic et al., 2007; Zeng et al., 2008). GSK3 is first recruited by the binding of Axin to LRP6 and then becomes engaged in the phosphorylation of LRP6, Fz, Dvl, Axin, and β-catenin, which contain multiple GSK3 sites, explaining the requirement of an intact GSK3 catalytic site for its relocalization (Taelman et al., 2010). Wnt receptor complexes containing Axin and GSK3 are then internalized into the cell by endocytosis (Blitzer and Nusse, 2006; Yamamoto et al., 2006) and subsequently sequestered by incorporation into the intraluminal vesicles (ILVs) of late endosomes that are produced by invagination and scission from the endosomal limiting membrane (Taelman et al., 2010; Dobrowolski and De Robertis, 2012). Sequestration of active GSK3 inside multivesicular endosomes (MVEs) leads to sustained stabilization of the half-life of many GSK3 protein substrates (Taelman et al., 2010), principal among which is newly synthesized β-catenin, which enters the nucleus to coactivate Wnt target genes.

The integration of cell signaling and endocytosis is critical for signal transduction outcomes (Sorkin and von Zastrow, 2009; Dobrowolski and De Robertis, 2012). Although most receptor complexes are negatively regulated by endocytosis (Katzmann et al., 2002), Wnt signal transduction requires endocytosis (Blitzer and Nusse, 2006). Inhibition of ILV formation in MVEs (also referred to in the literature as multivesicular bodies [MVBs]) by interfering with components of the endosomal sorting complex required for transport (ESCRT) (Katzmann et al., 2002; Wollert and Hurley, 2010) prevents canonical Wnt signaling (Taelman et al., 2010). Because endolysosomal function is essential for Wnt signaling, we decided to investigate the effect of inhibitors of lysosomal function on Wnt signaling. Lee et al. (2010) recently reported that presenilin 1 (PS1), an intramembrane protease that is mutated in early-onset familial Alzheimer's disease (FAD), is required for proper autophagosome digestion. These authors found that acidification of lysosomes was impaired in PS1-deficient cells, and proposed

a model in which PSs are required for lysosomal maturation. An extensive literature links autophagy defects with neurodegeneration (Nixon et al., 2008). Furthermore, toxic amyloid precursor protein (APP) peptides accumulate intracellularly specifically in MVBs in early-stage AD (Takahashi et al., 2002), and certain polymorphisms in the lysosomal protease cathepsin D increase the risk for AD (Nixon and Yang, 2011). Taken together, these observations motivated us to investigate whether PS deficiency might affect Wnt signaling through functional changes in the endolysosomal system.

In this study, we found that canonical Wnt signaling activity was significantly increased when lysosomal function was inhibited by chloroquine (CQ), a drug that raises lysosomal pH. Depletion of PS1 or PS2 also resulted in a significant increase of Wnt transcriptional activity in T cell factor (TCF)/β-catenin reporter gene assays. In both cases, lysosomal function was inhibited downstream of ILV formation. Indeed, the ESCRT machinery, which generates ILVs, and the small GTPase Rab7, which is involved in MVE/late-endosome formation (Bucci et al., 2000; Huotari and Helenius, 2011), were both required for Wnt transduction. In electron microscopy analyses, CQ-treated or PS-deficient cells displayed a striking accumulation of late-endosomal vacuoles containing ILVs. Immunofluorescence microscopy studies indicated that these MVEs were positive for Rab7 and the ILV membrane marker CD63 (Escola et al., 1998). The expanded late endosomes sequestered more GSK3/Wnt-receptor complexes compared with wild-type (WT) cells when the cells were treated with Wnt. Several GSK3 protein substrates, such as β-catenin, the previously developed GFP-GSK3-protein stability biosensor (Taelman et al., 2010), and the microtubule-associated protein Tau-GFP, became more stabilized by Wnt in PS1-depleted cells. The results presented here highlight the key role played by the endolysosomal pathway in Wnt signal transduction.

RESULTS

CQ Increases Wnt Signaling via MVEs

To investigate the role of the endolysosomal system in Wnt signaling, we used CQ, an antimalarial drug. CQ is a weak base that accumulates in lysosomes, causing their alkalinization and inhibiting the activity of acidic hydrolases (Wibo and Poole, 1974). Previous studies have described sensitive transcriptional reporters of Wnt activity in which multiple TCF binding sites (called SuperTopFlash and BAR reporters) activate transcription when β-catenin accumulates in the nucleus (Veeman et al., 2003; Biechele and Moon, 2008). As shown in Figures 1A and 1B, Wnt signaling was stimulated up to 7-fold by CQ treatment in HEK293T cells (brackets at left). Increases in Wnt signaling were also observed with the use of the lysosomal protease inhibitors leupeptin and E64 (increases of 2.6- and 2.1-fold, respectively; Figure S1A). Therefore, interference with lysosomal maturation and function potentiates Wnt signaling.

To test whether the amplification of Wnt signaling by CQ required the formation of ILVs in MVEs, we used small interfering RNA (siRNA) knockdown of hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), also known as vacuolar protein sorting 27 (Vps27), which is required for early stages of ILV formation (Katzmann et al., 2002; Taelman et al., 2010). HRS/Vps27 was required for the stimulation of Wnt signaling by CQ (Figure 1A, right bracket). Vps4 (another ESCRT component required for ILV formation) and Rab7 (a protein required for late-endosomal maturation) were also required (Figure 1B, right bracket; data not shown).

To determine whether CQ causes the accumulation of MVEs, we examined 3T3 cells treated overnight (Figures 1C–1E) or L-cells treated with CQ for 6 hr, 1 hr, or 15 min (Figures 1F and 1G) by transmission electron microscopy. In both cell lines, CQ treatment caused a striking increase in autolysosomes containing accumulations of electron-dense cytoplasmic materials, such as aggregated proteins, as well as small ILVs ~50 nm in diameter (which are characteristic of MVEs; Figures 1D and 1E). No double-membrane macroautophagic vacuoles were seen, even at early time points. However, the MVEs were clearly engaged in microautophagy (Sahu et al., 2011), because after only 15 min of CQ treatment, many showed large invaginations of the limiting membrane (Figure 1G, arrows) enclosing regions of cytoplasm containing fine electron-dense granules corresponding to ribosomes. After the limiting membrane is pinched off, these invaginations generate electron-dense deposits in late autolysosomes, which are then enveloped by single or multilaminar membranes depending on the stage of the individual autolysosomes (Figure 1E).

CQ treatment causes the rapid accumulation of endosomes marked in their outer membrane by Rab7 (Figures S1B–S1C‴ and Movie S1). These vacuoles correspond to MVEs because they colocalize with the tetraspanin protein CD63, a marker for ILV membranes (Figures S1D–S1E″; Escola et al., 1998). Flow cytometry confirmed that the CQ-treated cells had increased levels of Rab7 (by 25.2%) and CD63 (by 28.5%) antigens in the cell population as a whole (Figures 1H and 1I).

We conclude that the inhibition of lysosomal function caused by CQ does not prevent the formation of endosomal ILVs, but instead enhances it. The MVE expansion would enhance the sensitivity of cellular responses to Wnt, which are entirely dependent on the ESCRT machinery.

Dose-Dependent Response of Wnt Signaling to CQ and Bafilomycin

We next investigated the effects of inhibiting lysosomal function on Wnt signaling with different doses of CQ or the specific vacuolar ATPase (v-ATPase) inhibitor bafilomycin A (Figures 2A and 2B). The v-ATPase enzyme acidifies the entire endosomal pathway as vesicles traffic from the plasma membrane to lysosomes. We found that low concentrations of CQ or bafilomycin increased Wnt3a responses in LSL cells stably transfected with a TCF reporter, whereas at higher concentrations (>250 μM for CQ and >50 nM for bafilomycin) both drugs inhibited Wnt signaling, presumably by alkalinizing the early-endosomal compartment as well (Figures 2A and 2B). Because the generation of ILVs within the expanded endosomes can still proceed in the presence of CQ (Figure 1E), the accumulation of ILVs in MVEs may provide the basis for the increased sensitivity to Wnt signals after CQ treatment. At high CQ concentrations, the more-extensive alkalinization of the entire endolysosomal pathway may actually inhibit ILV formation or receptor activation.

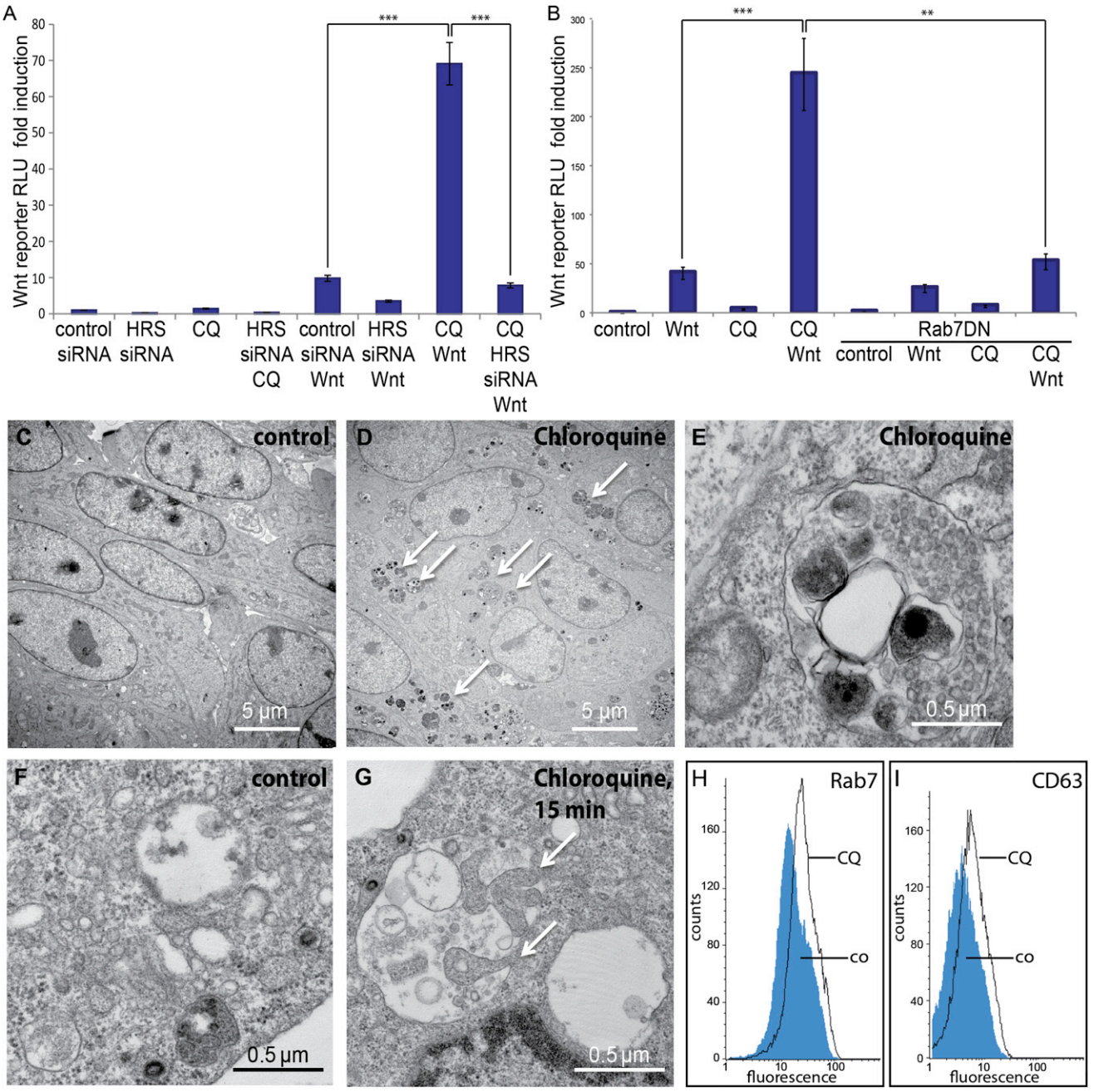


Figure 1. Lysosomal Inhibition by CQ Increases Wnt Signaling and Expands the MVE Compartment

