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Report

PI4K2β/AP-1-Based TGN-Endosomal Sorting Regulates Wnt Signaling

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Summary

Endosomal membrane traffic serves crucial roles in cell physiology, signaling, and development [1–4]. Sorting between endosomes and the *trans*-Golgi network (TGN) is regulated among other factors by the adaptor AP-1, an essential component of multicellular organisms [5]. Membrane recruitment of AP-1 requires phosphatidylinositol 4-phosphate [PI(4)P], though the precise mechanisms and PI4 kinase isozyme (or isozymes) involved in generation of this PI(4)P pool remain unclear [6, 7]. The Wnt pathway is a major developmental signaling cascade and depends on endosomal sorting in Wnt-sending cells [8–10]. Whether TGN/endosomal sorting modulates signaling downstream of Frizzled (Fz) receptors in Wnt-receiving cells is unknown. Here, we identify PI4-kinase type 2β (PI4K2β) as a regulator of TGN/endosomal sorting and Wnt signaling. PI4K2β and AP-1 interact directly and are required for efficient sorting between endosomes and the TGN. Zebrafish embryos depleted of PI4K2β or AP-1 lack pectoral fins due to defective Wnt signaling. Rescue experiments demonstrate requirements for PI4K2β-AP-1 complex formation and PI4K2β-mediated PI(4)P synthesis. Furthermore, PI4K2β binds to the Fz-associated component Dishevelled (Dvl) and regulates endosomal recycling of Fz receptors and Wnt target gene expression. These data reveal an evolutionarily conserved role for PI4K2β and AP-1 in coupling phosphoinositide metabolism to AP-1-mediated sorting and Wnt signaling.

Results and Discussion

PI4K2β Cycling between TGN and Endosomes

Strong evidence links PI(4)P synthesis to *trans*-Golgi network (TGN)/endosomal traffic [6, 7, 11], but the precise role of individual PI4-kinase isozymes remains unclear. For example, endosomal PI4K2α [12, 13] has been linked to AP-1 recruitment to the TGN [7], whereas other studies postulate its involvement in endolysosomal sorting via AP-3 and BLOC-1 [6, 14]. PI4K2β, an enzyme whose in vivo function has not been studied, is a likely candidate for regulating TGN/endosomal traffic. HA-tagged or endogenous PI4K2β was concentrated at the TGN/recycling endosomal interface (referred to below as TGN), where it overlapped with AP-1 and the TGN markers TGN46 (Figure 1A) or TGN38 (see Figures S1A and S1B available online). No significant colocalization was detected between PI4K2β and the early Golgi marker GM130, endosomal AP-3, AP-2, or the transferrin receptor (Figures S1A and S1B), or between PI4K2α and AP-1 or TGN38 (Figures S1C and S1D). Subpools of PI4K2β were found in late endosomes (Figure 1A), recycling endosomes, or enlarged early endosomes (Figure S1E). Thus, PI4K2β partitions between the TGN and endosomes.

PI4K2β contains an evolutionarily conserved acidic cluster dileucine (LL) motif that fits the AP adaptor complex binding consensus D/ExxxL[LI] (Figure 1B), which may underlie its partitioning between TGN and endosomes. Indeed, a PI4K2β mutant harboring an inactive LL motif (LLAA) was not retained at the TGN and instead accumulated on the plasma membrane and on LAMP1-positive endosomes (Figures 1C and 1D; Figure S1F). Kinase-inactive PI4K2β (D300A; Figures S1G and S1H) partitioned between the TGN and endosomes normally (Figures 1C and 1D).

PI4K2β Directly Associates with AP-1

As PI4K2β synthesizes PI(4)P (Figure S2E), colocalizes with the PI(4)P-binding [7, 11] AP-1 complex (Figure 1), and harbors a potential AP-1 interaction motif, we investigated a possible physical association. AP-1 coimmunoprecipitated with native PI4K2β, but not with PI4K2α (Figure 1E). Conversely, enzymatically active PI4K2β coimmunoprecipitated with AP-1, but not AP-2 (Figures 1F and 2A). Affinity chromatography, immunoprecipitation, and in vitro binding experiments revealed that active PI4K2β directly associates with the γ1σ1 subcomplex of AP-1 (Figures S2B and S2D) via its LL motif (Figure 1G; Figure S2A). Consistently, AP-1 was found on organelles immunoisolated with PI4K2β-specific antibodies (Figure S2C).

PI4K2β Localization and Function Depends on AP-1 and Vice Versa

The physical association of PI4K2β with AP-1 suggests an important role of AP-1 in regulating PI4K2β function. To explore this, we used immortalized mouse embryonic fibroblasts (MEFs) derived from AP-1-μ1A knockout animals ($\mu 1A^{-/-}$), which lack stable accumulation of heterotetrameric AP-1 complexes at the TGN [5]. PI4K2β was expressed at normal levels in $\mu 1A^{-/-}$ MEFs (Figure S2F) but redistributed from the TGN to peripheral puncta (Figure 2B). Thus, a functional AP-1 complex is required to properly localize PI4K2β. Similar phenotypes were seen in AP-1(γ1)-depleted cells (Figure 2C). Moreover, acute perturbation of AP-1 recruitment to the TGN by brefeldin A also resulted in loss of PI4K2β from the TGN and its accumulation in CD63-positive late endosomes (Figure 2D). Thus, AP-1 is required for proper localization of PI4K2β. Conversely, PI4K2β knockdown cells showed a specific loss of AP-1 (Figures 2E and 2F; Figure S2G) from membranes and cytosol (Figure S2H), whereas the levels of AP-2, AP-3, and GGA3 remained unchanged (Figure 2E). Loss of PI4K2β did not affect the subcellular localization of GGA3 (Figure S2J) or the Golgi PI(4)P sensor eGFP-FAPP1-PH (Figure S2K). Moreover,

