**Cell Reports**

**PLEKHA4/kramer Attenuates Dishevelled Ubiquitination to Modulate Wnt and Planar Cell Polarity Signaling**

**Graphical Abstract**

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**In Brief**
Regulation of Wnt signaling is critical to metazoan development. Shami Shah et al. identify a phosphoinositide-binding protein, PLEKHA4/kramer, that enhances Wnt signaling in mammalian cells and the non-canonical pathway, planar cell polarity, in *Drosophila*. Mechanistically, PLEKHA4 sequesters the Cullin-3 E3 ligase adaptor KLHL12 in plasma membrane clusters, preventing Dishevelled polyubiquitination.

**Highlights**
- PLEKHA4 promotes Wnt signaling by inhibiting the degradation of Dishevelled (DVL)
- PLEKHA4 binds to PI(4,5)P₂ and oligomerizes into plasma membrane clusters
- The clusters sequester a Cullin-3 adaptor, KLHL12, preventing DVL ubiquitination
- The *Drosophila* PLEKHA4 homolog, *kramer*, regulates planar cell polarity signaling
PLEKHA4/kramer Attenuates Dishevelled Ubiquitination to Modulate Wnt and Planar Cell Polarity Signaling

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https://doi.org/10.1016/j.celrep.2019.04.060

SUMMARY

Wnt signaling pathways direct key physiological decisions in development. Here, we establish a role for a pleckstrin homology domain-containing protein, PLEKHA4, as a modulator of signaling strength in Wnt-receiving cells. PLEKHA4 oligomerizes into clusters at PI(4,5)P_2-rich regions of the plasma membrane and recruits the Cullin-3 (CUL3) E3 ubiquitin ligase substrate adaptor Kelch-like protein 12 (KLHL12) to these assemblies. This recruitment decreases CUL3-KLHL12-mediated polyubiquitination of Dishevelled, a central intermediate in canonical and non-canonical Wnt signaling. Knockdown of PLEKHA4 in mammalian cells demonstrates that PLEKHA4 positively regulates canonical and non-canonical Wnt signaling via these effects on the Dishevelled polyubiquitination machinery. In vivo knockout of the Drosophila melanogaster PLEKHA4 homolog, kramer, selectively affects the non-canonical, planar cell polarity (PCP) signaling pathway. We propose that PLEKHA4 tunes the sensitivities of cells toward the stimulation of Wnt or PCP signaling by sequestering a key E3 ligase adaptor controlling Dishevelled polyubiquitination within PI(4,5)P_2-rich plasma membrane clusters.

INTRODUCTION

Wnt signaling controls key cell fate decisions in the development of multicellular eukaryotes, and its dysregulation can cause many human diseases (Clevers and Nusse, 2012). As such, Wnt signaling is subject to many points and types of regulation, both in cells that produce and in those that receive the secreted Wnt signals. In the Wnt-receiving cell, the engagement of Wnt proteins by the Frizzled family of cell-surface receptors (Janda et al., 2012) can activate different intracellular signaling pathways, including the canonical, β-catenin-dependent pathway and the non-canonical planar cell polarity (PCP) and Wnt-Ca^2+ routes (Cadigan and Peifer, 2009; Devenport, 2014; Gómez-Orte et al., 2013; MacDonald et al., 2009; Wallingford, 2012).

A feature common to all Wnt signaling pathways is the involvement of the cytoplasmic protein Dishevelled (DVL), whose recruitment to the plasma membrane upon Wnt binding to Frizzled initiates the intracellular signal transduction pathways. Because of this dynamic behavior, the DVL proteins represent key factors that Wnt-receiving cells can use to tune the strength of the Wnt signal (Gao and Chen, 2010; Mlodzik, 2016; Wallingford and Habas, 2005). DVL levels are modulated by ubiquitination (Angers et al., 2006; Ganner et al., 2009; Gao et al., 2010; Miyazaki et al., 2004; Sharma et al., 2012; Wei et al., 2012). In particular, the E3 ubiquitin ligase Cullin-3 (CUL3), in complex with one of its substrate adaptors, Kelch-like protein 12 (KLHL12), catalyzes the polyubiquitination of DVL3, leading to the proteasomal degradation of the latter (Angers et al., 2006). By lowering DVL3 levels, the CUL3-KLHL12 E3 ligase diminishes the strength of Wnt signaling. Given that cells must dynamically tune their Wnt ligand sensitivities to different physiological settings, a major unanswered question is how this activity of CUL3-KLHL12 toward DVL3 is regulated.

CUL3 interacts physiologically with adaptors other than KLHL12 (Dubiel et al., 2018) and, in complex with KLHL12, functions at intracellular locations that are distinct from the plasma membrane (Jin et al., 2012; Rondou et al., 2008). For example, at the endoplasmic reticulum, CUL3-KLHL12 mediates monoubiquitination of the COPII vesicle component SEC31 to facilitate the formation of enlarged COPII vesicles that transport large cargoes such as collagen to the Golgi complex; local bursts of calcium regulate this action of CUL3-KLHL12 via the calcium-binding adaptor proteins PEF1 and ALG2 (Jin et al., 2012; McGourty et al., 2016). It remains unknown how these distinct activities of CUL3-KLHL12 are regulated. What factors modulate the interaction of CUL3-KLHL12 with DVL3? Do such putative factors act analogously to how PEF1 and ALG2 regulate CUL3-KLHL12 activity at the endoplasmic reticulum (ER)? One possibility is that plasma membrane-localized factors control CUL3-KLHL12 activity toward DVL3 in this membrane, the site of DVL3 action in Wnt signaling.

Key signaling molecules at the plasma membrane are the phosphoinositides (PIPs) PI4P and PI(4,5)P_2, which function in part by recruiting soluble proteins to the plasma membrane and allosterically regulating their function (Balla, 2013; Hammond et al., 2012). The largest family of PIP-binding proteins in...
PLEKHA4 Localizes to the Plasma Membrane via Interactions with PI(4,5)P₂

Our interest in PLEKHA4 emerged from a motivation to understand the roles for phosphoinositides in directing signaling via the engagement of their head group by effector proteins bearing both PH domains and additional domains for mediating signaling. PH domain-containing proteins number ~250 in humans, and the majority have not been extensively characterized (Lemmon, 2007). In particular, the PH domain-containing protein PLEKHA4, also known as PEPP1, is part of a family that includes several mediators of intracellular signaling (e.g., FAPP1/2 [D’Angeło et al., 2007; Godi et al., 2004], TAPP1/2 [Li and Marshall, 2015], and PLEKHA7/Hadp1 [Shah et al., 2016]). Other than a single report suggesting that its PH domain binds to phosphatidylinositol 3-phosphate (PI3P) (Dowler et al., 2000) and a computational study predicting that its PH domain binds to phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃) (Jungmichel et al., 2014), PLEKHA4 is an unstudied protein with no known cellular functions. We thus set out to elucidate its molecular properties, subcellular localization, protein interaction partners, and cellular and physiological roles.

We began our studies of PLEKHA4 by examining the properties of the PH domain and how it influences the subcellular localization of the protein. We found that a fluorescent protein fusion to PLEKHA4 localized to the plasma membrane (Figure 1A). This result was surprising because protein-lipid overlay assays had previously suggested to other investigators that the PH domain of PLEKHA4 binds to PI3P, which localizes to endosomes and not to the plasma membrane (Dowler et al., 2000; Schink et al., 2013).

We revisited the PI(4,5)P₂ binding of the PLEKHA4 PH domain (residues 45–167) using liposome sedimentation assays that assess protein-lipid interactions in the context of intact lipid bilayers, which represent a more physiologically relevant environment (Zhao and Lappalainen, 2012). The PLEKHA4 PH domain partially co-sedimented with liposomes containing any one of the three bis-phosphorylated PI(4,5)Ps (PI(3,4,5)P₃, PI(3,5)P₃, and PI(4,5)P₂) and exhibited little affinity for PI3P or the other PI(4,5)P₃ species (Figure 1B). Although moderate, the observed binding was specific, as it was abolished by the mutation of either of two key Arg residues in the PH domain predicted by a crystal structure to contact the PI(4,5)P₃ reporter (Milburn et al., 2004) (Figures 1C and S1A).

