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Wnt Signaling Translocates Lys48-Linked Polyubiquitinated Proteins to the Lysosomal Pathway

Graphical Abstract

Highlights

- Wnt signaling causes accumulation of K48-polyUb proteins in endolysosomes/MVBs
- Wnt-induced accumulation of polyUb proteins is GSK3 phosphorylation dependent
- HRS and Axin1 are required for Wnt-induced lysosomal relocalization of polyubiquitin
- Wnt transiently reduces free ubiquitin levels in the cell

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In Brief
Kim et al. show that the lysosomal and proteasomal protein degradation systems crosstalk in a Wnt-regulated manner. Lys48-linked polyubiquitinated proteins, normally degraded in the proteasome, become localized in MVBs/lysosomes during Wnt signaling. By sequestering polyubiquitin chains inside endolysosomes, Wnt signaling causes a rapid and transient reduction of free mono-ubiquitin.

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Wnt Signaling Translocates Lys48-Linked Polyubiquitinated Proteins to the Lysosomal Pathway

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INTRODUCTION

Wnt signaling is intimately linked to the endolysosomal pathway in the receiving cell. Once bound to the Frizzled and LRP6 coreceptors, the Wnt ligand is rapidly endocytosed into early signalosomes (Blitzer and Nusse, 2006) and then into late endosomal multivesicular bodies (MVBs) (Taelman et al., 2010; Vinyoles et al., 2014). The activated receptor complex binds cytosolic proteins such as Glycogen Synthase Kinase 3 (GSK3) and Axin1, which become sequestered from the cytosol inside intraluminal vesicles of MVBs via microautophagy (Dobrowolski et al., 2012; Vinyoles et al., 2014). For this reason, canonical Wnt signaling requires the function of the endosomal sorting complexes required for transport (ESCRT) machinery (Taelman et al., 2010; Ploper et al., 2015) to sequester GSK3 and Axin, causing stabilization of its substrate β-catenin in the cytosol. In the absence of Wnt, phosphorylation of proteins by GSK3 generates “phosphodegrons” recognized by E3 ubiquitin ligases such as β-TrCP, triggering polyubiquitination and degradation in proteasomes (Acebron et al., 2014).

GSK3 is a major cellular serine/threonine kinase with many substrates (Kim et al., 2009). In bioinformatic analyses, about 20% of human proteins were found to contain three or more consecutive GSK3 sites, raising the possibility that GSK3 phosphodegrons might regulate the degradation of many cellular proteins (Taelman et al., 2010). Previous studies using radioactive pulse-chase experiments and biosensors containing GSK3 sites showed that Wnt signaling, through the inhibition of GSK3, stabilizes many proteins in addition to β-catenin (Taelman et al., 2010; Kong et al., 2013; Acebron et al., 2014). Because Wnt signaling is highest at the G2/M phase of the cell cycle, the important proposal was made that the resulting Wnt-stabilization of proteins (Wnt/STOP) slows down GSK3-dependent protein degradation in preparation for cell division (Acebron et al., 2014).

Protein degradation takes place in two different cellular compartments, proteasomes and lysosomes, but whether a physiologically regulated crosstalk between these protein degradation pathways exists is unknown (Ciechanover, 2005; Park and Cuervo, 2013). Lysosomal catabolism includes membrane proteins and endocytosed materials, as well as cytoplasmic domains enveloped by a double membrane via macroautophagy. Cytosolic proteins containing the Hsc70 recognition sequence KFERQ-like can also enter the lysosome through chaperone-mediated autophagy (CMA) (Park and Cuervo, 2013). In addition, proteins can enter the lysosome through the process known as microautophagy in which cytosol becomes entrapped inside the intraluminal vesicles of MVBs. Recently, it has been shown that some degree of selectivity exists in late endosome microautophagy for proteins containing KFERQ-like sequences (designated endosomal microautophagy or eMI) (Sahu et al., 2011). CMA and eMI do not involve ubiquitination, whereas proteasomal degradation and lysosomal targeting of cellular membrane proteins require ubiquitination.

There are seven lysines in ubiquitin, all of which can participate in the formation of polyubiquitin (polyUb) chains by forming an isopeptide bond between the hydroxyl group of Gly76 of a donor ubiquitin and the ε-amine of one of the lysine residues of an acceptor ubiquitin (Komander and Rape, 2012). It is thought...
that K48-linked polyubiquitin chains target proteins to proteasomal degradation, whereas monoubiquitination or Lys63-linked polyubiquitination target membrane proteins to lysosomal degradation (Nathan et al., 2013). Ubiquitin molecules are recycled back by proteasome-associated deubiquitinating enzymes (Lee et al., 2011), and proteins targeted for endolysosomal degradation are thought to be deubiquitinated before incorporation into MVBs (Piper and Katzmann, 2007). Thus, ubiquitin recycling plays an important role in regulating cellular homeostasis.

The starting point for this study was an unexpected observation: cultured mammalian cells treated with Wnt conditioned medium or purified Wnt3a protein showed an increase in levels of polyubiquitinated proteins in western blots of total cell extracts. This accumulation was striking in the first few hours of Wnt treatment but was lost at later stages. This increase in polyubiquitinated proteins was counterintuitive because, since Wnt inhibits GSK3 and consequently phosphodegron formation, the expectation was that total levels of polyUb proteins would be decreased, in accordance with the stabilization of cellular proteins in pulse-chase experiments (Taelman et al., 2010) and the decrease in polyubiquitination reported for a multitude of individual GSK3 target proteins during Wnt/STOP signaling (Acebron et al., 2014).

We found that Wnt caused accumulation of polyubiquitinated proteins in the MVB/lysosomal protein degradation pathway, and that their relocation to membrane vesicles required the ESCRT machinery components HRS/Vps27 (Hepatocyte growth factor Regulated tyrosine-kinase Substrate) and Vps4 (Vacuolar protein sorting 4), as well as GSK3 activity. Surprisingly, polyubiquitination via Lys48 was required and sufficient for Wnt-induced translocation of polyubiquitin into MVBs, while the other six lysines in ubiquitin were dispensable. We showed that three soluble GSK3 substrate proteins, Smad1, Smad4, and β-catenin became translocated to MVBs together with K48-linked polyUb during Wnt signaling by ESCRT-dependent microautophagy. Sequestration of K48-linked polyubiquitin proteins in endosomal organelles was accomplished by a transient decrease in free mono-ubiquitin, which may contribute to the stabilization of proteins during the first few hours of Wnt/STOP. We conclude that Wnt signaling causes a switch from proteasomal to lysosomal degradation of K48-linked polyubiquitinated GSK3 protein substrates.