(A) CQ (50 μM; Sigma) significantly increases the Wnt responsiveness of HEK293T cells in a Wnt reporter (SuperTopFlash) assay. Left bracket: 11.2 ± 2.1-fold increase (Luciferase/Renilla relative light unit [RLU]) in Wnt-treated control siRNA cells versus 69 ± 8.8 in Wnt-treated CQ cells. HRS ESCRT protein is required for this stimulation (left bracket).

(B) CQ stimulation of Wnt activity (BAR Wnt reporter) can be blocked by dominant-negative Rab7 cotransfection (cells treated with Wnt and CQ had a 243 ± 39-fold induction and 55 ± 8.9 RLU after coexpression of Rab7DN).

(C–E) Treatment of 3T3 fibroblasts with CQ for 12 hr leads to the formation of numerous endosomal and autolysosomal vesicles bounded by a single membrane, containing ILVs ~50 nm in diameter (characteristic of MVEs), and electron-dense material representing digested cytoplasmic components taken in by microautophagy.

(F and G) After CQ treatment for 15 min, MVEs frequently showed membrane invaginations containing ribosomes and aggregated cytoplasmic components (arrows), reflecting the process of microautophagy.

(H and I) Flow cytometry analyses showing an increase of the late-endosomal markers Rab7 and CD63 in cells treated with CQ for 8 hr.

All histograms are presented as mean ± SEM.

See also Figure S1 and Movie S1.

Our results agree with a previous study by Cruciat et al. (2010) showing that LRP6 phosphorylation requires acidification of early endosomes/signalosomes in HEK293T cells treated with high concentrations of bafilomycin that inhibit Wnt signaling. We now extend their findings by showing that at low doses v-ATPase inhibition increases Wnt signaling, presumably reflecting a higher requirement for the proton pump in the more-acidic late-endosomal compartment. These results highlight the link between Wnt signal transduction and membrane trafficking.

PS Deficiency Leads to Increased Sensitivity to Wnt Signals

A new function for PS in the maturation of v-ATPase was recently reported (Lee et al., 2010), motivating us to explore the impact of lysosomal malfunction caused by PS deficiency on Wnt signaling. We first confirmed that knock out or siRNA depletion of PS1 indeed generated less acidified endosomes, as was shown by reduced staining with LysoTracker which marks acidic organelles (Figures S2A–S2E′). Efficient knockdown of PS1 was demonstrated with an immunoblot using a Flag-tagged PS1 construct (Figure S2F). We then extended the observations of Lee et al. (2010) by showing that the fluid-phase endosomal compartment, measured by endocytosis of DextranRed (tetramethylrhodamine Dextran), was significantly expanded by PS1 knockdown with siRNA (Figures 2C and 2D). Flow cytometry confirmed the increase in DextranRed-accessible endosomes in the PS1-depleted cell population in a quantitative way (Figure 2E).

Endosomal expansion was accompanied by an increase in Wnt responsiveness in PS1-depleted HEK293T cells, particularly at higher Wnt3a doses (Figure 2F, bracket). The stimulation of Wnt signaling caused by PS1 siRNA-mediated depletion was entirely dependent on ILV formation in MVEs because, as shown in Figure 2G, HRS/Vps27 depletion strongly inhibited Wnt signaling in control siRNA or PS1 siRNA-treated HEK293T cells. We extended these findings to the *Xenopus* animal cap system (Taelman et al., 2010). We designed a PS1 morpholino oligo (MO) that reproducibly increased signaling by SuperTopFlash reporter in cells coinjected with *xWnt8* mRNA (Figure 2H), and this effect was rescued by human PS1 coinjection (Figure S2G). This increase in Wnt signaling was blocked by HRS/Vps27 MO (Figure 2H, right bracket). We conclude that the ESCRT machinery is required for the effects of PS1 knockdown both in cultured cells and in the in vivo animal cap system.

We also tested whether PS2 affected Wnt signaling using siRNA. Depletion of PS1 or PS2 led to increased responsiveness in Wnt luciferase assays (Figure 2I), indicating that both proteins have functional overlap, at least in HEK293T and HeLa cells (see also Figures 3A–3D″ below). Although the increase in Wnt signaling by PS1 siRNA was higher than that by PS2 siRNA, both together had additive effects (Figure 2I). The effects of PS2 siRNA on Wnt signaling, as well as those of PS1, could be rescued by overexpression of the human *PS1* gene (Figure S2H). Interestingly, FAD-associated PS1 mutations (M146V, A246E, and L392V) were much less effective at rescuing the Wnt signaling increased by PS1 depletion (Figure S2I). In contrast, PS1 mutations (D257A and D385A) in the aspartates required for proteolytic activity were as effective as WT PS1 in rescuing the effect on Wnt signaling (Figure S2I). This is in agreement with previous work showing that the effects on PSs on autolysosomes are independent of γ-secretase enzyme activity (Lee et al., 2010; Neely et al., 2011). The fact that FAD point mutations behave differently from protease-deficient PS1 could be relevant for disease.

Taken together, these data suggest that PS depletion increases the capacity of cells to respond to Wnt by promoting the generation of ESCRT-dependent ILVs in the endosomal pathway.

Late Endosomes Accumulate in PS1- and PS2-Deficient Cells

PS depletion had a dramatic impact on the endolysosomal system. In electron microscopy studies, depletion of PS1 or PS2, or the simultaneous depletion of both gene products, resulted in an increase in the number and size of single-membrane-bound vacuolar structures in HeLa cells (compare Figure 3A with Figures 3B–3D). These structures were autolysosomes and MVEs, as they contained a variable number of small and uniform ILVs (Figures 3B′–3D′) as well as electron-dense, undigested cytoplasmic material. Double-membrane macroautophagic vesicles were not observed. Some of the most striking MVEs are shown in Figures 3A″–3D″. Immunofluorescence analyses showed increased staining for the endosomal pathway markers Rab7 and LAMP1, as well as for the ILV marker CD63 when PS1 or PS1/2 were depleted with siRNA (Figure S3; data not shown). Flow cytometry showed increased levels of Rab7 (by 30.9%) and CD63 (by 35.7%) in the entire cell population after PS1 knockdown (Figures 3E and 3F).

We conclude that PS1 and PS2 play a fundamental role in endolysosomal biogenesis and function, and, as in the case of CQ, PS deficiency blocks the pathway downstream of ILV formation.

PS Depletion Leads to Increased Phospho-LRP6 and Nuclear β-Catenin upon Wnt Signaling

Given the expansion of the MVE compartment in PS1-depleted cells, we next asked whether trafficking of the activated Wnt coreceptor LRP6 was affected upon Wnt ligand binding. Phosphorylation of LRP6 marks the initial activation step in the Wnt signaling cascade (Niehrs and Shen, 2010). HEK293T cells stably expressing LRP6-GFP (Kategaya et al., 2009) were transfected with control or PS1 siRNAs and treated with high concentrations of Wnt3a overnight, and activation of LRP6 was detected with a phosphospecific anti-p-LRP6 antibody (Figures 4A–4F). Phospho-LRP6 immunostaining in vesicle-like cytoplasmic puncta (Bilic et al., 2007) was detected only in cells treated with Wnt (arrows), and was significantly higher in cells lacking PS1 (compare Figures 4D and 4B). Only a subset of cells displayed strong responses to Wnt, probably due to the cell-cycle dependence of Wnt signaling (Davidson et al., 2009; Niehrs and Acebron, 2012). Immunoblot analyses confirmed that PS1 knockdown caused a reproducible increase in LRP6 phosphorylation in the cell population as a whole, whereas total levels of nonphosphorylated LRP6-GFP were not affected (Figures 4E and 4E′). Accordingly, the ratio of pLRP6/LRP6-GFP was increased by Wnt in PS1-depleted cells (Figure 4F).