A GFP-tagged PH domain adopted a diffuse cytosolic localization, suggesting that a monomeric PH domain was not sufficient to confer the membrane targeting of PLEKHA4 (Figure 1D). We noticed that just upstream of the PH domain were two other motifs that could mediate membrane binding: a putative amphipathic helix (H, residues 28–41) and a basic peptide (BP, residues 42–50) (Figure S1A). The fusion of these motifs to the PH domain afforded a minimal construct (PLEKHA4-H-BP-PH, residues 28–167) that mediated both strong and specific co-sedimentation with PI(4,5)P₂-containing liposomes and localization to the plasma membrane within cells (Figures 1E and 1F). Either a single F40E mutation in the helix motif or a quadruple mutation of Arg/Lys residues within the basic peptide to Ala (4A) abolished binding to PI(4,5)P₂-containing liposomes and the plasma membrane localization of a GFP fusion to this minimal construct (Figures 1G, 1H, and S1A).

To further establish the requirement of PI(4,5)P₂ for the plasma membrane localization of the PLEKHA4-H-BP-PH construct, we transiently depleted this lipid by the stimulation of cells expressing the M₁ muscarinic receptor (M₁R) with its ligand, oxotremorine M, to induce phospholipase C-mediated PI(4,5)P₂ hydrolysis (Falkenburger et al., 2010a, 2010b). The activation of M₁R in HeLa cells expressing H-BP-PH caused shifts in localization of both a PI(4,5)P₂ reporter (PH domain of PLCβ1) and GFP-tagged PLEKHA4-H-BP-PH from the plasma membrane to the cytosol (Figure S1B).

PLEKHA4 Assembles into Higher-Order Structures at the Plasma Membrane

The above data establish a sequence of three N-terminal motifs responsible for PLEKHA4 plasma membrane localization. However, full-length PLEKHA4 is not uniformly distributed at the plasma membrane but is instead strikingly localized to puncta visible by conventional confocal microscopy (Figure 1A) and superresolution structured illumination microscopy (SR-SIM) (Figure 2A). Thus, additional factors beyond the N-terminal PI(4,5)P₂-binding domains may control the localization and/or assembly of the full-length protein.

The PLEKHA4 puncta did not colocalize with the markers of established assemblies at the plasma membrane, including clathrin-coated pits, caveolae, or endoplasmic reticulum-plasma membrane contact sites (Figures S2A–S2C). We also observed...
no colocalization with endosomal and lysosomal markers, which is consistent with our finding that the PH domain does not bind to PI3P (Figure S2 D). We thus hypothesized that the puncta were the result of PLEKHA4 self-association. PLEKHA4 has two adjacent domains at the C terminus that could be responsible for the oligomerization into higher-order structures: a coiled-coil (CC) domain and an intrinsically disordered region (IDR).

Biochemical and imaging experiments support a role for both of these domains in the formation of the PLEKHA4 clusters. First, the isolated CC domain was cytoplasmically localized but could be recruited to the plasma membrane by co-overexpression with full-length PLEKHA4, suggesting a role in dimerization or higher-order oligomerization (Figure 2B). Second, a version of PLEKHA4 lacking the IDR remained at the plasma membrane but no longer assembled into puncta (Figure 2A). Third, a fusion of the CC and IDR domains formed large puncta in the cytoplasm that could, like the isolated CC domain, be recruited to the plasma membrane by full-length PLEKHA4 (Figure 2B). To complement these imaging data, we found via co-immunoprecipitation (co-IP) assays that both the isolated CC domain and a CC-IDR fusion could physically interact with full-length PLEKHA4 (Figure 2C).

The requirement of the IDR for puncta formation and the failure of full-length PLEKHA4 to colocalize with known organelle markers led us to hypothesize that the PLEKHA4 puncta may represent liquid-liquid phase-separated domains. Also referred to as membraneless organelles, these structures form via controlled aggregation of proteins and other biological molecules and can lead to their sequestration from the bulk cytosol (Hyman et al., 2014; Li et al., 2012; Mitrea and Kriwacki, 2016; Shin and Brangwynne, 2017). A recently recognized mechanism of phase separation in biological systems is via non-covalent interactions between highly unstructured, intrinsically disordered protein domains (Elbaum-Garfinkle et al., 2015; Lin et al., 2015; Nott et al., 2015).

To test whether the IDR of PLEKHA4 can mediate assembly into higher-order structures within cells, we deployed an optogenetic method that capitalizes on the propensity of the protein Cryptochrome-2 (CRY2) to homo-oligomerize upon exposure to blue light. By fusing a putative IDR to mCherry (mCh)-tagged CRY2, light can be used to trigger the formation of highly fluorescent, spherical cytoplasmic aggregates termed optoDroplets, whose presence indicates that the IDR can mediate cluster formation (Park et al., 2017; Shin et al., 2017).
We generated mCh-CRY2 fusion constructs to either the PLEKHA4 IDR or the CC-IDR domains. The exposure of cells expressing these domains to blue light led to the rapid formation of intensely fluorescent cytoplasmic aggregates (Figure 2D; Videos S1 and S2). Removal of blue light stimulation led to a partial disaggregation, indicating that cluster formation was reversible. As a negative control, the irradiation of cells expressing mCh-CRY2 under identical conditions did not lead to aggregate formation (Figure 2D; Video S3).

These results indicate that the C-terminal domains of PLEKHA4 are capable of assembly into higher-order structures in a cellular context. To further bolster the notion that avidity and cluster formation is a strong driving force for PLEKHA4 assembly at the plasma membrane, we note that transient PI(4,5)P₂ depletion did not cause a substantial shift in the localization of full-length GFP-PLEKHA4 (Figure S1C). Attempts to purify the isolated C-terminal domains or full-length protein for in vitro analysis were not successful, and a comprehensive in vitro characterization would be necessary to fully understand the nature of the plasma membrane PLEKHA4 puncta. Nevertheless, it is interesting to speculate that the PLEKHA4-positive puncta at the plasma membrane may represent oligomeric, liquid-liquid phase-separated clusters.

**PLEKHA4 Associates with KLHL12, an Adaptor of the E3 Ubiquitin Ligase CUL3**

To explore possible additional components of the PLEKHA4 puncta and to ascertain a function for these assemblies, we searched for the protein-protein interaction partners of PLEKHA4. We generated stable HEK293 cell lines expressing GFP-PLEKHA4 or, as a negative control, GFP, and performed stable isotope labeling by amino acids in cell culture (SILAC)-enabled quantitative proteomics (Hoedt et al., 2014) of anti-GFP immunoprecipitates from each of these cell lines (Figure 3A; Table S1). The strongest hit from these experiments was KLHL12.
We validated the interaction of both PLEKHA4-GFP and GFP-PLEKHA4 with endogenous KLHL12 by co-IP followed by western blot (Figure 3B). To map the interacting regions, we performed co-IP of KLHL12 with several PLEKHA4 truncations and isolated domains (Figure 3C). The minimal region that interacted with KLHL12 is the Pro-rich domain (PRD) of PLEKHA4. A construct including both the PRD and the CC domains (PLEKHA4 PRD-CC) exhibited a much stronger interaction with KLHL12, which we attribute to the capacity of PLEKHA4PRD-CC to oligomerize.

We found that PLEKHA4 can control KLHL12 localization in cells. Whereas fluorescently tagged KLHL12 localizes to cytoplasmic puncta, consistent with previous studies (Mai et al., 2004) (Figure 3D, bottom cell), co-overexpression of PLEKHA4 with KLHL12 recruited KLHL12 to the plasma membrane (Figure 3D, top cell). Loss-of-function studies provide further evidence of a PLEKHA4-KLHL12 interaction. Knockdown of either protein by small interfering RNA (siRNA) resulted in a decrease in the level of the other, consistent with the idea that they mutually stabilize one another within a complex (Figure 3E).