RESULTS AND DISCUSSION

Wnt Signaling Induces Accumulation of PolyUb Proteins in MVB/Lysosomes

Unexpectedly, we observed that HEK293T cells treated with Wnt3a for 4 hr displayed an increase in total polyUb content in western blots using a monoclonal antibody (called FK1) that recognizes all possible forms of polyubiquitin chains (Figure 1A). The accumulation of polyUb was most striking between 30 and 120 min after addition of purified Wnt3a protein (Figure 1B). Use of a K48-polyUb-specific monoclonal antibody showed that K48-linked polyubiquitinated proteins, which are normally rapidly degraded in proteasomes, accumulated after 1 hr of treatment of HEK293T cells with Wnt3a conditioned medium (Figures 1C, S1A, and S1B). This accumulation was transient, decreasing after 24 hr of treatment, and becoming undetectable by 36 hr (Figure 1C). Accumulation of K48-linked polyubiquitinated proteins was not caused by proteasomal inhibition, as indicated by biosensor reporters of proteasomal activity, which were not affected by Wnt treatment (Figures S1C and S1D).

Given that Wnt signaling is intimately related to endosomal trafficking, we next investigated whether the transient accumulation of K48-linked polyUb led to protein degradation in the lysosomal pathway. Chloroquine (CQ) is a weak base that causes lysosome alkalization and inhibition of acidic hydrolases downstream of MVB formation (Dobrowolski et al., 2012). In the presence of Wnt3a, CQ treatment resulted in a robust accumulation of K48-linked polyUb after 6 hr (Figure 1D, cf. lanes 2–4). This indicates that the initial acute accumulation of polyUb is followed by degradation in the lysosomal pathway. Thus, Lys48-linked polyUb proteins that accumulate after Wnt signaling become CQ-sensitive lysosomal proteolytic substrates rather than proteasomal targets as is their normal fate.

Then, using immunolocalization, we examined the cellular compartment in which Wnt-induced polyUb accumulation took place. Wnt3a treatment caused a significant relocation of K48-linked polyUb antigen to vesicle-like puncta in untransfected HeLa cells (Figures 1E–1E”), suggesting translocation into endolysosomal vesicles. When cells were transfected with constitutively active LRP6 (CA-LRP6) (generated by deletion of its extracellular domain; Tamaik et al., 2004), total polyubiquitinated proteins labeled by the FK1 antibody colocalized with a phospho-LRP6 (Ser1490) antibody that marks Wnt signaling. Figure 1. Lys48-Linked Polyubiquitinated Proteins Accumulate in MVBs/Lysosomes during Wnt Signaling

(A) Total polyUb (FK1 antibody) accumulation in HEK293T cells after treatment with control (−) or Wnt3a (+) conditioned medium (CM) for 4 hr.

(B) Recombinant Wnt3a protein (80 ng/ml) causes rapid accumulation of polyUb.

(C) Wnt3a-induced K48-polyUb accumulation is transient.

(D) The lysosomal inhibitor Chloroquine (CQ) causes stabilization of Wnt3a-induced K48-polyUb in HEK293T cells (6-hr treatment).

(E–E”) K48-polyUb antigens localize to vesicular puncta (arrows) in HeLa cells treated with Wnt3a CM for 4 hr. Scale bars represent 10 μm. Error bars are SDs. (F–F”) Total polyUb accumulates in CA-LRP6 vesicles marked by p-LRP6 (arrows); arrowhead indicates a non-transfected cell.

(G–J”) K48-polyUb accumulates in endolysosomes enlarged by CQ treatment in L-cells treated with Wnt3a purified protein (arrows). Scale bars represent 10 μm.

(K–K”) Higher-power view of a large CQ-induced MVB (hatched line) showing K48-polyUb accumulation in puncta inside the MVB limiting membrane (arrowhead). Scale bar represents 2 μm.

(L) Accumulation of HA-Ub in CA-LRP6 vesicles requires HRS and Axin. HeLa cells were transfected with HA-ubiquitin (HA-Ub), CA-LRP6 and the indicated siRNAs. Error bars are SDs.

(M–Q) The MVB marker Vps4 colocalizes with polyUb accumulation in CA-LRP6 vesicles (arrows) in an HRS, Axin, and Vps4-dependent manner. Scale bars represent 10 μm.

See also Figure S1.
GSK3 Is Required for Wnt-Induced MVB Localization of Soluble PolyUb Proteins

Given that Wnt regulates GSK3, we asked whether this enzyme was required for polyUb protein translocation. We found that Wnt-induced polyUb accumulation was blocked by treatment with the GSK3 inhibitors BIO, SB-216763, and CHIR99021 (Figure 2A, cf. lanes 1–3 to 4–6; Figure S2A). This suggested that the polyUb proteins that accumulate after Wnt treatment may contain GSK3 phosphodegrons recognized by E3 ubiquitin ligases that mediate polyubiquitination. Treatment with the proteasome inhibitor MG132 indicated that the Wnt-stabilized polyUb represented only a fraction of all proteins degraded in the proteasome (Figure 2A, lanes 7 and 8). Accumulation of total polyUb after MG132 proteasomal inhibition was decreased by 90% (Figure 2A, lanes 7 and 8). This indicates that CA-LRP6 MVBs are able to incorporate cytosolic proteins via microautophagy.

To confirm that polyubiquitination of individual proteins was increased by Wnt, we carried out biochemical assays in HEK293T cells cotransfected with HA-ubiquitin, the E3 ligases β-TrCP or Smurf1, and epitope-tagged β-catenin, Smad1 or Smad4. Importantly, cells were not treated with proteasome inhibitors (a common practice in polyubiquitinylation assays that prevents rapid degradation in the proteasome, e.g., Acebron et al., 2014), and lysed in the presence of Triton X-100 and 1% SDS to solubilize MVBs. Immunoprecipitation studies showed that the polyubiquitinated fractions of GFP-β-catenin (Figure 2K), FLAG-Smad1 (Figure 2L), and FLAG-Smad4 (Figure 2M) were indeed stabilized by Wnt3a treatment for 2–4 hr in the absence of proteasome inhibitors. However, it is also known that these three proteins are normally stabilized by Wnt (MacDonald et al., 2009; Fuentealba et al., 2007; Demagny et al., 2014). This suggests that the polyubiquitinated fraction that accumulates after Wnt treatment may represent a small part of the total protein. In the case of β-catenin, the GSK3-phosphorylated form remains bound to the destruction complex (Li et al., 2012) and sequestered in MVBs (Taelman et al., 2010), while newly made protein accumulates in the cytoplasm and nucleus.