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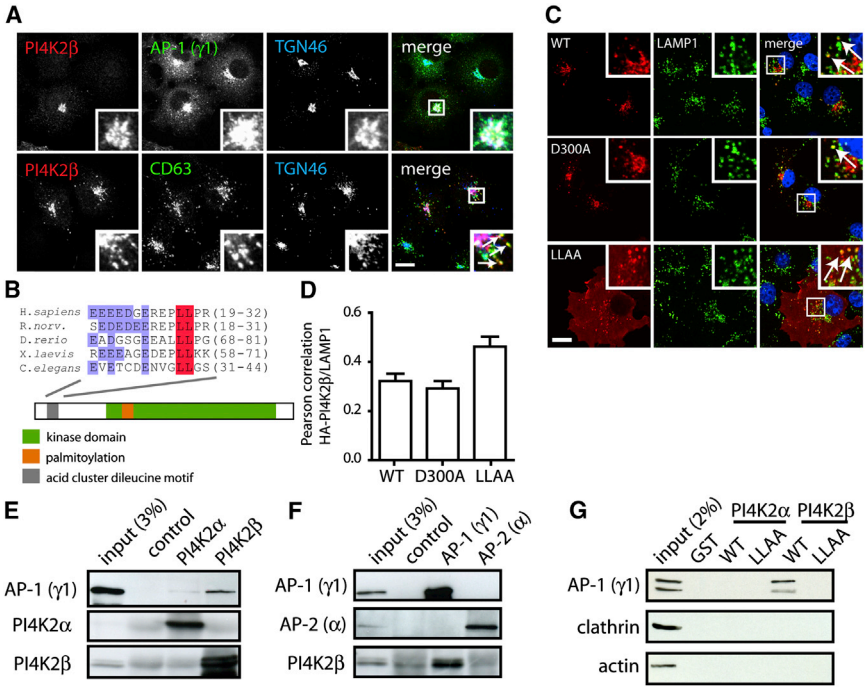

Figure 1. PI4K2β Interacts with AP-1 via a Conserved Acidic Cluster Dileucine Motif

(A) Colocalization of HA-PI4K2β (red) with AP-1, late endosomal CD63 (green), and TGN46 (blue) in COS7 cells. Arrows indicate TGN46-negative, CD63-positive PI4K2β structures.

(B) Conserved acidic cluster (blue) dileucine (red) motif within PI4K2β.

(C) Enhanced colocalization of dileucine-mutant HA-PI4K2β (LLAA, red) with LAMP1 (green) when compared to wild-type (WT) or kinase-inactive (D300A) forms. DAPI-labeled nuclei are in blue. Arrows indicate colocalization between PI4K2β and LAMP1. Scale bars represent 10 μm.

(D) Quantification of (C) (mean ± SEM).

(E) Antibodies to PI4K2β, but not PI4K2α, coimmunoprecipitate endogenous AP-1 from rat liver membrane extracts.

(F) Antibodies to AP-1, but not AP-2, coimmunoprecipitate endogenous PI4K2β.

(G) AP-1 from rat brain extracts is affinity purified by GST-PI4K2β (WT). PI4K2α or mutant PI4K2β (LLAA) fails to bind AP-1.

overexpression of wild-type (WT) PI4K2β in $\mu 1A^{-/-}$ MEFs was sufficient to recruit γ1-adaptin-containing AP-1 remnants [5] to eGFP-PI4K2β-positive puncta (Figure S2I, inset), whereas AP-1 binding defective PI4K2β (LLAA) or kinase-inactive PI4K2β (D300A; Figures S1G and S1H) failed to recruit γ1-adaptin (Figure S2I).

If PI4K2β and AP-1 form a functional complex, one would expect depletion of PI4K2β to partly mimic loss of function of AP-1. Indeed, PI4K2β knockdown resulted in loss of MPR46 from the TGN (Figure 2G) and secretion of lysosomal β-hexosaminidase (Figure 2H), hallmarks of AP-1 deficiency [5]. Thus, PI4K2β depletion phenocopies loss of AP-1 with respect to TGN/endosomal sorting. We conclude that PI4K2β is a major contributor to the synthesis of a PI(4)P pool required for AP-1 function.