The interaction of PLEKHA4 with KLHL12 provides a window into the potential roles of PLEKHA4 in the cell.
If PLEKHA4 is not a ubiquitination substrate of CUL3-KLHL12, then it instead may function as an adaptor to recruit CUL3-KLHL12 to a site of action at the plasma membrane. Among the established ubiquitination substrates of CUL3-KLHL12, the protein Dishevelled-3 (DVL3) can localize to the plasma membrane. We thus interrogated the levels of the total amount of DVL3 and the extent of DVL3 ubiquitination after modulating PLEKHA4 and/or KLHL12 levels.

We co-expressed FLAG-DVL3 with KLHL12, PLEKHA4, or both KLHL12 and PLEKHA4 (Figure 4A). As expected, the overexpression of KLHL12 decreased FLAG-DVL3 levels (Angers et al., 2006). Overexpression of PLEKHA4 along with KLHL12 led to higher levels of FLAG-DVL3 than overexpression of KLHL12 alone, suggesting that PLEKHA4 attenuates the effect of KLHL12 on DVL3 levels. The expression of PLEKHA4 alone had no significant effect relative to control, consistent with the idea that PLEKHA4 acts through CUL3-KLHL12.

Using a similar approach, we investigated how PLEKHA4 influences the ubiquitination of FLAG-DVL3 (Figure 4B). Here, we found that the overexpression of full-length PLEKHA4 along with KLHL12 attenuates the massive increase in FLAG-DVL3 ubiquitination caused by the overexpression of KLHL12 alone. We used as negative controls versions of PLEKHA4 and KLHL12 that are non-functional (i.e., PLEKHA4<sup>ΔPRD</sup>, which does not interact with KLHL12, and KLHL12<sup>Q405X</sup>, which does not engage CUL3; Angers et al., 2006). Thus, we conclude that the effect of overexpressed PLEKHA4 on FLAG-DVL3 ubiquitination requires its interaction with an active CUL3-KLHL12 E3 ligase.

Loss-of-function studies using siRNA and examining endogenous DVL proteins led to the same conclusions. Knockdown of PLEKHA4 decreased the levels of all three DVL isoforms (DVL1, DVL2, and DVL3), whereas, as expected (Angers et al., 2006), knockdown of KLHL12 led to increases in the levels of the three DVL proteins (Figure 4C). Furthermore, knockdown of PLEKHA4 led to the increased ubiquitination of endogenous DVL3, whereas knockdown of KLHL12 led to the decreased ubiquitination of DVL3 (Figure 4D). The effect of PLEKHA4 knockdown could be rescued by transfection with an siRNA-resistant GFP-PLEKHA4 construct, confirming the specificity of the PLEKHA4 siRNA and the functionality of the GFP-PLEKHA4 construct (Figure 4E). Collectively, these overexpression and knockdown studies support the hypothesis that PLEKHA4 negatively regulates CUL3-KLHL12-mediated ubiquitination of the DVL proteins.
PLEKHA4 Is a Positive Regulator of Canonical and Non-canonical Wnt Signaling in Mammalian Cells

DVL3 and its paralogs DVL1 and DVL2 are central intermediates in the canonical and non-canonical branches of Wnt signaling, which collectively can control many developmental processes (Clevers and Nusse, 2012; Mlodzik, 2016). We thus asked whether the modulation of PLEKHA4 levels would affect Wnt signaling strength. For these assays, we used a mouse fibroblast cell line (C57MG) responsive to Wnt stimulation that contained a Wnt-inducible GFP transcriptional reporter called WntRGreen (Brown et al., 1986; Santiago et al., 2012), as well as human cell lines such as HeLa cells.

PLEKHA4 knockdown decreased WntRGreen fluorescence, as visualized by microscopy (Figure 5A) and as quantified by flow cytometry (Figure 5B). As expected, KLHL12 knockdown increased WntRGreen fluorescence, which is consistent with a role for KLHL12 in mediating DVL3 ubiquitination (Figure 5B). We observed similar effects on DVL3 levels in these C57MG cells (Figure 5C) as we had previously seen in HeLa cells (Figure 4C). From these data, we conclude that PLEKHA4 functions in cells as a positive modulator of Wnt signaling via its effects on DVL3 levels.

To complement the β-catenin-dependent reporter, we also examined the effect of PLEKHA4 knockdown on endogenous Wnt levels using an siRNA-resistant GFP-PLEKHA4 FL construct. Western blot analysis of lysates from HeLa cells subjected to siPLEKHA4 or negative control siRNA (–), stimulation with Wnt3a-containing conditioned media, and transfection with either GFP only or an siRNA-resistant GFP-PLEKHA4 FL construct. *p < 0.05, **p < 0.01, ****p < 0.0001 (n = 3).

In graphs showing quantification, all of the levels were normalized to the loading control (GAPDH), except (F), in which levels were normalized to pan-JNK. Scale bar: 200 μm.

In graphs showing quantification, all of the levels were normalized to the loading control (GAPDH), except (F), in which levels were normalized to pan-JNK. Scale bar: 200 μm.
Finally, we assessed the effect of PLEKHA4 knockdown on a non-canonical Wnt signaling pathway. We found that knockdown of PLEKHA4 attenuated the Wnt5a-stimulated increase in the phosphorylation of c-Jun N-terminal kinase (JNK), which becomes activated upon the exposure of cells to Wnt5a, a stimulus of β-catenin-independent, non-canonical Wnt signaling (Boutros et al., 1998) (Figure 5F).

These data indicate that, in mammalian cells, PLEKHA4 is a positive regulator of both canonical, β-catenin-dependent, and non-canonical, β-catenin-independent, Wnt signaling.

**The Fly PLEKHA4 Homolog, kramer, Is a Specific Modulator of PCP Signaling**

To establish the physiological relevance of our in vitro findings linking PLEKHA4 to Wnt signaling, we investigated the loss of PLEKHA4 function in vivo in *Drosophila melanogaster*. Both canonical Wnt (Wingless) signaling and the PCP pathway are well established in this organism (Simons and Mlodzik, 2008; Swarup and Verheyen, 2012; Wodarz and Nusse, 1998). Although PCP signaling is not known to respond to Wnt ligands, it shares key intracellular signaling molecules and outputs with vertebrate non-canonical Wnt signaling and is considered the *Drosophila* counterpart of this pathway (Axelrod, 2009; Hale and Strutt, 2015). To simplify the analysis, the fly genome has only a single PLEKHA4 homolog, CG34383, which shares the overall domain architecture and 31% sequence identity with PLEKHA4 (including 53% identity between the PH domains).

We generated two CG34383 knockout alleles using CRISPR/Cas9-mediated mutagenesis, both carrying frame-shift mutations early in the coding sequence and thus predicted to be null (Figure S4). We first assessed the physiological effects of the loss of CG34383 in vivo by examining hair patterning in the adult wing. The uniform wing hair orientation (proximal to distal) is controlled by the PCP pathway, which depends on *dishevelled* (dsh), a core PCP signaling component (Devenport, 2014; Hale and Strutt, 2015). Disruption of PCP signaling leads to mis-oriented hair patterns—for example, as seen in a strain homozygous for *dsh*⁠⁻¹ (Figures 6A and S5A), a hypomorphic allele that is defective in PCP (Boutros et al., 1998). We found that knockout of CG34383 causes aberrant wing hair patterns similar to those in the *dsh*⁠⁻¹ flies (Figures 6A and S5A). Due to the irregular hair patterning in the CG34383 mutants, we named this locus *kramer* (*kmr*) and called our two knockout mutant alleles *kmr*⁠⁻¹ and *kmr*⁠⁻². In all of our experiments, both *kmr* alleles exhibited identical phenotypes and may be referred to interchangeably as *kmr*.