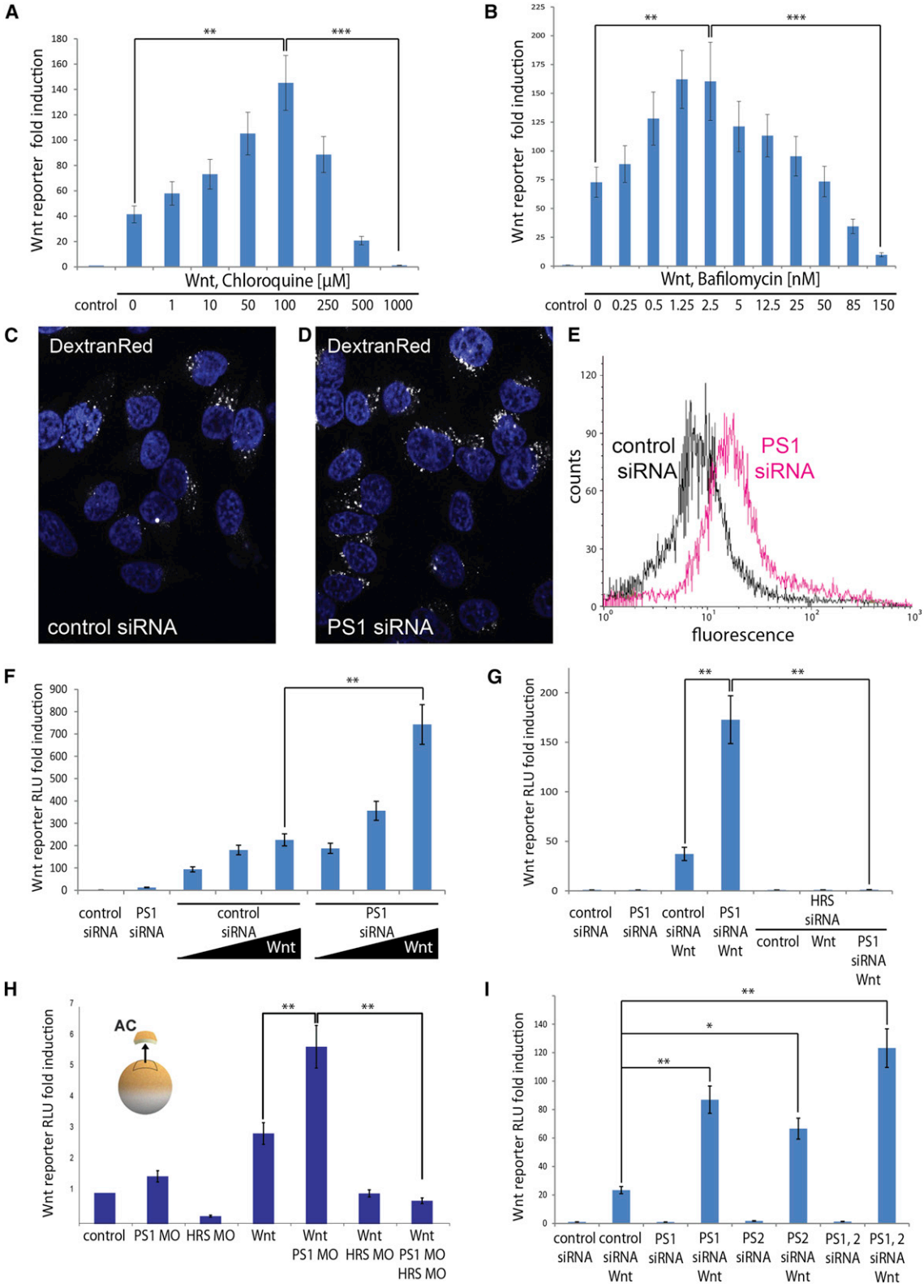


Figure 2. Wnt Sensitivity Increases when the Lysosomal Pathway Is Inhibited by CQ, Low-Dose Bafilomycin, or PS Depletion

(A and B) Concentration-dependent effects of CQ and bafilomycin A (Sigma) on Wnt luciferase reporter assays in LSL cells (SuperTopFlash reporter). Concentrations of 100 μM for CQ or 2.5 nM for bafilomycin A caused maximal increases in Wnt signals (145 ± 23 or 162 ± 34-fold increase in RLUs, respectively).

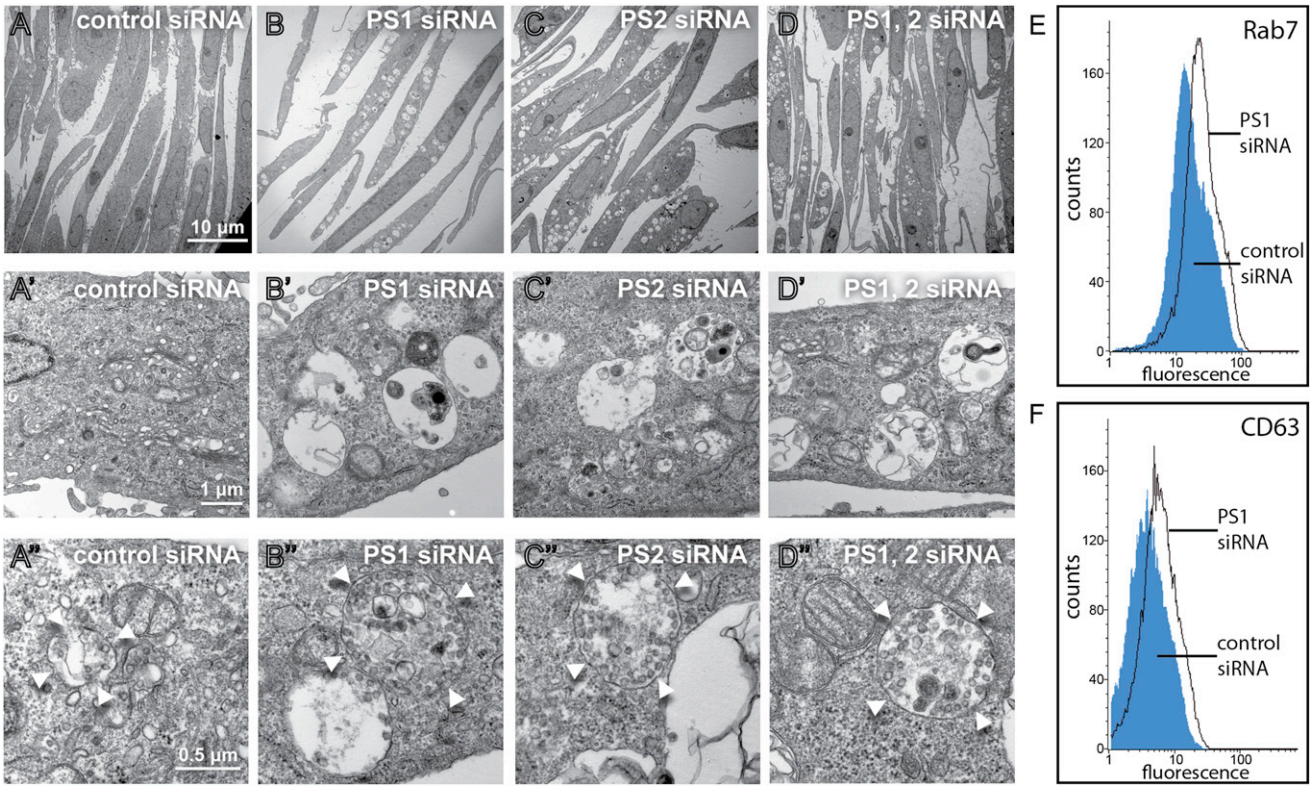


Figure 3. PS1- or PS2-Depleted Cells Accumulate MVE/Autolysosomal Vacuoles

(A–D) Low-power electron micrographs of HeLa cells transfected with control siRNA, or depleted of PS1 or PS2, or PS1 and PS2. Note the accumulation of MVEs and autolysosomes containing undigested electron-dense material. Higher-magnification views of the same cells showing autolysosomes containing electron-dense material, multilaminar membranes, and ILVs (A′–D′). Even higher-power views of endosomes and endolysosomal vesicles containing large numbers of ILVs (white arrowheads indicate limiting membranes) (A″–D″).

(E and F) Flow cytometry showing that PS1 depletion increases fluorescence intensity of the late-endosome-limiting membrane marker Rab7 (mean increase of 30.9%) or the ILV membrane protein CD63 (mean increase of 35.7%) in comparison with control siRNA.

See also Figure S3.

Accumulation of β-catenin in the nucleus constitutes one of the hallmarks of the Wnt signaling cascade. Nuclear accumulation of β-catenin was detectable only after Wnt treatment in HeLa cells (Figures 4G–4J′; arrows indicate the locations of individual nuclei). The increase in both nuclear and cytoplasmic β-catenin was highest in PS1-depleted cells treated with Wnt (Figure 4J). The increase in β-catenin levels was quantified in immunoblot analyses (Figures 4K and 4K′). A weak but significant increase in β-catenin was also observed in PS1-deficient cells even in the absence of the Wnt ligand (1.38 ± 0.08-fold increase over control; Figure 4K, lanes 1 and 3). However, this increase of β-catenin in PS1-depleted cells (see also Soriano et al., 2001, and Kang et al., 2002) did not generate a transcriptional Wnt signal (see Figures 2F, 2G, and 2I). This is explained by the observed accumulation in the cytoplasm (but not in the nucleus) in the absence of Wnt (compare Figures 4I and 4J).

Higher concentrations of either drug led to weaker increases and eventually inhibition of the signal. In the bafilomycin experiment, all samples contained a final concentration of 0.1% ethanol, which was used as vehicle.

(C and D) PS1 depletion expands the endosomal fluid-phase compartment in DextranRed endocytosis assays in HeLa cells.

(E) Flow cytometry quantification confirms expansion of the endocytic compartment marked by DextranRed after PS1 depletion.

(F) Wnt signaling is increased in PS1-depleted HEK293T cells (BAR luciferase assays). The most prominent difference between control and PS1 siRNAs resulted when cells received the highest concentration of Wnt3a (743 ± 89 versus 225 ± 27-fold induction; see bracket).

(G) The enhancing effect of PS depletion on Wnt signaling (by 4.6-fold in this experiment) is ESCRT dependent, because it is abolished when HRS is depleted by siRNA in HEK293T cells (173 ± 24-fold increase versus 1.1 ± 0.2 RLU; right bracket).

(H) The Wnt-enhancing effect of PS knockdown is also observed in *Xenopus* animal cap explant experiments. PS1 MO increased the Wnt signal by 2-fold (5.6 ± 0.8 versus 2.8 ± 0.3 RLU; left bracket) and was inhibited when HRS MO was coinjected into early *Xenopus laevis* embryos (right bracket).

(I) Depletion of PS1, PS2, or both, led to increases of Wnt signaling in Wnt luciferase reporter assays (BAR reporter) in HEK293T cells (24 ± 3.2-fold for Wnt in control siRNA cells, compared with 86 ± 9.2 for PS1, 69 ± 7.2 for PS2, and 124 ± 14.6 for both PS1 and PS2 siRNA).

All values in histograms are presented as the mean ± SEM of three independent experiments. See also Figure S2.