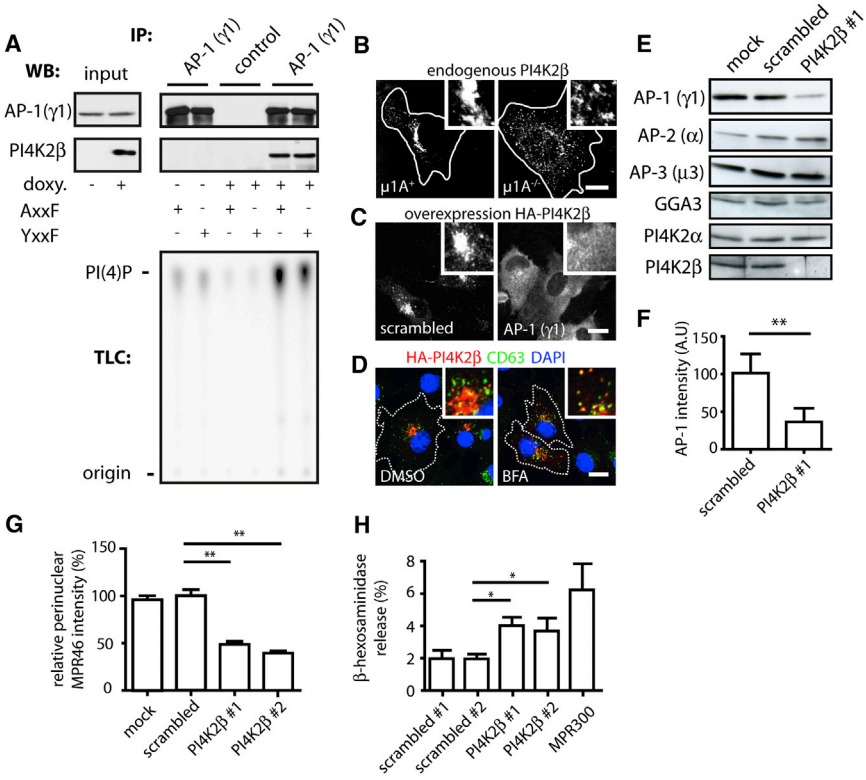

Figure 2. PI4K2β and AP-1 Functionally Depend On Each Other

(A) Active PI4K2β coimmunoprecipitates with AP-1(γ1) from doxycycline (doxy)-induced HEK293T FlpIn/TetR cells. Eluates were analyzed by immunoblotting (WB) or PI4-kinase activity assays. Yxxø or control Axxø sorting motif peptides [15] did not alter kinase activity.

(B) Immortalized mouse embryonic fibroblasts (MEFs) from $\mu 1A^{-/-}$ mice or stably rescued cells ($\mu 1A^{+}$) labeled for endogenous PI4K2β. Insets show zoom of perinuclear area. Dotted lines indicate cell boundaries.

(C) Disturbed localization of HA-PI4K2β (anti-HA) in AP-1(γ1)-depleted COS7 cells. Pearson's correlation (average ± SEM): scrambled, 0.250 ± 0.03; AP-1 (γ1), 0.480 ± 0.03.

(D) Disturbed localization of HA-PI4K2β (red) in brefeldin A-treated COS7 cells. Green, CD63-positive late endosomes; blue, DAPI-stained nuclei. Scale bars represent 10 μm.

(E) Western blot analysis of siRNA-treated HeLa cells.

(F) Quantitation of AP-1 levels shown in (E) (n = 5, **p < 0.002), normalized to actin.

(G) Amount of MPR46 within TGN46-positive area (mean ± SEM, 66 cells, n = 3, **p < 0.01).

(H) Increased secretion of β-hexosaminidase from COS7 cells depleted of PI4K2β or MPR300 (n = 3, three replicates per experiment; *p < 0.02).