The requirement of GSK3 activity for accumulation of Wnt-induced polyubiquitinated proteins and the Wnt-induced accumulation of polyubiquitinated Smad4, Smad1, and β-catenin were counterintuitive because Wnt is expected to inhibit GSK3 phosphodegrons. Taken together, our experiments suggest that acute Wnt-induced microautophagy of polyubiquitinated proteins might be confined to protein molecules already phosphorylated by GSK3, while the bulk of GSK3-unphosphorylated proteins remaining in the cytosol, or newly made, are stabilized via Wnt/STOP as GSK3 is translocated into MVBs and depleted from the cytosol (Taelman et al., 2010; Vinyoles et al., 2014). In agreement with the Wnt/STOP model of Acebron et al. (2014), cell size was increased after 24- or 48-hr treatment with Wnt3a in HeLa cells (Figures 2N–2P), C32 melanoma cells, and 293T cells (Figures S3A–S3C), and this cell size increase required HRS (Figure S3D) and Axin (Figures S3H–S3J).

Lys48-Linked Polyubiquitin Is Required and Sufficient for MVB Relocalization of Ub during Wnt Signaling

We next investigated whether a specific type of polyUb linkage was associated with MVB relocalization using ubiquitin point mutants (Lim et al., 2005). Overexpressed HA-Ub colocalized with phospho-LRP6 in CA-LRP6 vesicles (Figures 3A–3B, arrows). HA-Ub with all seven Lys mutated to Arg cannot form any type of polyubiquitin chains but can still mono-ubiquitinate substrates (Lim et al., 2005). This mutant, designated ubiquitin K0, did not localize in MVBs (Figure 3D). Remarkably, a single mutation of Lys48 to Arg (ubiquitin K48R), which leaves the other six Lys residues intact, was sufficient to prevent MVB localization (Figure 3F). Conversely, an ubiquitin mutant with all lysines mutated to arginine except for Lys48 (ubiquitin K0→K48) accumulated in pLRP6 vesicles (Figure 3H, arrows). Thus, an intact Lys48 residue of ubiquitin was required and sufficient for its translocation into CA-LRP6-positive vesicles (Figure 3I). Moreover, Wnt treatment increased protection of endogenous
Figure 2. GSK3 Is Required for Wnt-Induced Accumulation of PolyUb Proteins and the β-Catenin, Smad1, and Smad4 Phosphodegrons in MVBs
(A) The GSK3 inhibitor BIO blocked Wnt3a-induced accumulation of polyUb proteins in HEK293T cells.
(B) Inhibiting GSK3 decreased MG132-induced accumulation of total polyubiquitinated proteins, indicating that GSK3-induced phosphodegrons are responsible for a sizable fraction of protein catabolism in HEK293T cells.
(C–D0) Phospho-β-cateninGSK3 colocalized with RFP-GSK3 (arrows) in vesicles induced by CA-LRP6.
(E–F0) Phospho-Smad1GSK3 colocalized with RFP-GSK3 in CA-LRP6 MVBs. Scale bar represents 10 μm.
(G–H0) Phospho-Smad4GSK3 accumulated in the same vesicles as endogenous Axin (arrows) in CA-LRP6-transfected HeLa cells.
(I) Xenopus animal cap explants injected with EGFP-CA-LRP6 mRNA and immunostained for endogenous pSmad1GSK3 showing colocalization in vesicles. The dashed line delineates injected and un.injected cells. Scale bar represents 20 μm.
(J) Endogenous phospho-Smad4GSK3 localizes to EGFP-CA-LRP6-induced vesicles in dissociated Xenopus animal cap cells. Scale bar represents 2 μm.
(K–M) Wnt3a treatment increased HA-Ub levels bound to GFP-β-catenin, FLAG-Smad1, or FLAG-Smad4 after immunoprecipitation. HEK293T cells were cotransfected with HA-ubiquitin and β-TrCP or Smurfl.
(N–P) Forward scatter area (FSC-A) flow cytometry showing that HeLa cells increase in cell size via Wnt/STOP during 48 hr of treatment.
See also Figure S2.
polyubiquitin from Proteinase K digestion in HEK293T cells permeabilized with Digitonin (Figure 3J, lanes 3–6; Figure S3K), confirming its relocalization inside membrane-bounded organelles. In addition, endogenous K48-linked polyUb and microinjected HA-Ub accumulated in CA-LRP6 MVBs in Xenopus animal cap cells (Figures 3K–3L; Figures S3L–M). We conclude that Wnt signaling causes ubiquitin redistribution from the cytosol to the endolysosomal pathway specifically through the translocation of Lys48-linked polyUb proteins, which in the absence of Wnt would be targeted to proteasomes.

Wnt Causes a Transient Reduction in Free Mono-Ubiquitin

Ubiquitin homeostasis relies on the recycling of free ubiquitin from polyubiquitin chains upon proteolysis in both proteasomes and lysosomes (Lee et al., 2011; Piper and Katzmann, 2007). The finding that K48-linked polyUb proteins translocated to the lysosomal pathway during Wnt signaling raised an interesting question. Could Wnt regulate ubiquitin homeostasis by redistributing cellular ubiquitin from the cytosol to degradation in the endolysosomal pathway? We found that Wnt treatment for 30–120 min reduced free ubiquitin while simultaneously increasing polyUb proteins (Figures 4A and 4A). Using recombinant ubiquitin protein as a quantification standard, we found that free ubiquitin levels decreased by 70% after 60–120 min of Wnt treatment (Figures 4B–4B). HRS or Axin small interfering RNAs (siRNAs) prevented this decrease, indicating that MVB formation was necessary for this Wnt-induced acute reduction of free mono-ubiquitin in the cytosol (Figure 4C) and polyUb accumulation in the lysosomal compartment (Figure S3N). However, the reduction in free ubiquitin levels was restored to normal levels after 3 hr of Wnt treatment (Figures 4D and 4E), perhaps through the...
feedback regulation of ubiquitin gene expression (Kimura and Tanaka, 2010).

The transient decrease in free ubiquitin levels should enhance Wnt responses during the first few hours of Wnt/STOP signaling, since less ubiquitin is available to target proteins to proteasomes. The sequestration and degradation of K48-linked polyUb in the lysosomal compartment would help ensure a rapid switch from no Wnt to Wnt signaling status. Canonical Wnt signaling is maintained for many hours, so the marked decrease in free ubiquitin would only contribute to Wnt signaling during the initial hours, with GSK3 sequestration mediating sustained Wnt signaling at later stages, leading to the Wnt/STOP increase in cell size. Experiments involving Tet-inducible ubiquitin over- or under-expression indicate that transient ubiquitin depletion may be relevant to Wnt signaling (Figure S4).