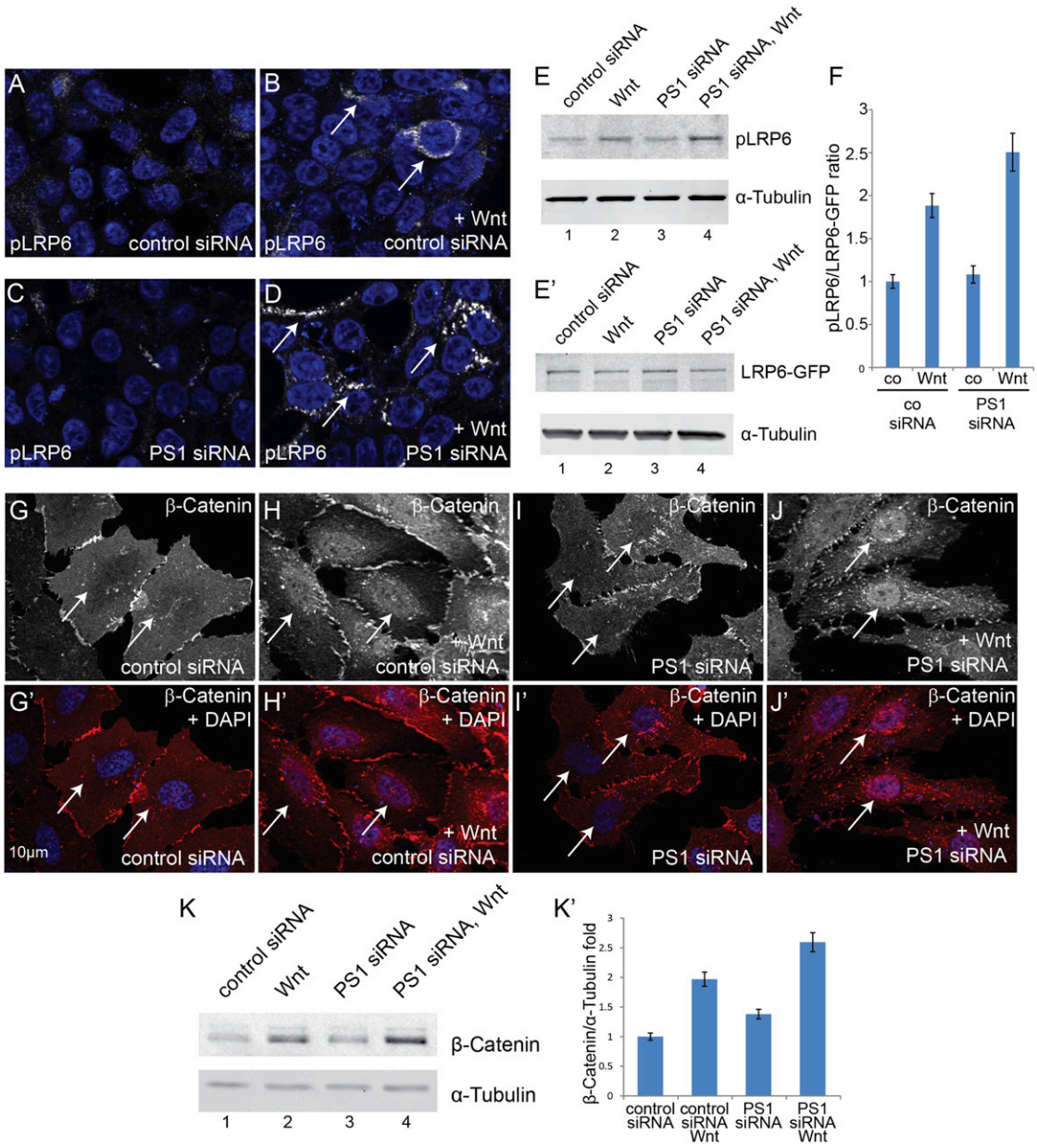


Figure 4. PS Depletion Leads to Accumulation of Phosphorylated-LRP6 Receptor and Nuclear β-Catenin upon Wnt Signaling

(A–D) The Wnt coreceptor LRP6 is phosphorylated specifically upon Wnt addition, and accumulates to a greater extent in PS1-depleted HEK293T cells stably expressing LRP6-GFP. Arrows indicate accumulation of phospho-LRP6 staining in cytoplasmic endosomes (called LRP6-signalosomes by Bilic et al., 2007).

(E) Phosphorylation of LRP6 increases after Wnt3a treatment of HEK293T cells while total LRP6-GFP levels remain unaffected.

(F) The ratio of pLRP6/LRP6-GFP (normalized to α-tubulin) was increased by Wnt3a treatment in control siRNA and PS1-depleted cells. Quantitative evaluation from three independent western blot experiments using ImageJ software.

(G–J) Total β-catenin increased with Wnt3a treatment and accumulated in the plasma membrane, cytoplasm, and inside the nucleus in HeLa cells. In the absence of Wnt3a addition, PS1-depleted cells showed a slight increase of β-catenin in the cytoplasm, but β-catenin was excluded from the nucleus. Wnt3a treatment of PS1-depleted cells led to a strong accumulation of β-catenin inside the nucleus and in cytoplasmic puncta. Nuclear localization is indicated by white arrows and is counterstained with DAPI.

(K) Immunoblot analyses showing significantly increased β-catenin levels in Wnt- and PS1 siRNA-treated cells (lanes 1, 2, and 4; 1.97 ± 0.12-fold and 2.59 ± 0.16-fold, respectively). The resulting mean values presented were obtained from five independent western blot experiments.

We conclude that PS1 depletion causes increased levels of activated phospho-LRP6 receptor in cytoplasmic puncta (presumably corresponding to endosomal vesicles of the type shown in Figure 3) when cells are exposed to Wnt, and that this correlates with increased nuclear β-catenin accumulation.

GSK3 Translocates to Endosomes in PS-Deficient Cells upon Wnt signaling

To investigate whether the increase in Wnt signaling observed in CQ-treated or PS1-depleted cells could be explained by the GSK3 sequestration mechanism, we used HeLa cells transiently expressing GSK3-RFP. Overnight exposure of these cells to Wnt-conditioned medium led to a relocalization of GSK3-RFP into cytoplasmic puncta (compare Figures 5A and 5B), which could be visualized when cells were washed with the mild detergent saponin to reveal the endocytic compartment prior to fixation (see Extended Experimental Procedures). This relocalization of GSK3 to cytoplasmic puncta upon Wnt signaling was strongly enhanced when cells were treated with CQ (Figures 5C and S4A). These Wnt-induced GSK3 puncta colocalized with the late-endosome marker Rab7 (Figures 5B″ and 5C″).

We next used an activated form of Rab5 (Rab5QL-DsRed), which induces the formation of large MVBs (Wegner et al., 2010), to show that GSK3-GFP is translocated to endosomes. HeLa cells were transfected with either control or PS1 siRNAs, treated with Wnt3a-conditioned medium overnight, and treated with saponin before fixation. We observed an accumulation of GSK3-GFP puncta inside and in the periphery of Rab5QL MVBs, specifically when PS1-depleted cells were treated with Wnt (compare Figures 5F–5F″ with Figures 5G–G″; see also Figure S4B). We also found that Wnt treatment led to the translocation of GSK3-GFP to MVBs in control siRNA cells (Figures 5D–5D″ and 5E–5E″; see also Figure S4B), although to a lesser degree.

These results suggest that depletion of PS1 with siRNA or inhibition of lysosome function with CQ enhanced the translocation of GSK3 to late endosomes/MVBs that is normally triggered by Wnt.

GSK3 Protein Substrates Are Stabilized by Wnt in PS1-Depleted Cells

Because GSK3 is more efficiently sequestered in MVEs of PS1-deficient cells, we investigated whether GSK3 phosphorylation substrates would be more stable in these cells. Consistent with the GSK3 sequestration model, we observed that cytosolic GSK3 substrates such as β-catenin (Figure 4K) and a GFP-GSK3 protein stability biosensor primed by a mitogen-activated protein kinase (MAPK) site (Figure 6A; Taelman et al., 2010) were more efficiently stabilized in PS knockdown cells upon receiving a Wnt signal (Figure 6A). As a control, we used a mutated GFP biosensor construct in which the three GSK3 phosphorylation sites were mutated into alanines. We found that the GSK3-resistant biosensor became stabilized and its levels not affected by Wnt or PS1 siRNA (compare Figures 6B and 6B′ with Figures 6A and 6A′). This indicates that the effects of Wnt and PS1 siRNA are exerted at the level of the protein half-life via GSK3 sites.

Using the same experimental design, we found that transfected Tau-GFP (Kwan and Kirschner, 2005) was stabilized by Wnt treatment, and that this stabilization was more marked when PS1 was depleted (Figures 6D and 6D′). Tau is a microtubule-associated protein that plays an important role in AD and contains multiple GSK3 phosphorylation sites. When we measured Tau phosphorylation by GSK3 in immunoblots using an anti-phospho-Tau (T181) antibody, we found that Wnt signaling decreased phosphorylation in both control and PS1-depleted cells (Figures 6C and 6C′). These results support the hypothesis that Wnt signaling removes active GSK3 enzyme from the cytosol, decreasing the protein phosphorylations that target GSK3 protein substrates for degradation. The increased protein stabilization caused by Wnt in PS1-deficient cells, in particular in the case of Tau, could provide a new link among Wnt signaling, protein stabilization, and neurodegeneration.