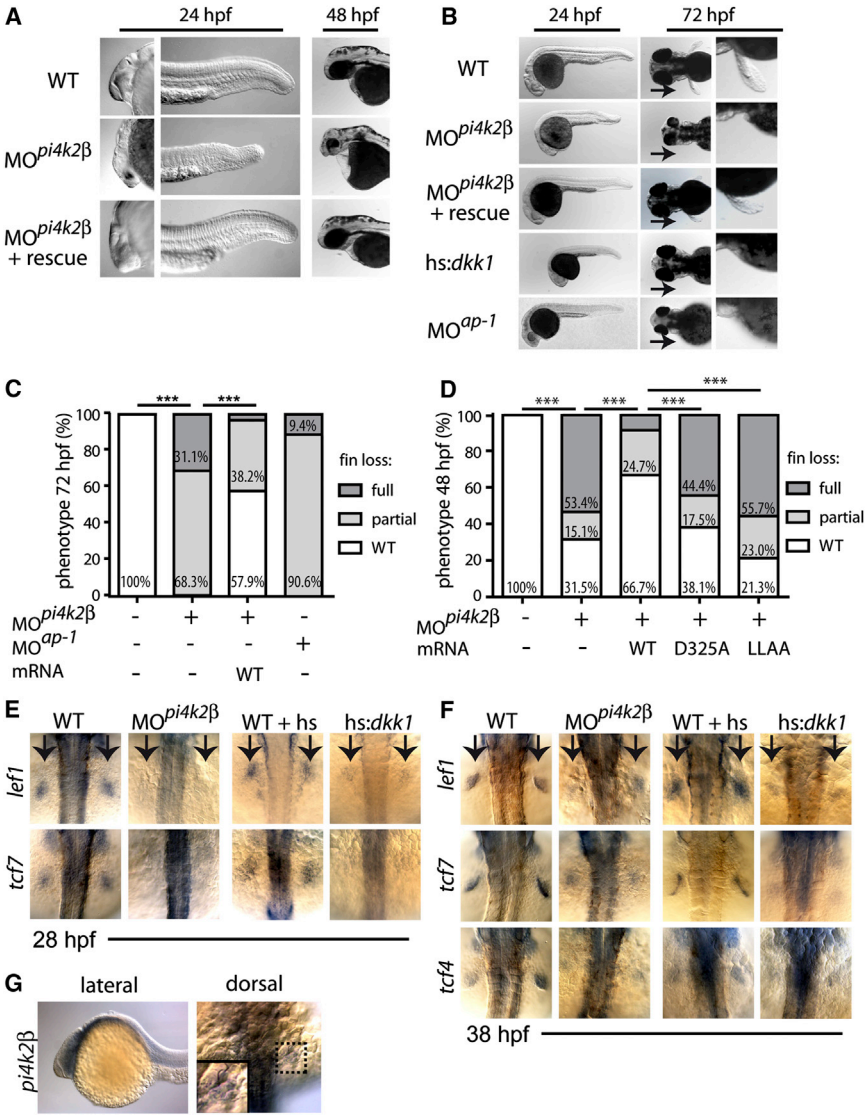

Figure 3. PI4K2β/AP-1 Complex Regulates Wnt-Dependent Fin Development

(A) Zebrafish embryos injected at the single-cell stage with a morpholino (MO) targeting *pi4k2β* (MO$^{pi4k2\beta}$). Detailed head and tail views of zebrafish noninjected (WT), injected with MO$^{pi4k2\beta}$, or injected with MO$^{pi4k2\beta}$ and MO-insensitive rescue mRNA (MO$^{pi4k2\beta}$ +rescue) at 24 and 48 hr postfertilization (hpf) are shown.

(B) At 24 hpf (lateral view), *pi4k2β* morphants display shortened body axes and head defects comparable to Tg[*hsp70:dkk1-GFP*]^{w32} fish heat-shocked at the one- to three-somite stage (hs:*dkk1*). Ventral views of 72 hpf *ap-1* and *pi4k2β* morphants and hs:*dkk1* embryos heat shocked at 24 hpf with defective pectoral fin development (arrows) are shown. *pi4k2β* morphant phenotypes are partially rescued by coinjection of MO-insensitive zebrafish *pi4k2β* mRNA (+ rescue).

(C) Quantification of MO phenotypes shown in (B) at 72 hpf (>130 embryos per condition; chi-square test: ***$p < 0.001$).

(D) Zebrafish embryos injected at the single-cell stage with a MO targeting *pi4k2β* lose pectoral fins. Coinjection of MO-insensitive *pi4k2β* mRNA encoding WT, but not dileucine (LLAA) or kinase-inactive (D325A) mutant zPI4K2β, rescued defective pectoral fin development in *pi4k2β* morphants at 48 hpf (see also Figure S3E).

(E) Dorsal view of in situ hybridization at 28 hpf for Wnt target genes *lef1* and *tcf7* in the developing pectoral fin bud (arrow). *pi4k2β* morphants have reduced expression of *lef1* (33 out of 36) and *tcf7* (28 out of 28) in the developing pectoral fin bud. 100% of Tg[*hsp70:dkk1-GFP*]^{w32} embryos heat shocked at 24 hpf show downregulation of *lef1* (n = 21) and *tcf7* (n = 26) expression.

(F) In situ hybridization shows downregulation of *lef1* (23 out of 27), *tcf7* (34 out of 36), and *tcf4* (27 out of 30) at 38 hpf upon MO$^{pi4k2\beta}$ injection. 100% of Tg[*hsp70:dkk1-GFP*]^{w32} transgenic fish heat shocked at 24 hpf (hs:*dkk1*) show downregulation of *lef1* (n = 32), *tcf7* (n = 43), and *tcf4* (n = 23). Arrows indicate developing pectoral fin.

(G) *pi4k2β* expression in pectoral fin buds (boxed area, inset) at 24 hpf visualized by in situ hybridization.

PI4K2β is Required for Pectoral Fin Development and Wnt Target Gene Expression in Zebrafish

Endosomal membrane traffic [8–10, 16] and PI(4)P-synthesizing enzymes [17, 18] are linked to Wnt signaling and development. To explore a possible function of PI4K2β/AP-1 in developmental signaling, we downregulated PI4K2β by morpholino oligonucleotide (MO) injection into zebrafish (Figure S3A). *Pi4k2β* morphant embryos showed a severely shortened body axis, a smaller head, and pericardial edema at 48 hr postfertilization (hpf) (Figure 3A). These phenotypes were rescued by MO-insensitive *pi4k2β* mRNA (Figures 3A–3D). Analysis at 48 hpf and 72 hpf revealed that the pectoral fin was poorly developed or absent in *pi4k2β* or *ap-1* morphants (Figures 3B and 3C; Figure S3E), similar to effects caused by impaired Wnt or FGF signaling [19, 20]. To support these data, we conducted rescue experiments using MO-resistant zebrafish *pi4k2β* mRNA encoding PI4K2β (zPI4K2β) mutants lacking kinase activity (D325A) or the ability to bind to AP-1 (Figure 1B; Figure S1G). All zPI4K2β variants were expressed at similar levels (Figure S3C) and behaved as predicted (Figure S3D). Whereas wild-type zPI4K2β effectively rescued pectoral fin development at 48 hpf, AP-1 binding-defective or kinase-inactive mutant zPI4K2β were functionally inactive (Figure 3D; Figure S3E). Hence, zPI4K2β and AP-1 exert a key function in zebrafish fin development.