**Conclusions**

This study has revealed a novel switch from proteasomal to lysosomal protein degradation governed by Wnt signaling. Although Wnt inhibits the proteasomal degradation of many proteins via the Wnt/STOP mechanism in which cytosolic proteins remain unphosphorylated by GSK3 (Acebron et al., 2014), we found that some proteins polyubiquitinated by K48-linked chains translocated together with GSK3 and Axin into MVBs. Inhibition of lysosomal activity with Chloroquine indicated that these proteins, which would normally be degraded in the proteasome, are instead degraded in the endolysosomal pathway. Lys63-polyUb or mono-ubiquitin, which target proteins to MVBs, are recycled back to the cytosol before proteins enter MVBs (Swarminathan et al., 1999; Piper and Katzmann, 2007). However, in the presence of Wnt, K48-linked polyUb is not removed from...
its targets before entering lysosomes for degradation, leading to a strong although transient reduction in free ubiquitin levels. The molecular mechanism by which soluble β-catenin, Smad1, and Smad4 are translocated from the cytosol to MVBs by microautophagy into Wnt endosomes remains a matter for future studies. However, the results so far show that it requires GSK3 activity, Axin, and the ESCRT machinery. In one speculative scenario, one could imagine that GSK3 might remain bound to its substrates and is carried by Axin bound to oligomerized Dishevelled (MacDonald et al., 2009) attached to the Wnt receptor complex in intraluminal vesicles. The three proteins studied here lack recognizable KFERQ-like motifs, indicating that their vesicular relocalization is not mediated by the previously described CMA and eMI mechanisms (Sahu et al., 2011). We conclude that a crosstalk between the proteasomal and lysosomal protein degradation pathways exists, and that it is regulated by Wnt signaling.

EXPERIMENTAL PROCEDURES

Wnt Protein and Antibodies

Purified recombinant Wnt3a protein (PeproTech #315-20) or Wnt3a conditioned medium were used in these experiments, with similar results. Control and Wnt3a conditioned medium were generated from control L cells (ATCC #CRL-2648) and L cells stably expressing mouse Wnt3a (ATCC #CRL-2647). Primary antibodies used were anti-polyUb (FK1) (Enzo #BML-PW8805), anti-ubiquitin (Santa Cruz Biotechnology #9133), anti-K48-PolyUb (Millipore #05-1307), anti-β-catenin (Santa Cruz #7199), anti-GAPDH (Cell Signaling Technology #2118), anti-p-LRP6 S1490 (Cell Signaling Technology #2568), anti-α-tubulin (Calbiochem #CP06), anti-HA (Sigma-Aldrich #H6908 and #05-1307), anti-β-catenin (Santa Cruz #1722), anti-p-Smad1 GSK3 (Demagny et al., 2014), anti-p-Smad4 GSK3 (Demagny et al., 2014), anti-p-Smad4 (Bengtson, 2007), and anti-p-Smad4ΔC (Demagny et al., 2014).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.04.048.

AUTHOR CONTRIBUTIONS

H.K. and E.M.D.R. designed research. H.K., P.V., J.H., and D.P. performed experiments and analyzed the data. H.K. and E.M.D.R. wrote the manuscript.

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REFERENCES


Wnt Signaling Translocates Lys48-Linked Polyubiquitinated Proteins to the Lysosomal Pathway

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Figure S1, Related to Figure 1: Wnt Signaling Does not Inhibit Proteasomal Activity, and Causes Accumulation of Polyubiquitinated Proteins in MVBs
(A) Wnt3a increased the amount of total polyUb proteins detected by FK1 antibody in western blot. Western blots from four independent experiments were quantified using ImageJ and graphed. *** indicates p<0.001. Error bars are standard deviations. 293T cells were treated with control (-) or Wnt3a (+) conditioned medium for 4 hours and subjected to western blot. A representative western blot is shown in Figure 1A.

(B) Wnt3a caused accumulation of K48-linked polyUb proteins. 293T cells were treated with control (-) or Wnt3a (+) conditioned medium for 1 hour and K48-linked polyUb levels from cell lysates were analyzed by western blot. α-Tubulin levels were used for normalization. ImageJ was used to quantify western blots from three independent experiments. * indicates p<0.05. Error bars are standard deviations. A representative western blot is shown in Figure 1C, lanes 1-2.

(C) Purified Wnt3a did not inhibit proteasomal activity assayed by Ub-G76V-GFP proteasomal reporter. Ub-G76V-GFP contains an uncleavable ubiquitin moiety fused to the N-terminus of GFP, generating an ubiquitin fusion degradation signal. This reporter is known to be rapidly degraded by the proteasome (Dantuma et al., 2000). HeLa cells were transfected with Ub-G76V-GFP and treated 24 hours later with either proteasomal inhibitor MG132 or recombinant Wnt3a protein for 4 hours. Note that the Ub-G76V-fused GFP proteasomal reporter was not stabilized by Wnt3a protein. Scale bars 50 µm.

(D) Wnt3a did not inhibit proteasomal activity assayed by the GFPµ proteasomal reporter. This reporter protein has a destabilizing sequence, called the CL-1 degron, fused to the C-terminus of GFP and is known to be rapidly degraded by the ubiquitin-proteasome system in an ubiquitin-dependent manner (Bence et al., 2001). HEK293T cells were transfected with GFPµ and treated 36 hours later with the lysosomal protease inhibitor Leupeptin, the proteasomal inhibitor MG132, or Wnt3a protein for 2 hours. Note that neither Wnt3a treatment nor lysosomal inhibition resulted in proteasomal inhibition.

(E-F) Endogenous K48-linked polyUb proteins and GSK3 colocalized in CA-LRP6 MVBs (arrows). HeLa cells were transfected with either CA-LRP6 or empty vector (pCS2) and immunostained for endogenous K48-linked polyUb and GSK3 after 36 hours of transfection. CA-LRP6 caused sequestration of GSK3 inside MVBs (Taelman et al., 2010). We note that nuclear K48-linked polyUb staining is lower in control than in CA-LRP6 transfected cells. This suggests the possibility that Wnt-induced relocalization of K48-linked polyUb to MVBs may lead to the deubiquitination of nuclear K48-linked polyUb proteins in order to maintain cellular ubiquitin homeostasis. Scale bars 10 µm.

(G-H) Endogenous K48-linked polyUb proteins were relocalized to Lamp1-positive lysosomes by CA-LRP6 (arrows). HeLa cells were transfected with either CA-LRP6 or pCS2 and immunostained for endogenous K48-linked polyUb and Lamp1 after 36 hours of transfection. Scale bars 10 µm.
(I) Axin siRNA efficiently reduced Axin protein levels in western blots. 293T cells were transfected with either control siRNA (si-Con) or Axin siRNA (si-Axin) and treated with control (-) or Wnt3a (+) conditioned medium for 2 hours. Anti-Axin1 antibody (Cell Signaling #2087) was used for detecting Axin1.

(J) Axin siRNA caused a depletion of Axin immunostaining in HeLa cells. HeLa cells were transfected with siRNAs and plasmids as indicated and stained with anti-Axin1 antibody (R&D Systems #AF3287). Note that CA-LRP6 induced accumulation of Axin in vesicular structures and that Axin siRNA effectively depleted Axin levels. Scale bars 10 µm.