We performed two experiments to demonstrate the specificity of these effects. First, we confirmed that the loss of function in *kmr* was responsible for the wing hair phenotype by generating flies hemizygous for the *kmr* alleles with a deletion covering *kmr* and 22 additional genes (Df(3R)Exel6170, called df). The *kmr^pm/kmr* and *kmr^uf/kmr^uf* hemizygotes exhibited wing hair polarity defects identical to those seen in the *kmr*⁻¹ and *kmr*⁻² strains (Figures 6B and S5B).

Second, we tested the genetic interaction of *kmr* with *dsh* by examining hair patterning in transheterozygotic strains containing one allele each of either *kmr*⁻¹ or *kmr*⁻² and one of three different *dsh* alleles, the hypomorphic *dsh*⁻¹ and the amorphic *dsh*⁠⁻² allele. Whereas heterozygotes carrying only one copy of these alleles (*kmr*⁻¹/*kmr*⁻¹, *kmr*⁻¹/*kmr*⁻², *dsh*/*dsh*⁻¹, *dsh*/*dsh*⁠⁻², and *dsh*⁻¹/*dsh*⁠⁻²) exhibited wild-type wing hair patterning (Figures 6C and S5C), transheterozygotes containing one copy each of mutant alleles *kmr* and *dsh* exhibited modest but consistent defects in wing hair patterning (Figures 6D and S5D). This kind of genetic interaction indicates that partial loss of the function of both proteins causes synthetic defects in PCP signaling, strongly suggesting that *kmr* and *dsh* function in the same pathway.

We analyzed defects in hair patterning in two additional adult tissues, the eye and the thorax, using scanning electron microscopy (SEM) imaging. Again, we found that homozygous *kmr*⁻¹ and *kmr*⁻² flies exhibited a loss of polarized hair patterning in these tissues, similar to the *dsh*⁻¹ homoygote (Figures 6E, 6H, S6A, and S7A). Transheterozygote analysis revealed synthetic defects between the two *kmr* alleles and the three *dsh* alleles in the PCP phenotypes in these tissues as well (Figures 6F, 6G, 6I, 6J, S6B, S6C, S7B, and S7C).

**kramer Modulates PCP Signaling by Affecting Dishevelled Levels and Polarized Localization in the Developing Wing**

Finally, to evaluate the mechanism by which *kmr* affects PCP signaling, we examined the levels of the Dsh protein in the...
homozygous kmr<sup>1</sup> and kmr<sup>2</sup> flies. Due to the unavailability of suitable antibodies for immunofluorescence, we used a strain expressing a functional, fluorescently tagged Dsh under the control of the endogenous <i>dsh</i> promoter (<i>dsh::Clover</i>) (Axelrod, 2001).

First, we examined Dsh-Clover expression in the wing imaginal disc, a larval tissue that gives rise to the adult wing. Dsh is moderately enriched at the apical membrane of wing disc epithelial cells (Axelrod, 2001; Wu et al., 2004), which are best visualized at the folds because these locations are where apical membranes of opposing epithelial cells meet (Figure 7A, arrows). Compared to the wild type, homozygous kmr<sup>1</sup> and kmr<sup>2</sup> flies exhibited reduced Dsh-Clover levels or enrichment within the epithelial folds (Figure 7B). These data suggest that the loss of kmr leads to a downregulation of Dsh levels in this tissue and at this developmental time point.

Second, we evaluated the subcellular localization of Dsh-Clover at 30 h after pupariation formation. At this stage, Dsh and other core PCP proteins adopt an asymmetric, polarized localization within the epithelium, in advance of the formation of actin bundles where the trichome (hair) will emerge. In the wild-type background, we observed apparent Dsh-Clover enrichment in the proximal-to-distal boundaries in wild-type background. This asymmetry is partially lost, causing a gain of symmetry in both kmr<sup>-</sup> strains. Two representative images are shown for each genotype, and enlarged images showing a single cell (i–vi) are indicated by the dotted line. The illustrations depict the Dsh subcellular localization pattern in wild-type and the kmr<sup>-</sup> mutant. Ant, anterior; Dist, distal; Post, posterior; Prox, proximal. Scale bars: 50 μm (A, z projection), 10 μm (A, orthogonal projection), 5 μm (C, full-size image), and 2.5 μm (C, enlarged images [i–vi]).

(D) The working model for PLEKHA4/Kmr function. It attenuates DVL ubiquitination by sequestering the CUL3 E3 ubiquitin ligase substrate adaptor KLHL12 in clusters at the plasma membrane to, depending on the context, enhance canonical Wnt signaling and/or non-canonical Wnt/PCP signaling pathways.
DISCUSSION

Phosphoinositides are present in low abundance but act as important constituents of eukaryotic membranes (Balla, 2013; Dickson and Hille, 2019; Schink et al., 2016). A major function of these lipids is to act as membrane identity markers by presenting their head groups as ligands to facilitate the recruitment of cytosolic proteins to the correct target membrane. The most prevalent PI-binding module is the PH domain, which is the 11th most abundant domain in the human proteome, and the physiological functions of the vast majority of PH domain-containing proteins remain unknown (Lemmon, 2008). Our studies reveal a link between PIPs and the control of ubiquitination and Wnt signaling pathways that is mediated by the PH domain-containing protein PLEKHA4.

We found that PLEKHA4 interacts specifically with P(4,5)P_2 at the plasma membrane within cells. Three motifs at the N terminus of PLEKHA4—an amphipathic helix, a basic peptide, and the PH domain—collectively confer the specificity of this recognition. The full-length PLEKHA4 protein assembles into higher-order structures at the plasma membrane that are visible by confocal microscopy as puncta. This assembly is mediated by two C-terminal domains of PLEKHA4: a coiled-coil region and an IDR. These C-terminal domains can self-associate in cells, as ascertained by colocalization, co-IP, and optoDroplet assays (Park et al., 2017; Shin et al., 2017). While the nature of the PLEKHA4 clusters remains unknown, the puncta fail to colocalize with markers of known plasma membrane assemblies, organelles, or membrane contact sites, and it is interesting to speculate they may represent liquid-liquid phase-separated domains (i.e., membraneless organelles) containing PLEKHA4 and other interaction partners (Banjade and Rosen, 2014; Hyman et al., 2014; Li et al., 2012; Mitrea and Kriwacki, 2016; Shin and Brangwynne, 2017).

We characterized a protein-protein interaction between PLEKHA4 and the CUL3 E3 ubiquitin adaptor KLHL12, to which PLEKHA4 binds via its central, proline-rich domain. Mechanistic studies in mammalian cells using both overexpression and RNAi-mediated knockdown of PLEKHA4 indicate a role for this protein as a positive regulator of DVL levels by preventing its polyubiquitination by the CUL3-KLHL12 E3 ligase. These in vitro studies also indicated that PLEKHA4 is a positive modulator of both canonical, β-catenin-dependent and non-canonical, β-catenin-independent Wnt signaling pathways. These in vitro studies support a model wherein PLEKHA4 recruits the CUL3-KLHL12 E3 ligase complex to the plasma membrane

A fascinating aspect of this model is that PLEKHA4 can bring CUL3-KLHL12 to the very membrane where its substrates, the DVL proteins, are activated in Wnt signaling, and yet this recruitment results in less DVL polyubiquitination. We propose that PLEKHA4 sequesters KLHL12 in plasma membrane-associated clusters, in effect creating an “exclusion zone” devoid of CUL3-KLHL12 E3 ligase activity and preventing the polyubiquitination of DVL pools at or near this membrane. DVL proteins form cytoplasmic phase-separated clusters (Sear, 2007), and it is possible that sequestration of KLHL12 at the plasma membrane by PLEKHA4 serves to spatially segregate the E3 ligase from these cytoplasmic clusters.