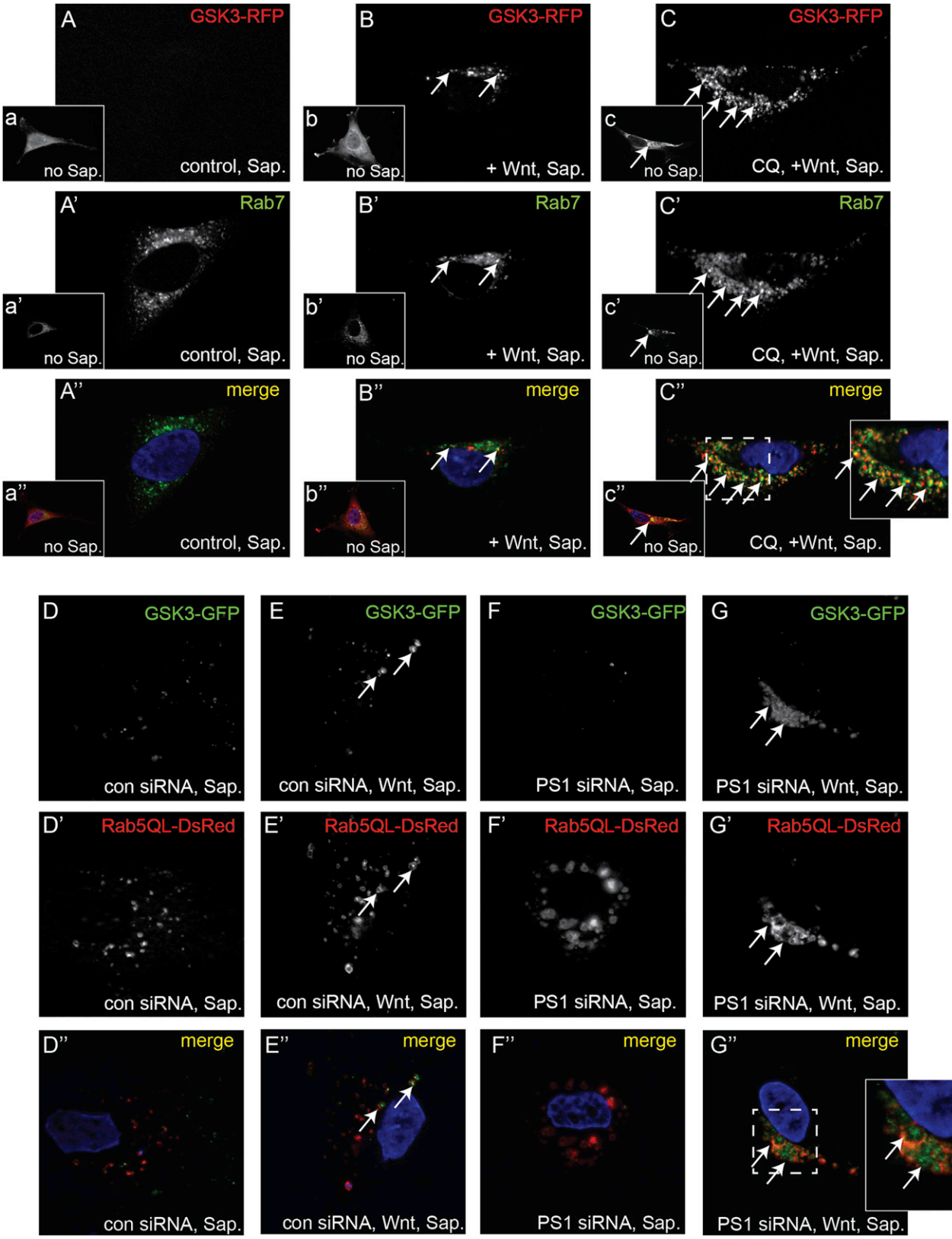
DISCUSSION

Late Endosomes Are Required for Wnt Signaling

Recently, we proposed a model for sustained Wnt signaling through sequestration of GSK3 in MVEs (Taelman et al., 2010). In the study presented here, we investigated how Wnt signal transduction is affected when the function of the endolysosomal pathway is altered by inhibiting lysosomal function with CQ, low doses of bafilomycin A, the lysosomal protease inhibitors leupeptin or E64, or PS1 depletion. We found that lysosomal inhibition caused an expansion of the late-endosomal compartment, leading to a more efficient sequestration of GSK3 and the generation of an enhanced Wnt signal. The CQ effect had an absolute requirement for the ESCRT machinery, which is essential for the formation of ILVs in the endosomal compartment. The enhanced Wnt signaling caused by PS depletion required Hrs/Vps27 not only in cell culture experiments but also in vivo in explants from *Xenopus* PS1 morpholino-injected embryos. Figure 7 shows a proposed model for how lysosomal inhibition increases Wnt/GSK3 signaling and protein stabilization.

The antimalarial drug CQ, which alkalinizes lysosomes but still allows the formation of ILVs, caused a great expansion of the late-endosomal compartment. Not all signaling pathways were increased by CQ treatment: in the case of Sonic Hedgehog (Shh), signaling measured by a luciferase reporter was inhibited by CQ, whereas Hrs-siRNA increased signaling instead of inhibiting signaling as in the case of Wnt (data not shown). An increase in the fluid phase of the endosomal compartment (marked by endocytosed DextranRed) was observed after siRNA-mediated depletion of PS1. This gene has been linked to AD and was recently found to be critical for lysosomal maturation (Lee et al., 2010; Neely et al., 2011; Zhang et al., 2012). PS1 and PS2 are intramembrane proteases that form the catalytic core of the γ-secretase complex that cleaves the transmembrane domains of a large number of proteins. PSs are found in most cellular membranes, including in the ILVs of MVEs (De Strooper and Annaert, 2010). In the experiments reported here, lysosomal inhibition or PS depletion increased Wnt signaling in an ESCRT-dependent manner, but did not generate a signal in the absence of Wnt ligand. This effect could be due to an expanded surface of the limiting membrane of MVEs, or perhaps to changes in pH within the endosome that could increase the sensitivity of the endosomal membrane to undergo invagination to generate ILVs.

Recently published work by Li et al. (2012) described the formation of active Wnt receptor complexes containing most of the components of the β-catenin destruction complex, except for the E3 ligase βTrCP. In agreement with that report, we observed that βTrCP does not translocate into LRP6 endosomes, whereas β-catenin phosphorylated by GSK3 is sequestered inside these Wnt-specific endosomes (Taelman et al.,


A
GSK3-RFP
a
no Sap.
control, Sap.
B
GSK3-RFP
b
no Sap.
+ Wnt, Sap.
C
GSK3-RFP
c
no Sap.
CQ, +Wnt, Sap.
A'
Rab7
a'
no Sap.
control, Sap.
B'
Rab7
b'
no Sap.
+ Wnt, Sap.
C'
Rab7
c'
no Sap.
CQ, +Wnt, Sap.
A''
merge
a''
no Sap.
control, Sap.
B''
merge
b''
no Sap.
+ Wnt, Sap.
C''
merge
c''
no Sap.
CQ, +Wnt, Sap.
D
GSK3-GFP
con siRNA, Sap.
E
GSK3-GFP
con siRNA, Wnt, Sap.
F
GSK3-GFP
PS1 siRNA, Sap.
G
GSK3-GFP
PS1 siRNA, Wnt, Sap.
D'
Rab5QL-DsRed
con siRNA, Sap.
E'
Rab5QL-DsRed
con siRNA, Wnt, Sap.
F'
Rab5QL-DsRed
PS1 siRNA, Sap.
G'
Rab5QL-DsRed
PS1 siRNA, Wnt, Sap.
D''
merge
con siRNA, Sap.
E''
merge
con siRNA, Wnt, Sap.
F''
merge
PS1 siRNA, Sap.
G''
merge
PS1 siRNA, Wnt, Sap.

2010; our own unpublished results). These findings underscore the deep relationship between endosomal trafficking and the Wnt signaling pathway.

Depletion of PS Increases Wnt Signaling

PSs have been proposed to be involved in maturation of lysosomes through N-glycosylation of the V0a1 subunit of the v-ATPase complex needed for endosomal acidification (Lee et al., 2010). However, this mechanism was recently questioned (Zhang et al., 2012), and it was suggested instead that PSs may be involved in regulating the gene network associated with lysosomal biogenesis (Sardiello et al., 2009), and affect autophagy/lysosomal proteolysis independently of lysosomal acidification (Neely et al., 2011). Lee et al. (2010) observed a decrease of endosomal acidification in LysoTracker staining of PS1 knockout cells and siRNA knockdowns, and we were able to confirm their observations. We also found significant increases of MVE markers such as CD63, Rab7, and endocytosed DextranRed in cells depleted of PS1. The effects on lysosomal maturation were independent of γ-secretase activity (Lee et al., 2010; Neely et al., 2011; Figure S2I), excluding an effect on Notch or other γ-secretase-dependent pathways.

The enhancement of Wnt signaling by PS depletion described here was entirely dependent on ESCRT machinery (Figures 1A, 2G, and 2H). Thus, formation of ILVs of late endosomes is required for the enhanced effects of the Wnt ligand. Our observations differ from a report by Kang et al. (2002), who proposed that PS functions as an alternative scaffold for β-catenin degradation. Importantly, they found that loss of PS1 stabilized β-catenin in a Wnt-independent way, whereas in our experiments, addition of Wnt protein was required to trigger Wnt transcriptional activity in cell lines with low levels of endogenous Wnt signaling (Figures 2F, 2G, and 2I). We note, however, that in *Xenopus* explants PS1 MO did slightly increase β-catenin signaling (Figure 2H). In addition, PS1 siRNA caused a small but reproducible increase of cytoplasmic β-catenin levels in HeLa cells (Figures 4I and 4K'). We suggest that the reported Wnt-independent accumulation of β-catenin (Kang et al., 2002) may be explained in part by an endogenous autocrine Wnt signal that became enhanced though MVE expansion in PS1 knockout cells.

PS Depletion Increases Protein Stabilization by Wnt

Proteins that are normally degraded in lysosomes are taken up from the extracellular space by endocytosis, are membrane proteins, or are ingested from the cytosol via autophagy. Upon Wnt signaling, cells experience an additional effect, namely the endosomal sequestration of cytoplasmic GSK3, which is translocated into MVBs together with activated Wnt receptor complexes (Taelman et al., 2010; Dobrowolski and De Robertis, 2012). Here, we provide evidence that three cytosolic GSK3 substrates—the microtubule-associated protein Tau, β-catenin, and a GFP-GSK3 biosensor—were stabilized by Wnt signaling in PS1-depleted cells.

The experiments suggest a mechanism for Wnt signaling in PS-deficient cells. We propose that upon Wnt signaling, activated receptor complexes are internalized in endosomes and sequester GSK3 inside the expanded late endosomes together with the activated Wnt receptor complex, which is composed of multiple protein components phosphorylated by GSK3 (Figure 7). Once cytoplasmic levels of active GSK3 are sufficiently decreased, GSK3 protein substrates become less phosphorylated, their phosphodegron domains are not recognized by E3 polyubiquitin ligases, and GSK3 target proteins become stabilized.

This mechanism could play a role in AD, because PS deficiency increases the stability of multiple GSK3 substrates upon Wnt signaling. Some of these substrates, such as the microtubule-associated protein Tau, play crucial roles in the pathogenesis of AD. An extensive literature links Tau with GSK3 signaling (Hall et al., 2000; de Calignon et al., 2012). A role for Wnt signaling in prevalent neuropsychiatric diseases, including AD, schizophrenia, and autism, has been proposed (De Ferrari and Moon, 2006); however, studies performed to elucidate the function of Wnt in neurodegeneration have often generated contradictory results (summarized by Boonen et al., 2009). It is possible that Wnt signaling can result in different outcomes at different stages of AD progression. At early stages of AD, increased sequestration of GSK3 may stabilize many proteins, as shown here for Tau. However, chronic protein stabilization in PS1-deficient cells might eventually lead to failure of the endosomal pathway and Wnt signaling as the disease progresses. Once GSK3 ceases to be sequestered, elevated levels of cytosolic Tau might be more readily phosphorylated by GSK3, triggering AD pathology. It is possible that repeated Wnt stimulation through the course of a lifetime aging process, in combination with the accumulation of MVEs caused by defective trafficking, could result in increased protein stabilization and the eventual accumulation of the neurotoxic Amyloid β42 (Aβ42) peptide (De Strooper and Annaert, 2010).