Pectoral fin development depends on canonical Wnt and FGF signaling [19, 20]. To elucidate the potential link between PI4K2β and canonical Wnt or FGF signaling, we compared early development of *pi4k2β* morphants with transgenic embryos expressing the Wnt repressor Dkk1 under a heat-shock-inducible promoter (Tg[*hsp70:dkk1-GFP*]^{w32}) [21], or with embryos treated with the FGF signaling inhibitor SU5402. Early heat shock between 3.5 and 4.5 hpf in Tg[*hsp70:dkk1-GFP*]^{w32} transgenic embryos caused a well-described [21] reduction of anterior and posterior development (data not shown). Heat shocks between 10.5 and 11.5 hpf (one to three somites) resulted in milder developmental defects similar to the *pi4k2β* morphant phenotype (Figure 3B). Transgenic embryos heat shocked at 24 hpf had a normal body form but lacked pectoral fins (Figure 3B; Figure S3B). In comparison, embryos treated from 10.5 to 11.5 hpf on with the FGF inhibitor SU5402 displayed a truncated trunk and tail [22], a

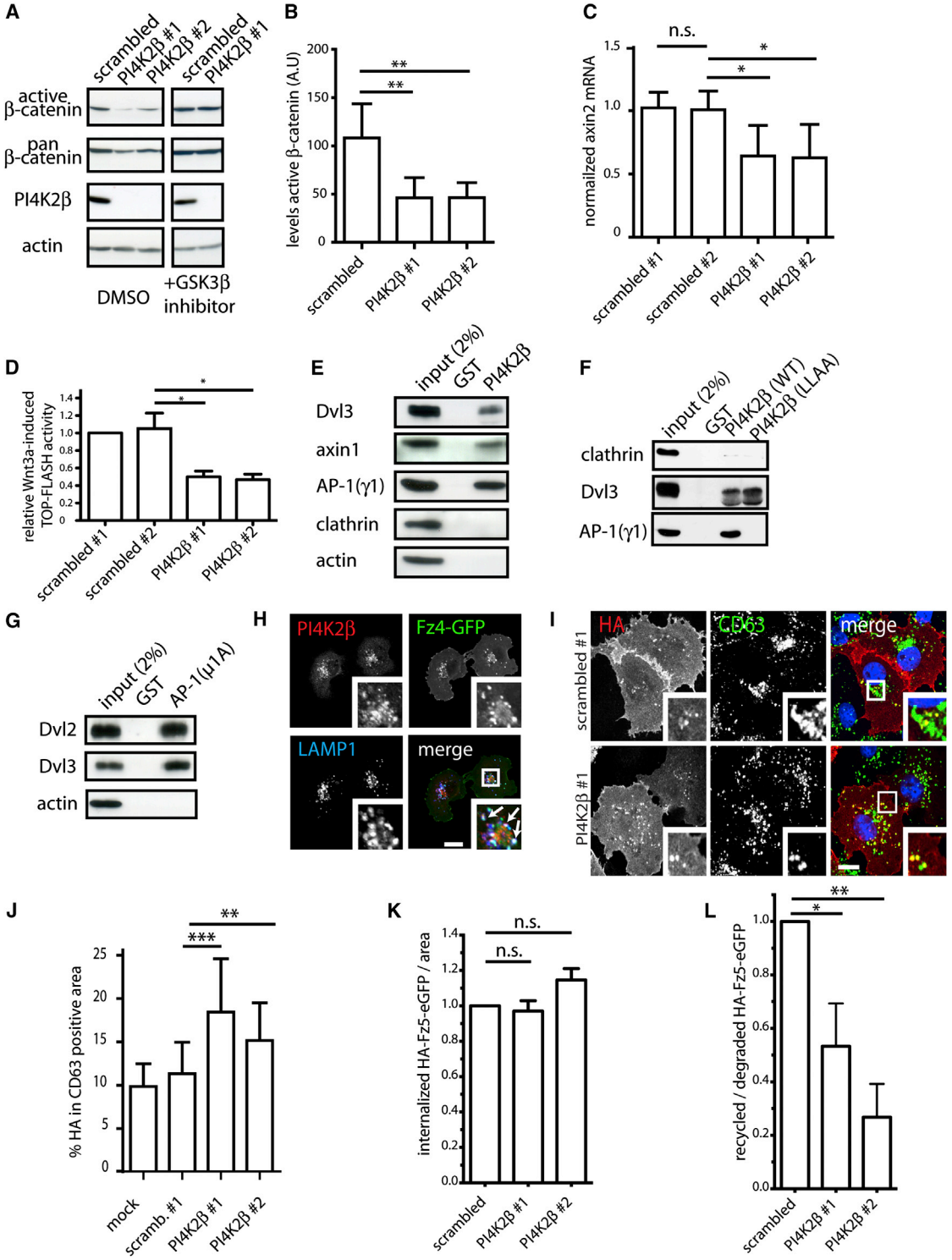

Figure 4. PI4K2β Regulates Wnt Signaling by Regulating Endosomal Fz Sorting

(A) Immunoblot analysis of PI4K2β-silenced cells treated with DMSO (control) or 3 μM GSK3β inhibitor CHIR99021 (1 hr).

(B) Quantification of active (dephospho) β-catenin levels shown in (A) (n = 6; **p < 0.005).

(C) Reduction in axin2 expression in PI4K2β-silenced cells. GAPDH was used for normalization. (n = 5; *p < 0.05).

(D) Super 8×TOPFlash reporter activity in PI4K2β-silenced CV1 cells or scrambled siRNA-treated control cells treated with Wnt3a-conditioned medium for 20 hr. Data were normalized to scrambled #1 (mean ± SEM; n = 5 independent experiments; *p < 0.05).

(E) AP-1 (γ1), Dvl3, and axin1 copurify from HEK293 cell extract on a GST-PI4K2β matrix.

(F) Same as (E), but using either wild-type (WT) or AP-1 binding-defective mutant (LLAA) GST-PI4K2β as baits.

(G) Dvl2 and Dvl3 copurify on GST-μ1A.