The knockout studies of the PLEKHA4 homolog in Drosophila melanogaster, kramer (kmr), provided both a validation of our in vitro model in a physiologically relevant setting and revealed layers of regulation of Dishevelled-dependent signaling in this organism. We found that knockout of kmr led to selective defects in PCP signaling, which corresponds to non-canonical Wnt signaling in Drosophila (Axelrod, 2009; Hale and Strutt, 2015; Simons and Mlodzik, 2008). Kmrr knockoout flies exhibited defects in hair patterning in the adult wing, eye, and thorax. Mechanistically, we established that kmr and dsh act in the same genetic pathway, as we observed synthetic defects in PCP signaling in transheterozygotic strains. Finally, we assessed the effect of kmr knockout on Dsh levels and localization in the developing wing. We found lower levels of Dsh enriched at the apical epithelium in the larval wing imaginal disc and a loss of asymmetric, polarized Dsh distribution at the plasma membrane in the pupal wing epithelium.

In contrast to these PCP phenotypes, we did not observe any phenotypes associated with Wingless signaling, which corresponds to the canonical, β-catenin-dependent pathway in flies, in kmr^1 and kmr^2 flies (Swapur and Verheyen, 2012). Given the substantial remaining levels of Dsh in the kmr^1 larval wing imaginal disc and pupal wing, it is possible that the partial, modulatory effect of kmr knockout on Dsh levels and localization is not sufficient to cause defects in Wingless signaling in Drosophila. In addition, kmr specifically regulates the localization of Dsh and thus affects PCP signaling in flies. Nonetheless, our results in cultured mammalian cell lines demonstrating the effect of PLEKHA4 knockdown on both the β-catenin-dependent and -independent signaling pathways suggest that the regulatory role of PLEKHA4 in vivo may go beyond the non-canonical Wnt signaling pathway in other organisms.

A detailed study of the Drosophila homologs of KLHL12 (diablo and kelch) established roles for the CUL3-Diablo (or Kelch) E3 ligase complex in modulating PCP signaling in vivo in this organism (Strutt et al., 2013). Notably, the study found that subtle changes to the levels of core PCP proteins such as Dsh led to a breakdown of their asymmetric localization in the pupal wing, leading to PCP phenotypes. They also found that Cull-Diablo/Kelch-mediated modulation of Dsh levels in Drosophila led to selective effects on PCP signaling, with no observed effects on Wingless signaling. By contrast, Moon and colleagues found that in vertebrate systems (mammalian cells and zebrafish embryos), the homologous CUL3-KLHL12 E3 ligase complex modulated DVL levels and the strength of canonical, β-catenin-dependent Wnt signaling (Angers et al., 2006).

Our data suggest that PLEKHA4/kmr acts as a tuner to attenuate the CUL3-4KLHL12-mediated polyubiquitination of DVL proteins by sequestration of KLHL12. We propose that, by creating different DVL setpoints, PLEKHA4/kmr can modulate the sensitivity of cells to stimulation by appropriate ligands that propagate canonical Wnt and/or non-canonical Wnt, or PCP, signaling pathways. In this model, the pathway most affected by PLEKHA4/kmr is determined by the relative dependence of
that pathway on changes in DVL levels in that tissue context. Furthermore, PLEKHA4/kmr may affect DVL levels to different extents in different contexts. Given the critical importance of Wnt signaling pathways in mammalian development and disease, PLEKHA4 may function as a regulator of these pathways in vivo in mammals as well.

Finally, the production and maintenance of PI(4,5)P2 at the plasma membrane is important for both canonical and non-canonical Wnt signaling pathways. PI(4,5)P2 enhances the strength of canonical Wnt signaling via effects on the Wnt co-receptor LRP6, and Wnt3a stimulation increases PI(4,5)P2 synthesis via the direct action of DVL on lipid kinases that synthesize PI(4,5)P2, effectively amplifying this lipid-based signal (Pan et al., 2008; Qin et al., 2009). PI(4,5)P2 is also a key determinant of cell polarity, aiding in both establishing the asymmetric spatial arrangement of polarity proteins and in activating actin-nucleating factors (Gassama-Diagne and Payrastre, 2009; Hassan et al., 1998; Shewan et al., 2011; Yin and Janmey, 2003). Given the central role of DVL in PCP signaling (Gao and Chen, 2010; Mlodzik, 2016), its ability to stimulate PI(4,5)P2 synthesis (Hu et al., 2015; Qin et al., 2009), and the critical role of actin dynamics in PCP signaling (Simons and Mlodzik, 2008; Yang and Mlodzik, 2015), changes to PI(4,5)P2 synthesis may directly affect this pathway as well. As a factor that binds to plasma membrane PI(4,5)P2 and affects DVL levels, PLEKHA4 adds a further layer of regulation to DVL-dependent pathways, including Wnt and PCP signaling.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.04.060.

**ACKNOWLEDGMENTS**

This work was supported in part by the NIH (R00GM110121 and R01GM131101 to J.M.B., R01NS099125 and R21OD023824 to C.H., R01GM084830 to M.L.G., and R01GM097272 and R01GM123018 to M.B.S.) and awards to J.M.B. from the Arnold and Mabel Beckman Foundation (Beckman Young Investigator) and the Alfred P. Sloan Foundation (Sloan Research Fellowship). Superresolution structured illumination microscopy (SR-SIM) imaging was performed at the Cornell University Biotechnology Resource Center, with support from the National Science Foundation (NSF) (DBI-1428922). SEM imaging was performed at the Cornell Center for Materials Research Facilities, with support from the NSF (DMR-1719875). We thank Jeffrey Axelrod and Song Song (Stanford University) for generously providing dsh:clover fly stocks and for helpful discussions; Anthony Brown (Weill Cornell Medicine) for generously providing C57MG-WntRGreen cells and for helpful discussions; the Barbash, Emr, Fromme, Lammerding, and Mao labs for sharing equipment and reagents; and Scott Emr, Chris Fromme, Shaogeng Tang, and Laura Thomas for helpful discussions and/or critical reading of the manuscript.

**AUTHOR CONTRIBUTIONS**

Conceptualization, A.S. and J.M.B.; Investigation, A.S., A.G.B., D.K., A.P., and W.C.; Writing – Original Draft, A.S. and J.M.B.; Supervision, C.H., M.L.G., M.B.S., and J.M.B.; Funding Acquisition, C.H., M.L.G., M.B.S., and J.M.B.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

Received: July 25, 2018
Revised: February 26, 2019
Accepted: April 11, 2019
Published: May 14, 2019

**REFERENCES**

Angers, S., Thorpe, C.J., Biechele, T.L., Goldenberg, S.J., Zheng, N., MacCoss, M.J., and Moon, R.T. (2006). The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-β-catenin pathway by targeting Dishevelled for degradation. Nat. Cell Biol. 8, 348–357.

Axelrod, J.D. (2001). Unipolar membrane association of Dishevelled mediates Frizzled planar cell polarity signaling. Genes Dev. 15, 1182–1187.

Axelrod, J.D. (2009). Progress and challenges in understanding planar cell polarity signaling. Semin. Cell Dev. Biol. 20, 964–971.

Ball, T. (2013). Phosphoinositides: tiny lipids with giant impact on cell regulation. Physiol. Rev. 93, 1019–1137.

Banerjee, S., and Rosen, M.K. (2014). Phase transitions of multivalent proteins can promote clustering of membrane receptors. eLife 3, e04123.

Bastos de Oliveira, F.M., Kim, D., Cussiol, J.R., Das, J., Jeong, M.C., Doerfler, L., Schmidt, K.H., Yu, H., and Smolka, M.B. (2015). Phosphoproteomics
reveals distinct modes of Mec1/ATR signaling during DNA replication. Mol. Cell 57, 1124–1132.

Bastos de Oliveira, F.M., Kim, D., Lanz, M., and Smolka, M.B. (2018). Quantitative Analysis of DNA Damage Signaling Responses to Chemical and Genetic Perturbations. Methods Mol. Biol. 1672, 645–660.

Bolatto, C., Parada, C., and Colmenares, V. (2017). A Rapid and Efficient Method to Dissect Pupal Wings of Drosophila Suitable for Immunodetections. Methods Mol. Biol. 1388, 313–343.