(K) HRS siRNA caused a significant reduction of HRS immunostaining in HeLa cells. HeLa cells were transfected either with control siRNA or with HRS siRNA as indicated and stained with anti-HRS antibody (Santa Cruz #30221). Scale bars 10 µm.

(L-O”) HeLa cells were transfected with HA-ubiquitin (HA-Ub), CA-LRP6 and siRNAs and immunostained for HA and Axin after 36 hours of DNA transfection; results are quantified in Figure 1L. In cells transfected with the pCS2 empty vector HA-ubiquitin was diffusely expressed throughout the cell, with stronger nuclear than cytoplasmic localization. CA-LRP6 induced vesicular relocalization of HA-ubiquitin into the same vesicles in which endogenous Axin protein was sequestered (arrows), as well as a decrease in HA-ubiquitin nuclear localization. Knockdown of HRS, which prevents MVB formation, prevented the sequestration of both HA-ubiquitin and Axin by CA-LRP6. Axin knockdown with siRNA also inhibited vesicular relocalization of HA-ubiquitin. Scale bars 10 µm.

(P-S”) CA-LRP6 induced relocalization of HA-ubiquitin to acidic vesicles marked by LysoTracker. HeLa cells were transfected with HA-ubiquitin, CA-LRP6 and siRNAs as indicated and stained with LysoTracker Red DND-99 (1:1000, Invitrogen, #L7528) before fixation and immunostaining for HA-ubiquitin. These acidic vesicles represent late endosomal MVBs and lysosomes. Knockdown of HRS (si-HRS) or Axin (si-Axin) prevented the sequestration of HA-ubiquitin into acidic vesicles. Scale bars 10 µm.

(T-W’) HRS and Axin are required for the Wnt-induced relocalization of HA-ubiquitin and the accumulation of polyubiquitin. HeLa cells were transfected with HA-ubiquitin and siRNAs as indicated. After 36 hours of transfection, cells were treated either with control or Wnt3a conditioned medium for 2 hours. Wnt3a caused vesicular relocalization of HA-ubiquitin and this was blocked by HRS siRNA and Axin siRNA. Additionally, total polyubiquitinated proteins (polyUb) stained by FK1 antibody were increased by Wnt3a, and this increase was suppressed by HRS siRNA and Axin siRNA.

(X) Histogram quantifying number of cells with vesicular HA-ubiquitin localization in Figure S1T-W.
Figure S2, Related to Figure 2: The Accumulation of Polyubiquitinated Proteins Induced by Wnt Treatment Requires GSK3 Activity; HRS is Required for CA-LRP6-induced MVB Relocalization of GSK3-phosphorylated Smad4

(A) Total PolyUb (FK1 antibody) accumulation induced by Wnt was inhibited by the GSK3 inhibitors BIO, SB-216763 and CHIR99021. This result indicates that the inhibitory effect of BIO on GSK3 in Figure 2A is specific to GSK3. 293T cells were treated with control (-) or Wnt3a (+) conditioned medium together with the indicated GSK3 inhibitors for 1 hour.

(B-B") CA-LRP6 caused accumulation of pSmad4GSK3 in LysoTracker-positive acidic vesicles. Together with Figure 2G-H", these results demonstrate that Wnt signaling relocates GSK3 phosphorylated Smad4 to GSK3 and Axin positive vesicles which are acidic late endolysosomes. Yellow arrowhead indicates a CA-LRP6 non-transfected cell (lacking pSmad4GSK3 accumulation) while the white arrowhead marks a CA-LRP6 transfected cell. White arrows show colocalization of pSmad4GSK3 with LysoTracker staining. Scale bars are 10 µm. We note that Smad4, as well as β-Catenin and Smad1, are devoid of any KFERQ-like motifs that mediate Chaperone Mediated Autophagy (CMA) and endosomal Microautophagy (eMI) (Majeski and Dice, 2004; Santambrogio and Cuervo, 2011), as ascertained using the ScanProsite tool (http://prosite.expasy.org/scanprosite/).

(C-D") HRS was required for the relocalization of phospho-Smad4GSK3 to EGFP-CA-LRP6 MVBs in Xenopus animal caps. Xenopus animal cap explants injected with EGFP-CA-LRP6 mRNA together with control morpholino (con-MO) or with HRS morpholino (HRS-MO) were immunostained for pSmad4GSK3. The dashed line delineates injected (Inj.) and uninjected (Uninj.) cells. Scale bars 20 µm.
Figure S3, Related to Figures 2 and 3: HRS and Axin are Required for the Wnt-induced Cell Size Increase, Ubiquitin Relocalization and PolyUb Accumulation

(A) Wnt increased size of C32 melanoma cells. C32 cells were treated with control buffer or purified Wnt3a protein for 24 hours, fixed and analyzed by flow cytometry for forward scatter (FSC-A) using LSR2 flow cytometer (BD Biosciences).
(B and C) Wnt/STOP increased the size of 293T cells. The Wnt-induced increase of cell size was observed both in total cells without G1 gating and in G1-gated cells. 293T cells were treated with control or Wnt3a conditioned medium for 48 hours, fixed and stained with Hoechst 33342 in order to gate cells in G1.

(D) HRS was required for the Wnt-induced cell size increase. 293T cells were transfected with control or HRS siRNAs and 24 hours after transfection, treated with Wnt3a conditioned medium for 48 hours. Cells were fixed and stained with Hoechst 33342 to gate G1 cells. Wnt increased 6.4% (not shown), and HRS siRNA decreased Wnt-increased cell size by 8.3%.

(E-F) Lysosomal inhibition by Chloroquine (CQ) augmented the Wnt-induced cell size increase. 293T cells were treated with control or Wnt3a conditioned medium together with Chloroquine (CQ), as indicated. Hoechst 33342 staining was used to gate for cells in the G1 phase of the cell cycle. CQ increased cell size by 7.4% in the absence of Wnt3a while it increased size by 13.7% in the presence of Wnt3a. These results demonstrate that lysosomal inhibition downstream of intraluminal MVB vesicle formation increases Wnt signaling, confirming our previous findings (Dobrowolski et al., 2012).

(G) Proteasomal inhibition increased cell size in HeLa cells. HeLa cells were treated with control ethanol or MG132 for 24 hours, fixed and analyzed by flow cytometry for cell size using forward scatter (FSC-A). This result suggests that the cell size increase by Wnt/STOP signaling is likely mediated by the stabilization of proteins that are normally proteasomal targets.

(H) Knockdown of Axin protein levels by a Doxycycline-induced shRNA targeting Axin1. 293T cells stably expressing Tet-inducible shAxin (shAxin-Tet-293T) were treated with Doxycycline for 48 hours and further treated with control or Wnt3a conditioned medium for the indicated times.