In early stages of AD, Aβ42 and its intermediates localize to MVBs (Takahashi et al., 2002) and are released from neurons via exosomes (Rajendran et al., 2006) to form extracellular amyloid deposits (Vella et al., 2008). According to the amyloid hypothesis for the onset of AD (Selkoe, 1991; Hardy and Higgins, 1992; Hardy, 2009), extracellular amyloid plaque deposits would lead to altered kinase/phosphatase activities and hyperphosphorylation of Tau. Other recent studies suggested that phosphorylated Tau spreads in a prion-like manner through neuronal synaptic circuits (Liu et al., 2012; de Calignon et al., 2012; Kfoury

Figure 5. Wnt-Induced Translocation of GSK3 Is Increased by CQ or PS siRNA Treatment

(A–C) GSK3-RFP-expressing HeLa cells were treated with saponin to visualize endosomal GSK3 and endogenous Rab7 staining. GSK3 signal without saponin treatment is shown in insets (a′–c″). Note that virtually no endosomal GSK3 staining was detected in control cells. Endosomal GSK3 staining was detected when cells were exposed to Wnt3a overnight, and was strongly increased when cells were treated with CQ and Wnt. Magnification of an area in C″ shows partial colocalization of GSK3-RFP with Rab7 antigen (arrows).

(D–G) GSK3-GFP relocalized to Rab5QL-DsRed MVBs in Wnt-treated cells, and this colocalization was strongest when PS1 is depleted (arrows). Experiments were carried out with saponin-treated HeLa cells.

See also Figure S4.

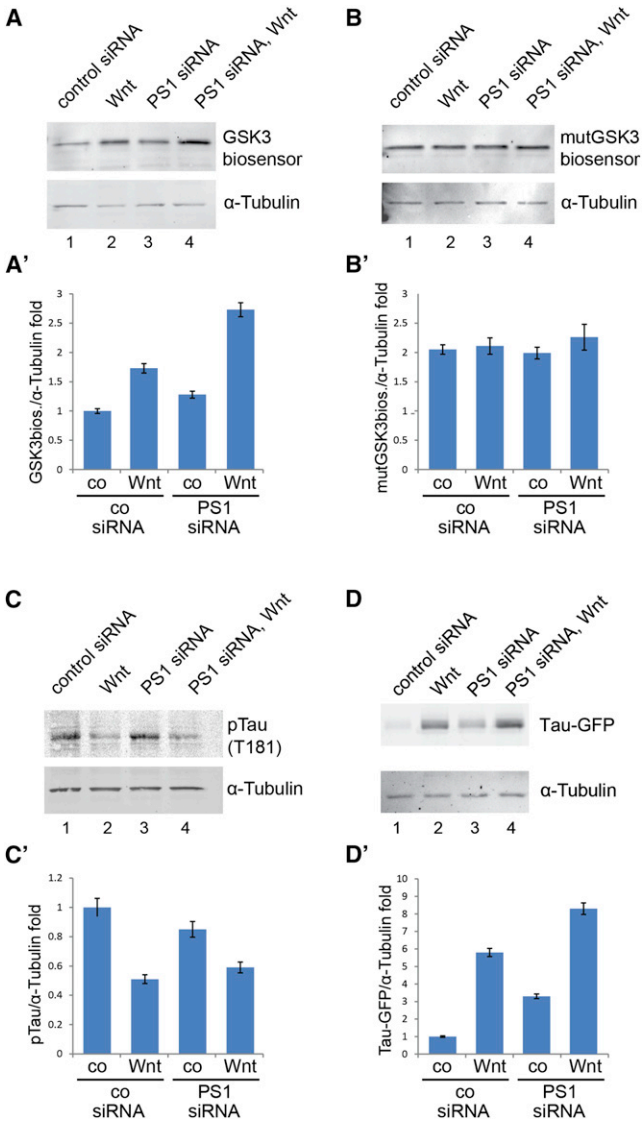


Figure 6. PS Depletion Causes Increased Stability of Wnt-Regulated GSK3 Substrates

(A) The GSK3-GFP biosensor consists of a Flag-tagged GFP containing MAPK-primed GSK3 phosphorylation sites (Taelman et al., 2010), and provides a measure of cytosolic GSK3 activity. Wnt treatment stabilized a GSK3-biosensor in control conditions (lane 2, 1.8 ± 0.08-fold increase over control siRNA) and was more efficiently stabilized in PS1-depleted cells when treated with Wnt (lane 4, 2.6 ± 0.16-fold increase over control).

(B) The control GFP biosensor (mutGSK3 biosensor) lacking GSK3 phosphorylation sites showed a higher stability than the GSK3-GFP biosensor protein, but no significant changes in stability were detected when cells were treated with Wnt or PS1 siRNA. This control shows that the stabilization of protein half-life by PS1 depletion and Wnt is mediated by the GSK3 sites.

(C) GSK3-specific Tau phosphorylation was decreased by Wnt treatment in control or PS1-depleted cells (decreased to 0.5 ± 0.03 in control or 0.59 ± 0.04 in PS1 siRNA lysates). Specific GSK3 phosphorylation of the endogenous microtubule-associated protein phospho-Tau was tested in immunoblots using the phospho-Tau (T181) antibody.

(D) Total Tau stability determined in Tau-GFP-expressing HeLa cells. Tau-GFP was stabilized in control siRNA-transfected cells by overnight treatment with Wnt3a (lane 2, 5.9 ± 0.23-fold increase over control). Accumulation of Tau without Wnt was also detected in PS1-depleted cells (lane 3, 3.2 ± 0.13-fold increase over control). The stability of Tau was further increased when PS1 siRNA cells were treated with Wnt (lane 4, 8.3 ± 0.33-fold increase).

For statistical evaluation, signals from three or more immunoblot analyses were quantified using ImageJ.

et al., 2012). In addition, autophagy and lysosomal proteolysis defects have also been proposed to be involved in the pathogenesis of AD (Nixon et al., 2008; Lee et al., 2010).

The experiments presented here demonstrate that decreased PS expression causes an increase in Wnt signaling. The results also show that PS1 depletion causes a considerable expansion of the endosomal system, resulting from the inhibition of lysosomal function downstream of ILV formation. The connection between Wnt signaling and increased stability of GSK3 phosphorylation protein targets in PS-deficient cells could explain why AD neurodegeneration is most severe in the hippocampus, especially in its dentate gyrus, and cerebral pyramidal neurons (Brundin et al., 2010), because it is in these particular cells that physiological Wnt signaling in the adult brain is maximal (Maretto et al., 2003). We propose that during the aging process, defects in membrane trafficking may increase protein stabilization in the cytosol after repeated stimulation by Wnt signaling, contributing to the formation and deposition of intracellular protein aggregates. Whether the proposed mechanisms play a role in human pathology remains to be determined. In the meantime, the results presented here strengthen the intimate connection between Wnt signaling and the cell biology of intracellular membrane trafficking.

EXPERIMENTAL PROCEDURES

Cell Culture and Knockdown Experiments

All cell lines used in this work (HeLa, 3T3, HEK293T, and L-cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) complete medium containing 10% fetal bovine serum (FBS), 1% glutamine, and 1% Pen/Strep. For knockdown experiments in cultured cells, the siRNAs targeting human PS1, PS2, and HRS were ON-TARGETplus SMARTpool siRNAs from Thermo Scientific (L-004998, L-006018, and L-016835, respectively). The nontargeting control-siRNA pool (D-001810) was from the same company. For RNAi depletion experiments, cells were transfected with siRNAs 24 hr prior to transfections with DNA. For the rescue experiments, we introduced a full-length human *PS1* gene (EHS1001-33034; Open Biosystems) into the pCS2+ expression plasmid and transfected 0.3 μg of this DNA into each well of a 12-well plate containing siRNA-pretreated cells. Knockdown experiments in *Xenopus laevis* were conducted using PS1 morpholino (sequence: TTCACTGGTGTCATTCAT ATTAGCT; Gene Tools, LLC) and Hrs morpholino (sequence: TGCCGCTTCCT CTTCCCATTGCGAA; Gene Tools, LLC).

Immunostaining, Flow Cytometry, and Western Blots

The following primary antibodies were used for immunostaining and flow cytometry: anti-Rab7 rabbit monoclonal (#9367; Cell Signaling) at 1:350, anti-CD63 mouse monoclonal (#556019; BD Biosciences) at 1:250, anti-β-catenin rabbit (H-102; SantaCruz) at 1:200, and anti-phospho-S1490-LRP6 rabbit (#2568; Cell Signaling) at 1:500.

For western blots, we used the following primary antibodies: anti-β-catenin (#C2206; Sigma) at 1:4000, anti-Flag monoclonal (#F1804; Sigma) at 1:1,500, anti-GFP (#A6455; Molecular Probes) at 1:500, anti-phospho-LRP6 (#2568; Cell Signaling) at 1:500, anti-phospho-Tau (T181, #5383; Cell Signaling) at 1:500, and anti-α-Tubulin monoclonal (#CP06; Calbiochem) at 1:1,500. Secondary antibodies coupled to Infrared Dyes (IRDye 680 and IRDye 800) at 1:3,000 (LI-COR) were used, and western blots were analyzed using a LI-COR Odyssey system.

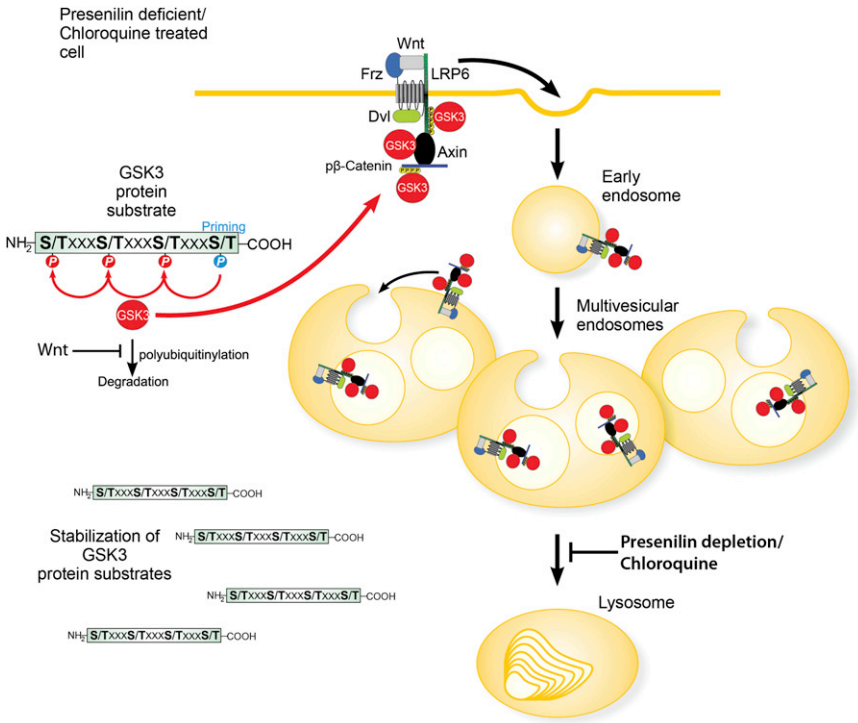


Figure 7. Model of How CQ or PS Depletion Affects Wnt Signaling by Expanding the Late-Endosomal Compartment

Lysosomal inhibition by CQ or depletion of PS leads to an accumulation of late-endosomal vesicles, which upon Wnt signaling cause increased sequestration of GSK3 in MVEs. Lower levels of active GSK3 in the cytosol during Wnt signaling result in increased stabilization of the half-life of GSK3 protein substrates. Note that the activated Wnt receptor complex consists of multiple GSK3 substrates (e.g., LRP6, Fz, Dvl, Axin, and phospho-β-catenin), leading to sequestration of active GSK3 enzyme molecules bound to its substrates.