Boutros, M., Paricio, N., Strutt, D.I., and Mlodzik, M. (1998). Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell 94, 109–118.

Brown, A.M.C., Wildin, R.S., Prendergast, T.J., and Varmus, H.E. (1986). A retrovirus vector expressing the putative mammary oncogene int-1 causes partial transformation of a mammary epithelial cell line. Cell 46, 1001–1009.

Cadigan, K.M., and Peifer, M. (2000). Wnt signaling from development to disease: insights from model systems. Cold Spring Harb. Perspect. Biol. 1, a002881.

Clevers, H., and Nusse, R. (2012). Wnt/iP-catenin signaling and disease. Cell 149, 1192–1205.

D’Angelo, G., Polischuk, E., Di Tullio, G., Santoro, M., Di Campi, A., Godi, A., West, G., Bielawski, J., Chuang, C.C., van den Spoel, A.C., et al. (2007). Glycosphingolipid synthesis requires FAPP2 transfer of glucosylceramide. Nature 449, 62–67.

Devenport, D. (2014). The cell biology of planar cell polarity. J. Cell Biol. 207, 171–179.

Dickson, E.J., and Hille, B. (2019). Understanding phosphoinositides: rare, dynamic, and essential membrane phospholipids. Biochem. J. 476, 1–23.

Dowler, S., Currie, R.A., Campbell, D.G., Deak, M., Kular, G., Downes, C.P., and Alessi, D.R. (2000). Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities. Biochem. J. 351, 19–31.

Dubiel, W., Dubiel, D., Wolf, D.A., and Naumann, M. (2018). Cullin 3-Based Ubiquitin Ligases as Master Regulators of Mammalian Cell Differentiation. Trends Biochem. Sci. 43, 95–107.

Elbaum-Garfinkle, S., Kim, Y., Szczepaniak, K., Chen, C.C.-H., Eckmann, C.R., Myong, S., and Brangwynne, C.P. (2015). The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics. Proc. Natl. Acad. Sci. USA 112, 7189–7194.

Falkenburger, B.H., Jensen, J.B., and Hille, B. (2010a). Kinetics of M1 muscarinic receptor and G protein signaling to phospholipase C in living cells. J. Gen. Physiol. 135, 81–97.

Falkenburger, B.H., Jensen, J.B., and Hille, B. (2010b). Kinetics of PIP2 metabolism and KCNQ2/3 channel regulation studied with a voltage-sensitive phosphatase in living cells. J. Gen. Physiol. 135, 99–114.

Furukawa, M., He, Y.J., Borchers, C., and Xiong, Y. (2003). Targeting of protein phosphatase 2A by the Drosophila kelch protein, involved in a ring-like structure. Exp. Cell Res. 290, 72–83.

Gao, C., and Chen, Y.G. (2010). Dishevelled: the hub of Wnt signaling. Cell. Signal. 22, 717–727.

Gao, C., Cao, W., Bao, L., Zuo, W., Xie, G., Cai, T., Fu, W., Zhang, J., Wu, W., Zhang, X., and Chen, Y.G. (2010). Autophagy negatively regulates Wnt signaling by promoting Dishevelled degradation. Nat. Cell Biol. 12, 781–790.

Gassama-Diagne, A., and Payrastre, B. (2009). Phosphoinositide Signaling Pathways. Promising Role as Builders of Epithelial Cell Polarity. Int. Rev. Cell. Mol. Biol. 273, 313–343.

Godi, A., Di Campi, A., Konstantakopoulos, A., Di Tullio, G., Alessi, D.R., Kular, G.S., Daniele, T., Marra, P., Lucocc, J.M., and De Matteis, M.A. (2004). FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P. Nat. Cell Biol. 6, 393–404.

Gómez-Orte, E., Sáenz-Narciso, B., Moreno, S., and Cabello, J. (2013). Multiple functions of the noncanonical Wnt pathway. Trends Genet. 29, 545–553.

Hale, R., and Strutt, D. (2015). Conservation of Planar Polarity Pathway Function Across the Animal Kingdom. Annu. Rev. Genet. 49, 529–551.

Hammond, G.R.V., and Balla, T. (2015). Polyphosphoinositide binding domains: key to inositol lipid biology. Biochim. Biophys. Acta 1851, 746–758.

Hammond, G.R.V., Fischer, M.J., Anderson, K.E., Holdich, J., Kotecki, A., Balla, T., and Irvine, R.F. (2012). PI(4)P and PI(4,5)P2 are essential but independent lipid determinants of membrane identity. Science 337, 727–730.

Hassan, B.A., Prokopenko, S.N., Breuer, S., Zhang, B., Paululat, U., and Bellen, H.J. (1998). skittles, a Drosophila phosphatidylinositol 4-phosphate 5-kinase, is required for cell viability, germine development and bristle morphology, but not for neurotransmitter release. Genetics 150, 1527–1537.

Hoedt, E., Zhang, G., and Neubert, T.A. (2014). Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) for Quantitative Proteomics. In Advances of Mass Spectrometry in Biomedical Research, A.G. Woods and C.C. Darie, eds. (Springer), pp. 93–106.

Hu, J., Yuan, Q., Kang, X., Qin, Y., Li, L., Ha, Y., and Wu, D. (2015). Resolution of structure of PIUS51A reveals molecular mechanism for its regulation by dimerization and dishevelled. Nat. Commun. 6, 8205.

Hyman, A.A., Weber, C.A., and Jülicher, F. (2014). Liquid-liquid phase separation in biology. Annu. Rev. Cell Dev. Biol. 30, 39–58.

Idevall-Hagren, O., Dickson, E.J., Hille, B., Toomre, D.K., and De Camilli, P. (2012). Optogenetic control of phosphoinositide metabolism. Proc. Natl. Acad. Sci. USA 109, E2316–E2323.

Janda, C.Y., Waghray, D., Levin, A.M., Thomas, C., and Garcia, K.C. (2012). Structural basis of Wnt recognition by Frizzled. Science 337, 59–64.

Jin, L., Pahuja, K.B., Wickliffe, K.E., Gorur, A., Baumgartel, C., Schekman, R., and Rape, M. (2012). Ubiquitin-dependent regulation of COPII coat size and function. Nature 482, 495–500.

Jungmichel, S., Sylvestersen, K.B., Choudhary, C., Nguyen, S., Mann, M., and Nielsen, M.L. (2014). Specificity and commonality of the phosphoinositide-binding proteome analyzed by quantitative mass spectrometry. Cell Rep. 6, 578–591.

Lemmon, M.A. (2007). Pleckstrin homology (PH) domains and phosphoinositides. Biochem. Soc. Symp. 74, 81–93.

Lemmon, M.A. (2008). Membrane recognition by phospholipid-binding domains. Nat. Rev. Mol. Cell Biol. 9, 99–111.

Li, H., and Marshall, A.J. (2015). Phosphatidylinositol (3,4) bisphosphate-specific phosphatases and effector proteins: a distinct branch of PI3K signaling. Cell. Signal. 27, 1789–1798.

Li, P., Banjade, S., Cheng, H.C., Kim, S., Chen, B., Guo, L., Liaguno, M., Hollows, J.V., King, D.S., Banani, S.F., et al. (2012). Phase transitions in the assembly of multivalent signalling proteins. Nature 483, 336–340.

Lin, Y., Potter, D.S.W., Rosen, M.K., and Parker, R. (2015). Formation and Maturation of Phase-Separated Liquid Droplets by RNA-Binding Proteins. Mol. Cell 60, 208–219.

MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/iP-catenin signaling: components, mechanisms, and diseases. Dev. Cell 17, 9–26.

Mai, A., Jung, S.K., and Yonehara, S. (2004). hDKIR, a human homologue of the Drosophila kelch protein, involved in a ring-like structure. Exp. Cell Res. 300, 72–83.