(I and J) Axin is required for the Wnt-induced increase of cell size. A stable 293T cell line expressing Tet-inducible shRNA against Axin1 was generated (shAxin-Tet-293T) (see supplemental experimental procedures for detail). shAxin-Tet-293T cells were treated with Doxycycline for 48 hours then treated with Wnt3a conditioned medium for an additional 24 hours. Without doxycycline, Wnt signaling increased cell size by 6.8%. Axin knockdown by doxycycline-induced expression of shAxin abolished the Wnt-induced size increase (0.08%). These results demonstrate that Axin is required for Wnt/STOP signaling.

(K) The mitochondrial matrix protein HSP60 was effectively protected against proteinase K digestion in digitonin-permeabilized cells, while α-Tubulin, a cytoplasmic protein, was not protected. However, HSP60 was digested in the presence of Triton X-100. This experiment serves as a positive control for the proteinase K protection assay shown in Figure 3J.
(L-M”) HRS is required for the relocalization of HA-Ub to CA-LRP6 MVBs in *Xenopus* animal caps. *HA-Ub* and *EGFP-CA-LRP6* mRNAs together with control morpholino (co-MO) or HRS morpholino (HRS-MO) were introduced by microinjection into *Xenopus* animal blastomeres at 4-8 cell stage. Note that HA-Ub sequestration in CA-LRP6 MVBs was blocked by HRS-MO. Scale bars 20 µm.

(N) Wnt-induced total PolyUb (FK1 antibody) accumulation is inhibited by HRS depletion (compare lanes 1-3 to 4-6) or by Axin depletion (compare lanes 1-3 to 7-9). HEK293T cells were transfected with control siRNA, HRS siRNA or Axin siRNA for 2 days, and Wnt3a protein was added for 30 or 60 min. This result correlates with the observation in Figure 4C that inhibiting MVB formation with si-HRS or Axin knockdown prevents the transient decrease in free ubiquitin levels caused by Wnt3a treatment. The decrease in free ubiquitin could contribute to the stabilization of proteins during Wnt/STOP.
Figure S4, Related to Figure 4: Ubiquitin Overexpression Decreased, and Ubiquitin Depletion Increased Wnt Signaling

This figure tests the hypothesis that the decrease in free mono-ubiquitin is relevant for the economy of Wnt signal transduction. While a decrease or increase by 50% may seem unimpressive, please keep in mind that Wnt reporter assays using luciferase require 8-10 hours, while the transient decrease in free ubiquitin lasts for only 2 hours after Wnt3a addition. We also note that the shRNA we designed targets only 3 of the 4 ubiquitin genes in humans.

(A) Ubiquitin overexpression partially inhibited Wnt signaling. 293T cells were transduced with a Doxycycline-inducible HA-Ub and with Wnt reporters (BAR and tk-Renilla, gift from Dr. Randall Moon) lentiviruses to generate stable cell lines (HAUb-Tet-293T BAR/Renilla). Cells were treated with Doxycycline for 24 hours and then with conditioned...
medium (either control or Wnt3a) for 10 hours before luciferase activity was measured and analyzed. Statistical significance was determined using t-test; p<0.001. Error bars are standard deviations.

(B) Mono-HA-ubiquitin is efficiently induced by doxycycline in HAUb-Tet-293T cells. A stable cell line harboring Tet-inducible HA-ubiquitin (HAUb-Tet-293T) was generated. Cells were treated with Doxycycline for 24 hours and further treated with Wnt3a conditioned medium for the indicated time. Western blot of mono-ubiquitin shows efficient induction of HA-ubiquitin by the addition of doxycycline. Note also that β-Catenin stabilization by Wnt signaling was inhibited by ubiquitin overexpression (compare lanes 1-3 to 4-6). This lends support to the idea that the transient decrease in mono-ubiquitin is involved in canonical Wnt signaling.

(C) Partial ubiquitin depletion increased Wnt signaling. A 293T cell line stably expressing Doxycycline-inducible shUb (targeting UBA, UBB and UBC but not Rps27a) and a Wnt reporter (shUb-Tet-293T BAR/Renilla) was generated using lentiviruses. Cells were treated first with Doxycycline for 2 days and then with control or Wnt3a conditioned medium for an additional 8 hours before luciferase activity was measured and analyzed. Statistical significance was determined by t-test; p<0.01. Error bars are standard deviations.

(D) Knockdown of mono-ubiquitin levels by shRNA targeting three ubiquitin genes. HeLa cells were transduced with a lentivirus harboring doxycycline-inducible shRNA targeting human ubiquitin genes (UBA, UBB and UBC but not Rps27a) to generate a stable cell line (shUb-Tet-HeLa). Cells were first treated with Doxycycline for 2 days and then with Wnt conditioned medium for the indicated time. Note that 1-2 hours of Wnt signaling caused a reduction of mono-ubiquitin and that shUb also reduced the level of mono-ubiquitin (compare lanes 1-3 to 4-6).

(E) Knockdown of ubiquitin by shRNA decreased K48-linked polyUb immunostaining in cells. HeLa cells harboring Tet-inducible shUb (shUb-Tet-HeLa) were treated with doxycycline for 48 hours before fixation and staining with anti-K48-linked polyUb antibody. Scale bars 10 µm.
Supplemental Experimental Procedures

Proteins and Chemicals

Recombinant human ubiquitin protein from Boston Biochem (#U-100H) was used as a standard for measuring mono-ubiquitin levels. The proteasomal inhibitor, MG132 (Sigma #C2211) was dissolved in DMSO as a 10 mM stock and used at 10 µM. A 10 mM solution of GSK3 inhibitor IX (BIO, Calbiochem #361550) was used at 10 µM. Other GSK3 inhibitors, SB-216763 (Sigma, #S3442) and CHIR99021 (abcam, #ab120890) were dissolved in DMSO as 10 mM stocks and used at 10 µM. The lysosomal inhibitor Chloroquine diphosphate (Sigma #C6628) was dissolved in water as a 100 mM stock solution and diluted 1:4000 to 25 µM. Leupeptin hemisulfate (Sigma #L2884), a lysosomal protease inhibitor, was dissolved in water as a 10 mM stock and used at 10 µM.