(H) Partial colocalization (arrows) of Fz4-eGFP with HA-PI4K2β (red) on LAMP1-positive late endosomes (blue) in COS7 cells. Scale bars represent 10 μm in (H) and (I).

(legend continued on next page)

phenotype clearly different from *pi4k2β* morphants. Thus, loss of pectoral fins seen in *pi4k2β* morphants is mimicked by inhibition of Wnt signaling and therefore may reflect impaired canonical Wnt signaling. In further support of this conclusion, expression of the Wnt target genes *lef1*, *tcf4*, and *tcf7* was reduced in the developing fin bud of *pi4k2β* morphants at 28 hpf (Figure 3E) and 38 hpf (Figure 3F). Consistent with the *pi4k2β* morphant fin phenotype, *pi4k2β* mRNA is expressed within the pectoral fin bud (Figure 3G). Thus, *pi4k2β* morphants closely resemble the induced Dkk1 overexpression phenotype but much less resemble the phenotypes caused by inhibited FGF signaling.

To further discriminate between FGF and Wnt signaling, we analyzed activation of Tbx5.1. Pectoral fin formation depends on activation of Tbx5.1 by Wnt signaling at 20 hpf, whereas the subsequent maintenance phase initiated at 24 hpf requires both Wnt and FGF signaling [20]. At 20 hpf, 28% of the *pi4k2β* morphants displayed reduced *tbx5.1* expression, a phenotype that became more pronounced at 24 hpf and was similar to heat-shocked Tg[*hsp70:dkk1-GFP*]^{w32} zebrafish (Figures S3F–S3H). By contrast, FGF inhibition did not reduce *tbx5.1* expression at 20 hpf (Figure S3G). Instead, it resulted in an elongation of the *tbx5.1* expression domain at 24 hpf (Figures S3F–S3I), as previously reported [20]. To control for efficient zPI4K2β downregulation, MO^{pi4k2β}-injected embryos were allowed to develop up to 72 hpf, and 77% of these showed a fin development defect (n = 26). We conclude that zPI4K2β/AP-1 is critical for zebrafish fin development via affecting canonical Wnt signaling.

PI4K2β Regulates Canonical Wnt Signaling in Mammalian Cells

To mechanistically explore the putative role of PI4K2β in canonical Wnt signaling, we silenced PI4K2β expression in mammalian cells and sampled the activation state of canonical Wnt signaling. Depletion of PI4K2β resulted in reduced levels of active phosphorylated LRP6, a specific component of canonical Wnt signaling (Figures S4A and S4B). PI4K2β-depleted cells also contained reduced amounts of active dephosphorylated β-catenin, a phenotype rescued by inhibition of GSK3β (Figures 4A and 4B). Moreover, expression of the Wnt target gene *axin2* was diminished in PI4K2β-depleted cells (Figure 4C).

Defective Wnt signaling may result from defects in Wnt secretion [8–10] or impaired signal transduction in Wnt-receiving cells [16, 18, 23]. PI4K2β depletion did not affect secretion of Wnt3a (Figures S4C and S4D). Thus, defects in Wnt target gene expression in PI4K2β-silenced cells likely originate from defective signal processing and/or trafficking of signaling components in Wnt-receiving cells lacking PI4K2β. Consistent with the latter possibility, luciferase reporter gene expression measured by TOPFlash assays was significantly reduced in Wnt3a-treated PI4K2β-depleted cells (Figure 4D). Furthermore, PI4K2β associated with the Wnt signaling components axin1 and Dvl2/3 (Figure 4E; Figure S4F), independent of PI4K2β binding to AP-1 (Figure 4F). Conversely, AP-1(μ), a subunit that does not bind to PI4K2β, also bound to Dvl2/3 (Figure 4G). These data suggest the existence of a trimeric AP-1/PI4K2β/Dvl complex.

Internalization of the Wnt coreceptor LRP6 may tune Wnt-induced β-catenin signaling [24], and inhibition of endocytosis results in impaired signal transduction in receiving *D. melanogaster* cells [25]. These observations, together with the fact that Dvl binds to AP-2 [23], AP-1 (Figure 4G), and the cytoplasmic tail of Fz receptors, suggest that altered Fz endocytosis and/or endosomal sorting may underlie defective Wnt signaling in absence of PI4K2β. Confocal imaging revealed that in addition to its plasma membrane pool, a fraction of Fz4-eGFP colocalized with PI4K2β on late endosomes (Figure 4H; similar to Fz5-eGFP, Figure S4G). Tracing of internalized HA-tagged Fz receptors confirmed Fz cycling between the cell surface and PI4K2β-positive endosomes (Figure S4H). We therefore hypothesized that PI4K2β may regulate Wnt signaling by altering (1) surface expression, (2) internalization, or (3) endosomal sorting of Fz. PI4K2β depletion did not affect Fz5 surface expression (Figure S4E) or internalization (Figure 4K), indicating that PI4K2β is dispensable for Fz surface trafficking or endocytosis.