McGourty, C.A., Akopian, D., Walsh, C., Gorur, A., Werner, A., Schekman, R., Bautista, D., and Rape, M. (2016). Regulation of the CUL3 Ubiquitin Ligase by a Calcium-Dependent Co-adapter. Cell 167, 525–536.e14.

Milburn, C.C., Komander, D., Deak, M., Alessi, D.R., and Van Aalten, D.M.F. (2004). 1UPR: Crystal structure of the PEPP1 pleckstrin homology domain in complex with inositol 1,3,4,5-tetrakisphosphate. https://www.ncbi.nlm.nih.gov/Structure/pdb/1UPR.
Mitrea, D.M., and Kriwacki, R.W. (2018). Phase separation in biology: functional organization of a higher order. Cell Commun. Signal. 14, 1.

Miyazaki, K., Fujita, T., Ozaki, T., Kato, C., Kurose, Y., Sakamoto, M., Kato, S., Goto, T., Itoyama, Y., Aoki, M., and Nakagawa, A. (2004). NEDL1, a novel ubiquitin-protein isopeptide ligase for dishevelled-1, targets mutant superoxide dismutase-1. J. Biol. Chem. 279, 11327–11335.

Mlodzik, M. (2016). The Dishevelled Protein Family: Still Rather a Mystery After Over 20 Years of Molecular Studies. Curr. Top. Dev. Biol. 117, 75–91.

Nott, T.J., Petsalaki, E., Farber, P., Jervis, D., Fussner, E., Plchowitz, A., Craggs, T.D., Bazett-Jones, D.P., Pawson, T., Forman-Kay, J.D., and Baldwin, A.J. (2015). Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles. Mol. Cell 57, 936–947.

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 LRPI phosphorylation. Science 321, 1350–1353.

Park, H., Kim, N.Y., Lee, S., Kim, N., Kim, J., and Heo, W.D. (2017). Optogenetic protein clustering through fluorescent protein tagging and extension of CRY2. Nat. Commun. 8, 30.

Qin, Y., Li, L., Pan, W., and Wu, D. (2009). Regulation of phosphatidylinositol kinases and metabolism by Wnt3a and Dvl. J. Biol. Chem. 284, 22544–22548.

Rondou, P., Haegeman, G., Vanhoenacker, P., and Van Craenenbroeck, K. (2008). BTB Protein KHL12 targets the dopamine D4 receptor for ubiquitination by a Cul3-based E3 ligase. J. Biol. Chem. 283, 11083–11096.

Santiago, F., Oguma, J., Brown, A.M.C., and Laurence, J. (2012). Noncanonical Wnt signaling promotes osteoclast differentiation and is facilitated by the human immunodeficiency virus protease inhibitor ritonavir. Biochem. Biophys. Res. Commun. 417, 223–230.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schind, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.

Schink, K.O., Raitborg, C., and Stemmer, H. (2013). Phosphatidylinositol 3-phosphate, a lipid that regulates membrane dynamics, protein sorting and cell signalling. BioEssays 35, 900–912.

Schink, K.O., Tan, K.-W., and Stemmer, H. (2016). Phosphoinositides in Control of Membrane Dynamics. Annu. Rev. Cell Dev. Biol. 32, 143–171.

Sear, R.P. (2007). Dishevelled: a protein that functions in living cells by phase separating. Soft Matter 3, 680–684.

Shah, J., Guerrera, D., Vasilevva, E., Sluyssmans, S., Bertels, E., and Citi, S. (2016). PLEKHA7: cytoskeletal adaptor protein at center stage in junctional organization and signaling. Int. J. Biochem. Cell Biol. 75, 112–116.

Sharma, J., Mulherkar, S., Mukherjee, D., and Jana, N.R. (2012). Malin regulates Wnt signaling pathway through degradation of dishevelled2. J. Biol. Chem. 287, 6830–6839.

Shewan, A., Eastburn, D.J., and Mostov, K. (2011). Phosphoinositides in cell architecture. Cold Spring Harb. Perspect. Biol. 3, a004796.

Shin, Y., and Brangwynne, C.P. (2017). Liquid phase condensation in cell physiology and disease. Science 357, aaaf4382.

Shin, Y., Berry, J., Panucci, N., Haataja, M.P., Toettcher, J.E., and Brangwynne, C.P. (2017). Spatiotemporal Control of Intracellular Phase Transitions Using Light-Activated optoDroplets. Cell 168, 159–171.e14.

Simons, M., and Mlodzik, M. (2008). Planar cell polarity signaling: from fly development to human disease. Annu. Rev. Genet. 42, 517–540.

Sprott, C.M., and Kumar, J.P. (2014). Dissection and immunostaining of imaginal discs from Drosophila melanogaster. J. Vis. Exp. 91, 51792.

Strutt, H., Searle, E., Thomas-Macarthur, V., Brookfield, R., and Strutt, D. (2013). A Cul-3-BTB ubiquitylation pathway regulates junctional levels and asymmetry of core planar polarity proteins. Development 140, 1693–1702.

Swarup, S., and Verheyen, E.M. (2012). Wnt/Wingless signaling in Drosophila. Cold Spring Harb. Perspect. Biol. 4, a007930.

Wallington, J.B. (2012). Planar cell polarity and the developmental control of cell behavior in vertebrate embryos. Annu. Rev. Cell Dev. Biol. 28, 627–653.

Wallington, J.B., and Habas, R. (2005). The developmental biology of Dishevelled: an enigmatic protein governing cell fate and cell polarity. Development 132, 4421–4436.

Wei, W., Li, M., Wang, J., Nie, F., and Li, L. (2012). The E3 ubiquitin ligase ITCH negatively regulates canonical Wnt signaling by targeting dishevelled protein. Mol. Cell. Biol. 32, 3900–3912.

Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. Annu. Rev. Cell Dev. Biol. 14, 59–88.

Wu, J., Klein, T.J., and Mlodzik, M. (2004). Subcellular localization of frizzled receptors, mediated by their cytoplasmic tails, regulates signaling pathway specificity. PLoS Biol. 2, E156.

Xu, L., Wei, Y., Reboul, J., Vaglio, P., Shin, T.H., Vidal, M., Elledge, S.J., and Harper, J.W. (2003). BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. Nature 425, 316–321.

Yang, Y., and Mlodzik, M. (2015). Wnt-Frizzled/planar cell polarity signaling: cellular orientation by facing the wind (Wnt). Annu. Rev. Cell Dev. Biol. 31, 623–646.

Yin, H.L., and Janmey, P.A. (2003). Phosphoinositide regulation of the actin cytoskeleton. Annu. Rev. Physiol. 65, 761–789.

Zhao, H., and Lappalainen, P. (2012). A simple guide to biochemical approaches for analyzing protein-lipid interactions. Mol. Biol. Cell 23, 2823–2830.
# STAR Methods

## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-PLEKHA4 | Abcam | Cat#ab170537 |
| Mouse monoclonal anti-KLHL12 | ProMab Biotechnology | Cat#30058 |
| Mouse monoclonal anti-DVL1 | Santa Cruz Biotechnology | Cat#sc-8025 [3F12] |
| Rabbit polyclonal anti-DVL2 | Cell Signaling Technology | Cat#3216 |
| Mouse monoclonal anti-DVL3 | Santa Cruz Biotechnology | Cat#sc-8027 [4D3] |
| Rabbit monoclonal anti-Axin2 | Cell Signaling Technology | Cat#2151 [76G6] |
| Rabbit monoclonal anti-Axin2 | Abcam | Cat#ab109307 |
| Mouse monoclonal anti-p-JNK | Santa Cruz Biotechnology | Cat#sc-7345 [D2] |
| Mouse monoclonal anti-GFP | Takara Bio | Cat #632375 Living Colors |
| Mouse monoclonal anti-mCherry | Abcam | Cat#ab125096 [1C51] |
| Rabbit polyclonal anti-FLAG | Millipore Sigma | Cat#F7425 |
| Rat monoclonal anti-HA | Roche | Cat#1186742001 [3F10] |
| Mouse monoclonal anti-Ubiquitin | Santa Cruz Biotechnology | Cat#sc-8017 [P4D1] |
| Mouse monoclonal anti-GAPDH | GeneTex | Cat#GTX78213 [1D4] |
| Rabbit polyclonal anti-Caveolin 1 | BD Biosciences | Cat#610059 |
| Rabbit polyclonal anti-EEA1 | Thermo Fisher Scientific | PA1-063A |
| Mouse monoclonal anti-Rab3 | Pietro De Camilli, Yale | CL42.1 ascites |
| Rabbit monoclonal anti-Rab8 | Cell Signaling Technology | Cat#6975S [D22D8] |
| Rabbit monoclonal anti-Rab11 | Thermo Fisher Scientific | Cat#700184 [3H18L5] |