Statistics

The results of three or more independent experiments are given as the mean ± SEM. Statistical analyses were performed with Excel (Microsoft), applying the two-tailed t test as appropriate. Significant differences of means are indicated as * ≤ 0.05, ** ≤ 0.01, and *** ≤ 0.005.

Additional Methods

Details regarding the methods used for luciferase assays, endolysosomal staining, in vivo time-lapse movies, and electron microscopy are provided in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.09.026.

LICENSING INFORMATION

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EXTENDED EXPERIMENTAL PROCEDURES

Luciferase Assays, LysoTracker and DextranRed Staining, hPS1 Mutant Constructs, and Time-Lapse Movies

For luciferase assays, cells were incubated with control or Wnt3a conditioned medium overnight, washed, lysed and measured using the Dual Luciferase Renilla Reporter Assay kit (#E1960) in the Promega Glomax plate luminometer following manufacturer's instructions. For a single well of a standard 12 well plate cells were grown up to a 80% confluency and transfected with 0.2 μg of BAR-Luciferase, 0.02 μg of CMV Renilla, and 0.3 μg of constructs such as GSK3 biosensor or Tau-GFP supplemented with carrier PCS2 empty vector to a final DNA amount of 1 μg.

Wnt3a conditioned medium, as well as control conditioned medium, were harvested from stably transfected L-cells according to the ATCC protocol (Willert et al., 2003), except for using a lower 2% FBS concentration, which we found to be more reliable and active. For the experiments in Figure 4, Wnt conditioned medium was supplemented by the addition of 80 ng/ml of purified Wnt3a protein (PeproTech, NJ). CQ diphosphate (Sigma) was dissolved in H_2O as a 100 mM stock solution and added to cell cultures at about 80% confluency. Transfections of DNA constructs were performed using BioT (Bioland); for siRNAs reverse transfections Lipofectamine 2000 (Invitrogen) was placed first into the plates and mixed with trypsinized cells. For visualization of the endocytic compartment, living HeLa cells were treated with 0.01% (weight/vol) saponin in PBS, gently shaking for 5 min at room temperature prior to fixation (Bishop and Woodman, 2000). Endosomal staining (Figure S4) was quantified using the ImageJ software and ITCN plugin that measures intensity maxima from images based on individual parameters (http://www.bioimage.ucsb.edu). For LysoTracker staining, living cells were incubated in pre-warmed culture medium containing diluted LysoTracker Red DND-99 reagent (1:1000, Invitrogen #L7528) for 1 to 3 min at 37°C (depending on the cell line), immediately and quickly washed twice with PBS and fixed with 4% PFA for 15 min at room temperature. Other procedures were as described in Taelman et al. (2010).

For DextranRed staining, HeLa cells were grown on clean glass coverslips, transfected with the appropriate siRNAs and incubated with 2 mg/ml dextran Tetramethylrhodamine (D1868, Invitrogen, NY) diluted in DMEM complete medium for 30 min. Subsequently, cells were washed, fixed with 4% Paraformaldehyde in PBS and mounted on glass slides for microscopic evaluation.

Stably transfected LSL-cells containing SuperTopFlash-Luciferase constructs (Blitzer and Nusse, 2006) and HEK293T cells stably expressing LRP6-GFP (Kategaya et al., 2009) fusion proteins were generously provided by Drs. Roel Nusse and Randall Moon, respectively.

Human *Presenilin 1* (PSEN1, transcript variant 1) from an EST was subcloned, together with a C-terminal Flag-tag into the expression vector pCS2 for rescue experiments. The hypomorphic FAD mutations A246E, L392V and M146V (Lee et al., 2010; Boonen et al., 2009), and the aspartate protease-inactive mutants D257A and D385A (Lee et al., 2010) were introduced by site-directed mutagenesis using Quick-Change II XL kit from Stratagene.

For filming in vivo time-lapse movies with Rab7-GFP and CQ, transfected NIH/3T3 cells were transferred to Lab-Tek coverglass chambers (#155380, Nalge Nunc International, New York). Pictures were taken every 5 min using a Zeiss Observer .Z1, equipped with Apotome.2, fully automated-stage and Temp/CO_2Module S, using a Colibri 488 LED or DIC optics with an EC Plan-Neofluar 40x/0.75 M27 objective. CQ was added just before acquisition started.

Electron Microscopy

Confluent cultures of 3T3 cells in 10 cm dishes treated with 50 μM CQ for the indicated times or of HeLa-cells depleted of PS1 and/or PS2 with siRNA were fixed by replacing the medium with fixative solution (2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4). After incubation in cold fixative for one hour, the cells were scraped from the dishes using a Teflon scraper, collected in Eppendorf tubes and recovered by centrifugation for 10 min 10,000 rpm. Cell pellets remained under the fixative for additional 3 hr at RT and were carefully washed three times for 10 min each with 0.1 M Cacodylate buffer. The pellets were post fixed with 2% Osmium tetroxide and processed for embedding in epoxy resin LX 112. Thin sections were stained with Uranyl acetate and Lead citrate and examined by TEM in a JEOL EX 1200 electron microscope.

SUPPLEMENTAL REFERENCES

Bishop, N., and Woodman, P. (2000). ATPase-defective mammalian VPS4 localizes to aberrant endosomes and impairs cholesterol trafficking. Mol. Biol. Cell *11*, 227–239.

Blitzer, J.T., and Nusse, R. (2006). A critical role for endocytosis in Wnt signaling. BMC Cell Biol. *7*, 28.

Kategaya, L.S., Changkakoty, B., Biechele, T., Conrad, W.H., Kaykas, A., Dasgupta, R., and Moon, R.T. (2009). Bili inhibits Wnt/beta-catenin signaling by regulating the recruitment of axin to LRP6. PLoS ONE *4*, e6129.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., 3rd, and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature *423*, 448–452.

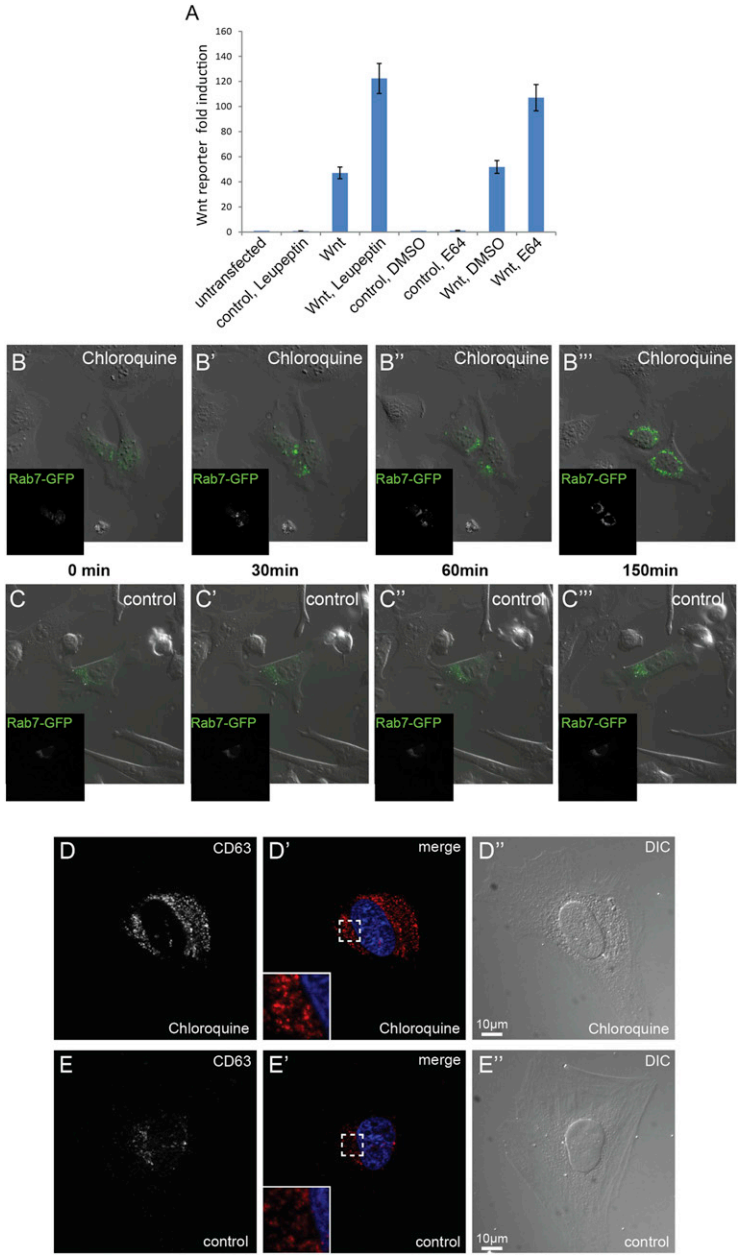

Figure S1. CQ Treatment Expands the Rab7- and CD63-Positive Endosomal Compartment, Related to Figure 1

(A) Wnt signaling is increased in cells treated overnight with the lysosomal proteases inhibitors leupeptin (122 ± 12-fold induction) or E64 (107 ± 10; both obtained from Sigma and used at a concentration of 200 μM or 6 μM in DMSO, respectively) as measured in LSL cells stably transfected with the Wnt reporter SuperTopFlash.

(B–B''') Time-lapse microscopy of HeLa cells showing an accumulation of the late-endosomal marker Rab7-GFP visible after ~30 min of CQ treatment.

(C–C''') No changes in Rab7 were detected in control cells. Insets show the signal detected in the Rab7-GFP channel, without Nomarski optics.

(D–D'') Endogenous CD63 protein marks the membranes of ILVs in MVBs and to lesser extent lysosomes (Escola et al., 1998). Note that CD63 puncta are increased in CQ-treated cells. Small endosome-like structures could be detected simply by differential interference contrast (DIC) optics when cells were treated with CQ.

(E–E'') Control HeLa cells showing low endogenous levels of CD63 antigen.