As internalized Fz partially colocalizes with PI4K2β in late endosomes (Figure 4H), we analyzed late endosome distribution in PI4K2β-knockdown cells. Late endosomes in PI4K2β-silenced cells were reduced in intensity and displayed a more scattered peripheral distribution compared to controls (Figures S4I–S4K), a phenotype rescued by siRNA-resistant wild-type (Figure S4K), but not AP-1 binding-defective (LLAA) (Figures S4K and S4L) or kinase-inactive mutant PI4K2β (Figure S4L). A similar dispersion was seen for Fz4 (Figures S4M and S4N). Further quantitative analysis of Fz endosomal sorting in PI4K2β-silenced cells revealed an accumulation of endocytosed Fz5 in late endosomes (Figures 4I and 4J), perhaps en route to degradation. Thus, PI4K2β is required for endosomal membrane homeostasis and hence may regulate partitioning of internalized Fz between degradative sorting and endosomal recycling to the surface. Consistent with this, loss of PI4K2β impaired Fz5 recycling while favoring its late endosomal degradation (Figure 4L). These collective data are consistent with a model wherein PI4K2β modulates canonical Wnt signaling by facilitating recycling of internalized Fz receptors, thereby preventing their degradative sorting to late endosomes.

We unravel here the association of AP-1 and PI4K2β, which provides a pool of PI(4)P required for AP-1 stability [7, 11] and function in TGN/endosomal sorting [5]. Given the known role of AP-1 in cell fate decisions, positioning of cilia, and melanosome biogenesis [26–28], we predict that PI4K2β may also regulate at least some of these processes. Association of PI4K2β and AP-1 mirrors complex formation of AP-2 with PI(4)P 5-kinase I [15] and of AP-3 with PI4K2α [6], suggesting a general model whereby AP complexes associate with PI kinases that synthesize the PI species to which they bind. These findings support the concept that membrane traffic is regulated by subpools of PIs synthesized by distinct PI kinase isoforms [29].

(I) Colocalization of internalized HA-Fz5-eGFP with CD63-positive late endosomes (green) in PI4K2β-silenced or control COS7 cells.
(J) Quantification of (I), percentage of total HA labeling in CD63-positive compartment (n = 2, >60 cells per condition, $^{**}p < 0.01$; $^{***}p < 0.001$).
(K) PI4K2β is dispensable for Fz5 endocytosis. Depletion of PI4K2β does not affect the relative amount of HA-Fz5-eGFP internalization when compared to control CV1 cells (taken for normalization and set to 1) (mean ± SEM; >250 cells per condition; n = 3 independent experiments; n.s., not significant).
(L) PI4K2β regulates Fz5 recycling. Depletion of PI4K2β reduces the ratio of plasmalemmal recycling versus degradation of HA-Fz5-eGFP when compared to control CV1 cells (mean ± SEM; >250 cells per condition; n = 3 independent experiments; $^{*}p < 0.05$; $^{**}p < 0.01$).

Our results identify a hitherto unknown molecular link between PI4K2β-dependent PI(4)P synthesis, PI4K2β/AP-1-mediated endosomal sorting of Fz in Wnt-receiving cells, and developmental Wnt signaling, which may also underlie the requirement for AP-1 in early embryonic development [5]. The function of PI4K2β and AP-1 in Fz recycling in Wnt-receiving cells reported here is distinct from the known role of endosomal sorting in Wnt secretion from signal-sending cells [8–10] or in Wnt-induced endosomal GSK3β sequestration [16]. We suggest that PI4K2β/AP-1 acts as a gatekeeper that regulates partitioning of internalized Fz between recycling and degradative endosomal sorting, thereby modulating the cellular responsiveness to Wnt. As human cancer is often linked to aberrant Wnt-β-catenin signaling [30], our data also identify PI4K2β as a novel potential target for anticancer therapies.

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.09.017.