Plasmids and siRNAs

pRK5-HA-ubiquitin WT, K48R, K0 and K48 (Addgene plasmids # 17608, 17604, 17603 and 17605, respectively) were gifts from Dr. T. M. Dawson (Lim et al., 2005). The proteasomal activity biosensor Ub-G76V-GFP was from N. Dantuma (Addgene plasmid # 11941) (Dantuma et al., 2000). The proteasomal reporter GFPµ was a gift from Dr. R. Kopito (Bence et al., 2001). The CA-LRP6 construct was kindly provided by Dr. X. He (Tamai et al., 2004), Vps4-EQ construct by Dr. P. Woodman (Bishop and Woodman, 2000), and GFP-β-Catenin by Dr. R. Moon. EGFP-Vps4, EGFP-Vps4EQ, RFP-GSK3β, GFP-β-Catenin and EGFP-CA-LRP6 have been described (Taelman et al., 2010) and are available from Addgene. Constructs for Flag-Smad1, xSmurf1, Flag-Smad4 and β-TrCP (Fuentenalba et al., 2007; Zhu et al., 1999; Demagny et al., 2014) have been described. pEN_TTmcs, pEN_TTmiRc2, pSLIK-zeo and pSLIK-neo (Addgene plasmids # 25755, 25752, 25735, 25735) were from Dr. Iain D.C. Fraser. Short hairpin RNA (shRNA) targeting human Axin1 was cloned into pSLIK-zeo. Briefly, oligonucleotides for sh-Axin cloning were synthesized (ValueGene) as follows:

F:5’AGCGATTGTTATCAAGAATGTACTAGTAGCAGGACACAGATGACCATTTTGA

R:5’GGCAATTGTTATCAAGAATGTACTACATCTGTGGCTTCACTAGTACATTCTTGAT

The oligonucleotides were annealed and cloned into pEN_TTmiRc2 then recombined in pSLIK-zeo vector using GATEWAY® LR Clonase® II Enzyme mix (Invitrogen) according to the manufacturer’s protocol. Ubiquitin from pRK5-HA-Ubiquitin were PCR amplified and first subcloned into pEN_TTmcs and recombined into the pSLIK-zeo vector using
GATEWAY® LR Clonase® II Enzyme mix (Invitrogen). An shRNA that targets three of the four human ubiquitin genes, UBA52, UBB and UBC, designated sh-Ub was cloned into pSLIK-zeo or pSLIK-neo. Briefly, oligonucleotides for shRNA cloning were synthesized as follows:

F:5’AGCGAACTCTTTCTGGATGTTGTAGTCTAGTGAAGCCACAGATGTAGACTACAA CATCCAGAAAGAGGT3’;
R:5’GGCAGACTCTTTCTGGATGTTGTAGTCTACATCTGTGGCTTCACTAGACTACAA CATCCAGAAAGAGGT3’.

The oligonucleotides were annealed and cloned first into pEN_TTmiRc2 then recombined to pSLIK-zeo or pSLIK-neo vectors using GATEWAY® LR Clonase® II Enzyme mix (Invitrogen). The siRNAs used for knockdown experiments, si-Control (Thermo Scientific, #L-001810), si-HRS (Thermo Scientific, #L-016835) or si-Axin1 (Thermo Scientific, #L-009625) were as described previously (Taelman et al., 2010).

**Cell Culture and Transfection, Lentiviral Gene Transduction**

HEK293T, L-cells and HeLa cells were cultured in DMEM containing 10% FBS/Glutamine/Penicillin/Streptomycin. Plasmid DNAs (1 µg/well of 12-well plates) were transfected with BioT (Bioland Scientific #B01-01) 24 hours after plating cells. siRNAs (20 pmol/ml in 12-well plate) were transfected with Lipofectamine 2000 (Invitrogen #11668-027) using the reverse transfection protocol provided by the manufacturer. For experiments involving plasmid and siRNA transfection, cells were first transfected with siRNAs using reverse transfection and 24 hours later transfected with plasmids. Lentiviral supernatants were generated by the UCLA Vector Core (supported by CURE/P30DK04130) and used to establish stable cell lines. Briefly, 293T or HeLa cells were transduced with sh-Axin-pSLIK-zeo, HA-Ubiquitin-pSLIK-zeo or Sh-Ub-pSLIK-zeo (or –neo) containing lentiviral supernatants together with protamine sulfate. 24 hours after infection, cells were washed with fresh medium and selected for ~ 2 weeks with medium containing 0.2 mg/ml of Zeocin™ (Invitrogen) or 0.2 mg/ml of Geneticin (Invitrogen). Doxycycline (Sigma, #D9891) was used to induce expression of lentivirally transduced genes or shRNAs.

**Western Blots**

For Western blots cells were lysed with a buffer containing 20 mM HEPES, pH 7.2, 150 mM NaCl, 1% Triton X-100, 10 mM EDTA, pH 8.0, 1 mM DTT and 1 mM NaF, together with protease inhibitor cocktail tablet (1 tablet per 20 ml of lysis buffer) (Roche #4693132001). In some experiments, 8 mM of N-ethylnaleimide (Thermo Scientific #23030) was included in the lysis buffer. Nitrocellulose membrane blots were washed
with TBST (TBS containing 0.1% Tween-20), blocked with TBST containing 3% BSA for 1 hour, and incubated with primary antibodies overnight. Primary antibodies were all used at 1:1000 dilution except anti-ubiquitin (Santa Cruz #9133, 1:500). Membranes were then washed three times with TBST and incubated with secondary antibodies for two hours at room temperature. Secondary antibodies were from Li-Cor (IRDye 680 and IRDye 800, 1:10,000) for infrared imaging of proteins using the LiCor Odyssey system. For western blots of mono-ubiquitin, blotted membranes were placed between wet blotting papers and autoclaved for 5 minutes using liquid cycle as described previously (Stanke et al., 2005) before blocking with 3% BSA/TBST. For quantification of mono-ubiquitin, recombinant ubiquitin protein (5-200 ng) was loaded on SDS gels side-by-side with cell lysates, blotted, autoclaved and analyzed by western blot analysis as described above. Standard curves were generated using arbitrary units read by the Li-Cor Odyssey system and by the software embedded in Excel (Microsoft Corp.).

Statistics

Results were presented as mean ± standard deviation (S.D.). Student t-test was used to determine statistical significance using Microsoft Excel.