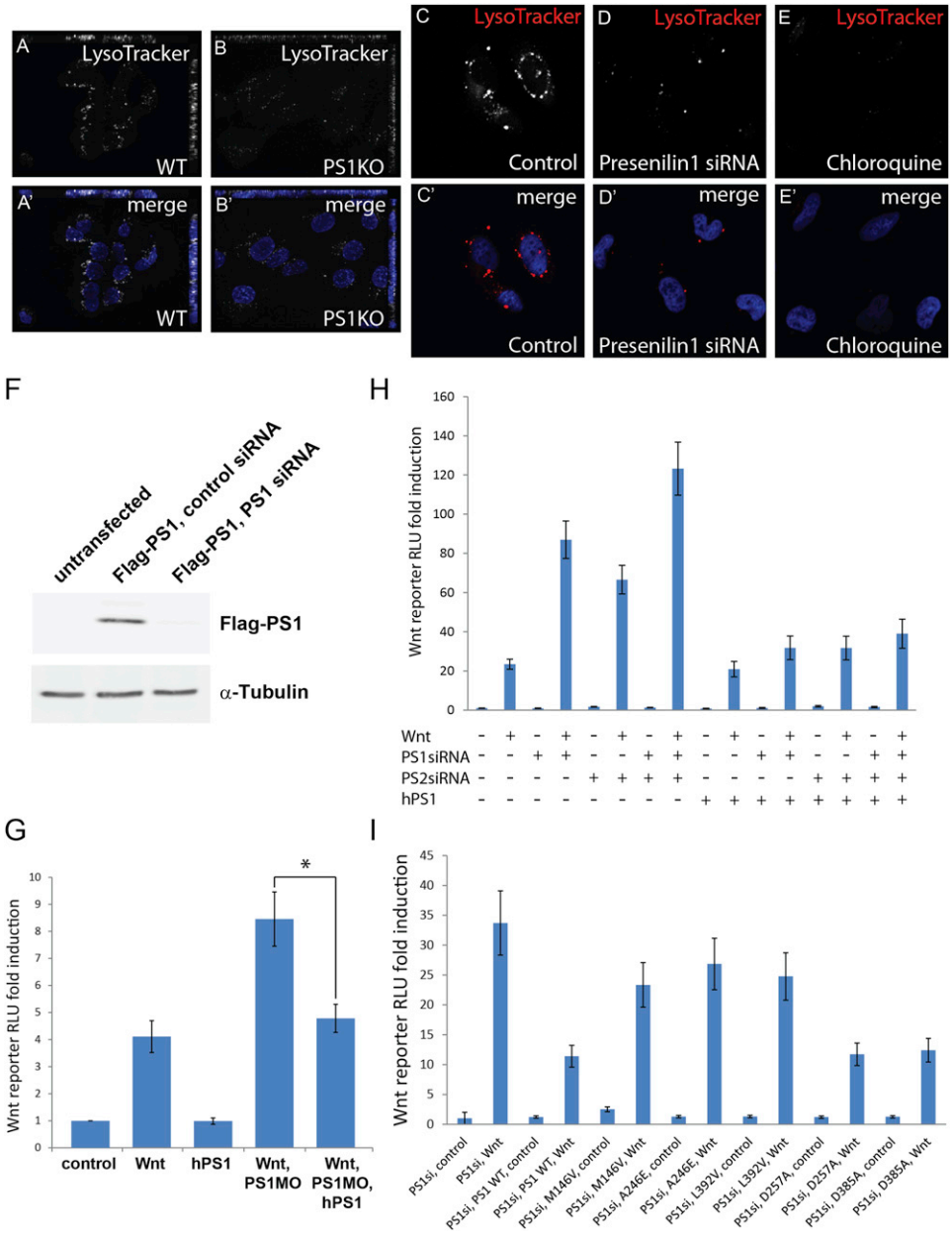

Figure S2. Decreased Acidification in PS-Deficient Cells and Rescue of PS Knockdown by Overexpression of WT or Protease-Inactive hPS1 but Only Partially by Hypomorphic FAD PS1 Mutations, Related to Figure 2

(A–B') LysoTracker staining showing a marked decrease of acidified endolysosomal vesicles in blastocyst-derived PS1 knockout cells (PS1KO). This result confirms the observations of Lee et al. (2010).

(C–E') LysoTracker staining was weaker in PS-depleted cells, indicating impaired acidification, and was virtually absent in cells treated with CQ.

(F) Flag-PS1 protein was effectively depleted by PS1 siRNA, but not by control siRNA, in transfected HeLa cells; α-tubulin served as the loading control.

(G) The increase in Wnt signaling caused by PS1 MO in *Xenopus* animal cap explants was rescued by coinjection of 20 pg of human PS1 DNA (bracket). RLUs were obtained using the SuperTopFlash reporter (n = 2).

(H) The increase in Wnt signaling caused by siRNA depletion of PS1, PS2, or both, could be rescued by overexpression of human PS1 (Wnt luciferase BAR reporter assays in HEK293T cells). Note that the effect of PS2 or PS1, PS2 depletion on Wnt signaling could be reversed by overexpression of PS1. This experiment shows that the effects of the PS siRNAs used in this study are specific.

(I) Overexpression of PS FAD mutations associated with early-onset FAD (M146V, A246E, and L392V) did not efficiently rescue the effect of PS depletion in cultured cells, whereas the aspartate mutations D257A and D385A reversed the effect of PS1 siRNA. All cells were transfected with PS1 siRNA and treated with either control or Wnt-conditioned medium and analyzed using SuperTopFlash-transfected HeLa cells. These results indicate that FAD mutations were less effective than WT or protease-inactive PS1 mutants. The protease activity of PS1 is not required for its effect on Wnt signaling.

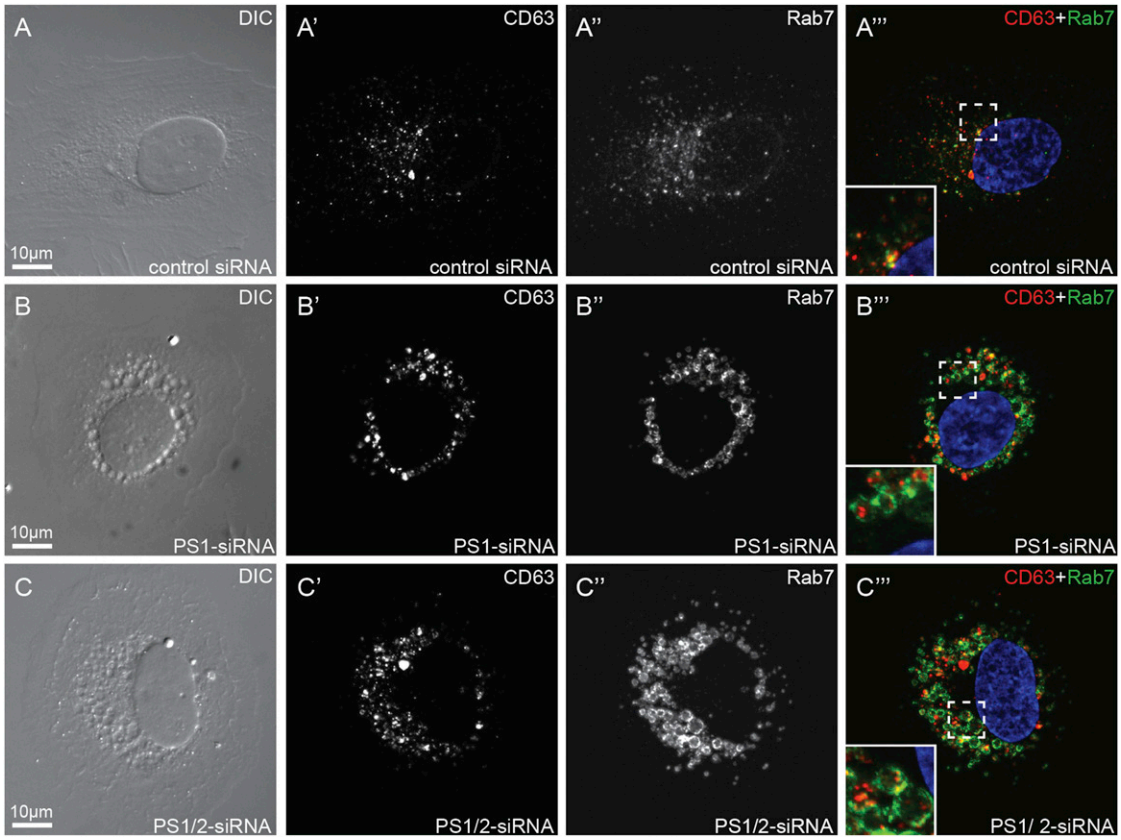

Figure S3. PS Depletion Enlarges the MVB Compartment, Related to Figure 3

(A–A''') CD63 and Rab7 puncta colocalize in control untreated HeLa cells.

(B–B''') PS1 depletion with siRNA causes enlarged Rab7-positive vacuoles, some of which contain the ILV marker CD63 in their lumen. Note that the MVEs enlarged by PS1 depletion are readily visualized by DIC optics.

(C–C''') Greatly enlarged vacuoles by double knockdown of PS1 and PS2. Note in the inset Rab7 staining surrounding the enlarged endosomes and CD63 localizing to the lumen of these vesicles.

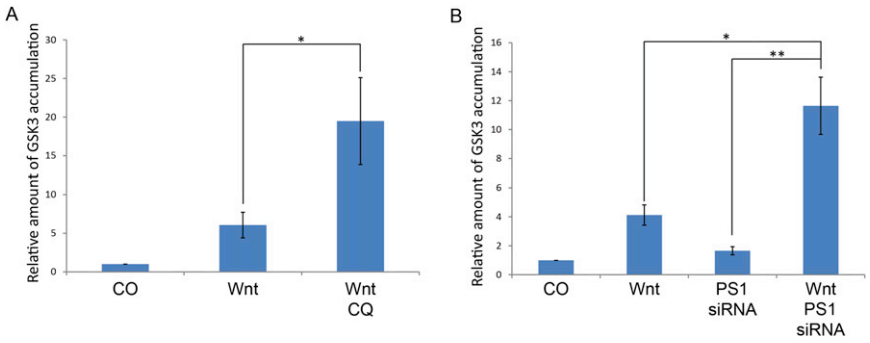

Figure S4. Quantification of Wnt-Dependent Relocalization of GSK3 after Lysosomal Inhibition by CQ or PS1-siRNA in Saponin-Permeabilized HeLa Cells, Related to Figure 5

(A) Endosomal GSK3 staining was increased (6.1 ± 1.6-fold) following Wnt3a treatment. In the presence of CQ, the GSK3 relocalization caused by Wnt treatment was further increased (19.5 ± 5.6-fold relative to control).

(B) Wnt treatment increased endosomal GSK3 (4.1 ± 0.7-fold), and further enhanced GSK3 translocation in PS1-depleted cells (11.6 ± 1.9-fold).

GSK3 signals were quantified as described in Extended Experimental Procedures.