Acknowledgments

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References

1. Bonifacino, J.S., and Hurley, J.H. (2008). Retromer. Curr. Opin. Cell Biol. *20*, 427–436.
2. Gould, G.W., and Lippincott-Schwartz, J. (2009). New roles for endosomes: from vesicular carriers to multi-purpose platforms. Nat. Rev. Mol. Cell Biol. *10*, 287–292.
3. Johannes, L., and Popoff, V. (2008). Tracing the retrograde route in protein trafficking. Cell *135*, 1175–1187.
4. Platta, H.W., and Stenmark, H. (2011). Endocytosis and signaling. Curr. Opin. Cell Biol. *23*, 393–403.
5. Meyer, C., Zizioli, D., Lausmann, S., Eskelinen, E.L., Hamann, J., Saftig, P., von Figura, K., and Schu, P. (2000). μ1A-adaptin-deficient mice: lethality, loss of AP-1 binding and rerouting of mannose 6-phosphate receptors. EMBO J. *19*, 2193–2203.
6. Craige, B., Salazar, G., and Faundez, V. (2008). Phosphatidylinositol-4-kinase type II alpha contains an AP-3-sorting motif and a kinase domain that are both required for endosome traffic. Mol. Biol. Cell *19*, 1415–1426.
7. Wang, Y.J., Wang, J., Sun, H.Q., Martinez, M., Sun, Y.X., Macia, E., Kirchhausen, T., Albanesi, J.P., Roth, M.G., and Yin, H.L. (2003). Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. Cell *114*, 299–310.
8. Belenkaya, T.Y., Wu, Y., Tang, X., Zhou, B., Cheng, L., Sharma, Y.V., Yan, D., Selva, E.M., and Lin, X. (2008). The retromer complex influences Wnt secretion by recycling wntless from endosomes to the trans-Golgi network. Dev. Cell *14*, 120–131.
9. Franch-Marro, X., Wendler, F., Guidato, S., Griffith, J., Baena-Lopez, A., Itasaki, N., Maurice, M.M., and Vincent, J.-P. (2008). Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. Nat. Cell Biol. *10*, 170–177.
10. Pan, C.-L., Baum, P.D., Gu, M., Jorgensen, E.M., Clark, S.G., and Garriga, G. (2008). C. elegans AP-2 and retromer control Wnt signaling by regulating mig-14/Wntless. Dev. Cell *14*, 132–139.
11. Baust, T., Czupalla, C., Krause, E., Bourel-Bonnet, L., and Hoflack, B. (2006). Proteomic analysis of adaptor protein 1A coats selectively assembled on liposomes. Proc. Natl. Acad. Sci. USA *103*, 3159–3164.
12. Balla, A., and Balla, T. (2006). Phosphatidylinositol 4-kinases: old enzymes with emerging functions. Trends Cell Biol. *16*, 351–361.
13. Minogue, S., Waugh, M.G., De Matteis, M.A., Stephens, D.J., Berditchevski, F., and Hsuan, J.J. (2006). Phosphatidylinositol 4-kinase is required for endosomal trafficking and degradation of the EGF receptor. J. Cell Sci. *119*, 571–581.
14. Salazar, G., Zlatic, S., Craige, B., Peden, A.A., Pohl, J., and Faundez, V. (2009). Hermansky-Pudlak syndrome protein complexes associate with phosphatidylinositol 4-kinase type II alpha in neuronal and non-neuronal cells. J. Biol. Chem. *284*, 1790–1802.
15. Krauss, M., Kukhtina, V., Pechstein, A., and Haucke, V. (2006). Stimulation of phosphatidylinositol kinase type I-mediated phosphatidylinositol (4,5)-bisphosphate synthesis by AP-2μ-cargo complexes. Proc. Natl. Acad. Sci. USA *103*, 11934–11939.
16. Taelman, V.F., Dobrowolski, R., Plouhinec, J.-L., Fuentealba, L.C., Vorwald, P.P., Gumper, I., Sabatini, D.D., and De Robertis, E.M. (2010). Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. Cell *143*, 1136–1148.
17. Ma, H., Blake, T., Chitnis, A., Liu, P., and Balla, T. (2009). Crucial role of phosphatidylinositol 4-kinase IIIalpha in development of zebrafish pectoral fin is linked to phosphoinositide 3-kinase and FGF signaling. J. Cell Sci. *122*, 4303–4310.
18. Pan, W., Choi, S.-C., Wang, H., Qin, Y., Volpicelli-Daley, L., Swan, L., Lucast, L., Khoo, C., Zhang, X., Li, L., et al. (2008). Wnt3a-mediated formation of phosphatidylinositol 4,5-bisphosphate regulates LRP6 phosphorylation. Science *321*, 1350–1353.
19. Kawakami, Y., Capdevila, J., Büscher, D., Itoh, T., Rodríguez Esteban, C., and Izpisúa Belmonte, J.C. (2001). WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. Cell *104*, 891–900.
20. Mercader, N., Fischer, S., and Neumann, C.J. (2006). Prdm1 acts downstream of a sequential RA, Wnt and Fgf signaling cascade during zebrafish forelimb induction. Development *133*, 2805–2815.
21. Stoick-Cooper, C.L., Weidinger, G., Riehle, K.J., Hubbert, C., Major, M.B., Fausto, N., and Moon, R.T. (2007). Distinct Wnt signaling pathways have opposing roles in appendage regeneration. Development *134*, 479–489.
22. Griffin, K., Patient, R., and Holder, N. (1995). Analysis of FGF function in normal and no tail zebrafish embryos reveals separate mechanisms for formation of the trunk and the tail. Development *121*, 2983–2994.
23. Yu, A., Rual, J.-F., Tamai, K., Harada, Y., Vidal, M., He, X., and Kirchhausen, T. (2007). Association of Dishevelled with the clathrin AP-2 adaptor is required for Frizzled endocytosis and planar cell polarity signaling. Dev. Cell *12*, 129–141.
24. Yamamoto, H., Sakane, H., Yamamoto, H., Michiue, T., and Kikuchi, A. (2008). Wnt3a and Dkk1 regulate distinct internalization pathways of LRP6 to tune the activation of β-catenin signaling. Dev. Cell *15*, 37–48.
25. Seto, E.S., and Bellen, H.J. (2006). Internalization is required for proper Wingless signaling in Drosophila melanogaster. J. Cell Biol. *173*, 95–106.
26. Benhra, N., Lallet, S., Cotton, M., Le Bras, S., Dussert, A., and Le Borgne, R. (2011). AP-1 controls the trafficking of Notch and Sanpodo toward E-cadherin junctions in sensory organ precursors. Curr. Biol. *21*, 87–95.
27. Delevoye, C., Hurbain, I., Tenza, D., Sibarita, J.-B., Uzan-Gafsou, S., Ohno, H., Geerts, W.J.C., Verkleij, A.J., Salamero, J., Marks, M.S., and Raposo, G. (2009). AP-1 and KIF13A coordinate endosomal sorting and positioning during melanosome biogenesis. J. Cell Biol. *187*, 247–264.
28. Kaplan, O.I., Molla-Herman, A., Cevik, S., Ghossoub, R., Kida, K., Kimura, Y., Jenkins, P., Martens, J.R., Setou, M., Benmerah, A., and Blacque, O.E. (2010). The AP-1 clathrin adaptor facilitates cilium formation and functions with RAB-8 in C. elegans ciliary membrane transport. J. Cell Sci. *123*, 3966–3977.
29. Balla, T. (2013). Phosphoinositides: tiny lipids with giant impact on cell regulation. Physiol. Rev. *93*, 1019–1137.
30. Moon, R.T. (2005). Wnt/beta-catenin pathway. Sci. STKE *2005*, cm1.