Immunofluorescence

For immunofluorescence L-cells or HeLa cells were grown on 12 mm round coverslips (Fisher Scientific #12-545-82) in 24-well plates. Coverslips were washed with absolute ethanol, transferred into wells, and rinsed at least three times with culture medium without serum before seeding cells. When using transfected cells, cells were first transfected in multi-well plates without coverslip and then seeded into coverslip-containing 24-well plates after 24 hours of transfection. Cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature, permeabilized with PBST (PBS containing 0.1% Triton X-100) and blocked with PBST containing 3% BSA or 5% donkey or goat serum for 1 hour before primary and secondary antibody incubation. Primary antibodies were incubated at 4°C overnight. After extensive washing with PBST, secondary antibodies were incubated for two hours at room temperature. All primary antibodies were used at 1:300 dilution. Secondary antibodies were from Jackson Laboratory (Alexa Fluor 488 or Cy3 conjugated anti-mouse IgG, anti-mouse IgM, anti-rabbit IgG or anti-goat IgG, 1:500). After extensive washes with PBST, coverslips were mounted in Prolong Gold antifade reagent with DAPI (Life Technologies #P-36931). For LysoTracker staining, cells were incubated with culture medium containing LysoTracker Red DND-99 (1:1000, Invitrogen, #L7528) for 3 minutes before fixation.
Protease protection assay

Proteinase K protection was performed as described previously (Taelman et al., 2010). HEK293T cells in 6-well plates were treated with control or Wnt3a conditioned medium for 2 hours. Cells were trypsinized and pelleted at 1000 rpm in a Sorvall 6000 tabletop centrifuge. Cell pellets were resuspended in 1 ml of buffer (100 mM potassium phosphate, pH 6.7, 5 mM MgCl₂, 250 mM sucrose) containing 6.5 µg/ml Digitonin (Sigma #D141) and incubated for 5 minutes at room temperature followed by 30 minutes on ice in Eppendorf tubes. Permeabilized cells were pelleted at 13,000 rpm in an Eppendorf centrifuge 5415R, resuspended in the same buffer without Digitonin, and divided into tubes containing 1 µg/ml Proteinase K (Invitrogen #25530-049) or Proteinase K with 0.3% Triton X-100, or water. After incubation at room temperature for 10 minutes, preheated 20 mM PMSF (Sigma #P7626) in 5X SDS loading buffer was added to each tube to stop the reaction and incubated for 5 minutes at 95°C before SDS-PAGE and western blot.

Xenopus Embryo Microinjection and Animal Cap Imaging

*Xenopus laevis* embryos were collected for microinjections as described (Sheng et al., 2014). Briefly, 600 units of Human chorionic gonadotropin (Sigma #CG10) were injected into a female *Xenopus laevis* 12 hours before collecting eggs. Eggs were obtained in 1X MMR (Marc’s Modified Ringer) solution containing 100 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES, pH 7.4. Collected eggs were in vitro fertilized using excised testis. Staging was as described (Nieuwkoop and Faber, 1967). In vitro synthesized mRNAs were introduced into embryos by microinjection using an IM 300 Microinjector (Narishige International USA, Inc). Embryos were injected in 1X MMR and cultured in 0.1X MMR.

Animal cap explants imaging was as described previously (Kim et al., 2012). Briefly, mRNAs were injected into one or two blastomeres at the four- or eight-cell stage. Animal cap explants were dissected at stage 9 or 10 and fixed with 4% paraformaldehyde for 2-3 hours at room temperature. After fixation, caps were blocked with 3% BSA/0.1% Triton X-100 in PBS or CAS-Block (Invitrogen #00-8120) for 1 hour and then incubated with primary antibody overnight at 4°C, followed by secondary antibody incubation for 2 hours at room temperature. Primary antibodies were used at 1:200 dilution and secondary antibodies from Jackson Laboratory were used at 1:300 dilution. After extensive washes with PBST, animal cap tissues were placed on glass slides (Fisher Scientific #12-550-15), positioned so that the inner layer (less pigmented layer) faced upward, and mounted with Prolong Gold antifade mounting medium with DAPI (Life Technologies #P-36931).
**Ubiquitination assays**

Ubiquitination assays were performed, with slight modification, according to the protocol of Zhu et al., 1999. For ubiquitination assays of β-Catenin, Smad1 and Smad4, HEK293T cells were transfected with GFP-β-Catenin, Flag-Smad1 or Flag-Smad4 together with HA-ubiquitin and either β-TrCP or Smurf1. After 48 hours of transfection, cells were treated with control or Wnt3a conditioned medium for 2 to 4 hours, lysed on the plate with a buffer containing 20 mM HEPES, pH 7.2, 150 mM NaCl, 1% Triton X-100, 10 mM EDTA, pH 8.0, 1mM DTT and 1 mM NaF together with a protease inhibitor cocktail tablet per 20 ml (Roche #4693132001) and incubated in Eppendorf tubes at 95°C in the presence of 1% SDS to disrupt protein-protein interactions. The lysates were then diluted 10-fold to reduce SDS concentration to 0.1% before immunoprecipitating with GFP or Flag antibody conjugated agarose beads (Santa Cruz #9996 AC or Sigma #A2220, respectively). Bead-lysate mixtures were incubated overnight at 4°C using an end-over-end rotator. Beads were then washed three times with cold buffer (10 mM Tris, pH 9.0, 100 mM NaCl, 0.5% NP40) followed by elution with SDS loading buffer at 95°C for 5 minutes. Note that proteasome inhibitors were not used in these assays.

**Flow Cytometric Measurement of Cell Size**

For flow cytometric measurements of cell size, cells were plated in 6 well plates. For HeLa cells, 80 ng/ml of purified Wnt3a protein (Peprotech) was added for the indicated incubation times. For C32 melanoma cells, cells were cultured in RPMI 1640 (with L-glutamine) (Life Technologies) containing 10% Tetracycline-free fetal bovine serum (Omega Scientific) and 1% Pen/Strep. C32 cells were treated with 80 ng/ml of Wnt3a protein (Peprotech) for 24 hours. 293T cells were treated with Wnt3a conditioned medium for 48 hours. For analyzing the requirement of HRS, 293T cells were treated with control or HRS siRNA, then split into 6-well plates, and treated with Wnt3a conditioned medium for 48 hours. In order to study the effects of lysosomal inhibition on the Wnt-mediated cell size increase, 293T cells were treated with or without Wnt3a conditioned medium for 48 hours, and then with or without Chloroquine (CQ) (dissolved in water) at a final concentration of 25 µM plus Wnt3a medium for the next 24 hours. To measure the effects of proteasome inhibition on cell size, HeLa cells were treated with MG132 (Sigma #C2211) at 10 µM for 24 hours. To assess the effects of Axin depletion on Wnt-mediated cell size increase, shAxin-Tet-239T cells were treated with (or without) Doxycycline for 48 hours to deplete Axin levels, and then with or without Wnt3a conditioned medium for the next 24 hours. Cells were then collected and fixed in solution with 4% paraformaldehyde for 20 minutes, washed in PBS, and stained with Hoechst 33342 (Invitrogen H3570) for 30 minutes. Cell size was quantified by flow cytometry in an LSRII flow cytometer (BD Biosciences). 10000 events per sample were collected by FACSDiva Version 6.0 (BD
Biosciences) and the forward scatter (FSC-A) was analyzed and histogram overlays plotted using FlowJo software.

**Imaging**

All imaging was performed with a Z1 Imager upright microscope from Zeiss (Germany), equipped with an Apotome using the corresponding filter sets. Images were collected with the Zeiss Axiovision software.
Supplemental References