**Chemicals, Peptides, and Recombinant Proteins**

| Name                                           | Source                          | Identifier |
|------------------------------------------------|---------------------------------|------------|
| Osmium Tetroxide (4% solution)                  | Electron Microscopy Sciences    | Cat#RT 19140 |
| Protein G-Sepharose resin                       | BioVision Inc.                  | Cat#6511-5 |
| DPX                                             | Millipore Sigma                 | Cat#06522 |
| L-α-phosphatidylinositol (Liver, Bovine) (sodium salt) (PI) | Avanti Polar Lipids           | Cat#640042 |
| 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DOPS) | Avanti Polar Lipids           | Cat#64003C |
| 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) | Echelon Biosciences             | Cat#L-1182 |
| Dil'®;DiIC<sub>4</sub>(7) (1,1'-Diocadecyl-3,3',3'-Tetramethylindocarbocyanine iodide) | Thermo Fisher Scientific       | Cat#D12731 |
| L-α-myophosphatidylinositol 3-monophosphate, 3-O-phospho linked, D(+)-sn-1,2-di-O-hexadecanoylglyceryl (PI3P) | CellSignals Inc.              | Cat#910    |
| L-α-myophosphatidylinositol 4-monophosphate, 3-O-phospho linked, D(+)-sn-1,2-di-O-hexadecanoylglyceryl (PI4P) | CellSignals Inc.              | Cat#912    |
| L-α-myophosphatidylinositol 5-monophosphate, 3-O-phospho linked, D(+)-sn-1,2-di-O-hexadecanoylglyceryl (PI5P) | CellSignals Inc.              | Cat#914    |
| L-α-myophosphatidylinositol 4,5-bisphosphate, 3-O-phospho linked, D(+)-sn-1,2-di-O-hexadecanoylglyceryl (PI(4,5)P<sub>2</sub>) | CellSignals Inc.              | Cat#902    |
| L-α-myophosphatidylinositol 3,4-bisphosphate, 3-O-phospho linked, D(+)-sn-1,2-di-O-hexadecanoylglyceryl (PI(3,4)P<sub>2</sub>) | CellSignals Inc.              | Cat#904    |
| L-α-myophosphatidylinositol 3,5-bisphosphate, 3-O-phospho linked, D(+)-sn-1,2-di-O-hexadecanoylglyceryl (PI(3,5)P<sub>2</sub>) | CellSignals Inc.              | Cat#906    |
| L-α-myophosphatidylinositol 3,4,5-trisphosphate, 3-O-phospho linked, D(+)-sn-1,2-di-O-hexadecanoylglyceryl (PI(3,4,5)P<sub>3</sub>) | CellSignals Inc.              | Cat#908    |

(Continued on next page)
### Experimental Models: Cell Lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Human: Flp-In T-REx HeLa | Pietro De Camilli, Yale | N/A |
| Human: Flp-In T-REx HeLa GFP-PLEKHA4 | This paper | N/A |
| Human: Flp-In T-REx HeLa PLEKHA4-GFP | This paper | N/A |
| Human: HEK293 TN | Tony Bretscher, Cornell | N/A |
| Human: Flp-In HEK293–GFP (SILAC Heavy/Light) | This paper | N/A |
| Human: Flp-In HEK293–PLEKHA4-GFP (SILAC Heavy/Light) | This paper | N/A |
| Mouse: C57MG–WntRGreen | Anthony Brown, Weill Cornell Medicine | N/A |
| Mouse: MV7 Rat2a (control) MV7 Rat2a–Wnt1 | Gerlinde Van De Walle, Cornell | N/A |
| Mouse: L (control) L–Wnt3a | Anthony Brown, Weill Cornell Medicine | N/A |

### Experimental Models: Organisms/Strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| D. melanogaster: y[1] M[w[+mC] = Act5C-Cas9.P]ZH-2A w[*] | Bloomington Drosophila Stock Center | Stock# 54590 |
| D. melanogaster: y[1] P[ry[17.7] nos-phiC31 \.int.NLS]; P(CaryFattP40 | Bloomington Drosophila Stock Center | Stock# 25709 |
| D. melanogaster: y v; TM3, Sb / TM6B, Tb Hu | This paper | N/A |
| D. melanogaster: sp/CyoW (II); TM2/TM6B, Tb (III) | This paper | N/A |
| D. melanogaster: w[1] dsh[1] | Bloomington Drosophila Stock Center | Stock# 5298 |
| D. melanogaster: w[1] dsh[3] P[ry+[17.2] = neoFRT]19A/FM7a | Bloomington Drosophila Stock Center | Stock# 6331 |
| D. melanogaster: dsh[75] P[ry+[17.2] = neoFRT]19A/FM7a | Bloomington Drosophila Stock Center | Stock# 68165 |
| D. melanogaster: w[1118]; Df(3R)Exel6170, P[w[+mC] = XP-U]Exel6170/TM6B, Tb1[1] | Bloomington Drosophila Stock Center | Stock# 7649 |
| D. melanogaster: pCasper4-Dsh-clover2 | Jeffrey Axelrod, Stanford | N/A |

### Oligonucleotides

See Table S2 for oligonucleotide information

### Recombinant DNA

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| PLEKHA4 cDNA | DNASU | BC024157 |
| pEGFP-C1 | Clontech | Cat#6084-1 |
| pEGFP-N1 | Clontech | Cat#6085-1 |
| mCherry-N1 | Clontech | Cat#632523 |
| mCherry-C1 | Clontech | Cat#632524 |
| GFP-PLEKHA4 | This paper | N/A |
| PLEKHA4-GFP | This paper | N/A |
| mCherry-PLEKHA4 | This paper | N/A |
| PLEKHA4-mCherry | This paper | N/A |
| GFP-PLEKHA4PH (45–167) | This paper | N/A |
| GFP-PLEKHA4PRD (167–357) | This paper | N/A |
| GFP-PLEKHA4PRD-CC (167–495) | This paper | N/A |
| GFP-PLEKHA4PRD-CC-IDR (167–779) | This paper | N/A |
| GFP-PLEKHA4CC (357–495) | This paper | N/A |
| GFP-PLEKHA4CC-IDR (357–779) | This paper | N/A |
| GFP-PLEKHA4IDR (495–779) | This paper | N/A |
| GFP-PLEKHA4H-BP-PH (28–167) | This paper | N/A |
| PLEKHA4SN-IDR (28–495)-GFP | This paper | N/A |
| GFP-PLEKHA4ΔCC-IDR (1–357) | This paper | N/A |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| GFP-PLEKHA4<sup>ΔN+ΔBP</sup> (54–779) | This paper | N/A |
| GFP-PLEKHA4<sup>ΔPH</sup> (1–45, 167–779) | This paper | N/A |
| GFP-PLEKHA4<sup>ΔH+ΔBP+ΔPH</sup> (1–27, 168–779) | This paper | N/A |
| GFP-PLEKHA4<sup>ΔPRD</sup> (1–167, 357–779) | This paper | N/A |
| GFP-PLEKHA4<sup>ΔDR</sup> (1–495) | This paper | N/A |
| pCDNA5-FRT | Thermo Fisher | Cat#K601002 |
| pCDNA5-FRT-GFP-PLEKHA4 | This paper | N/A |
| pCDNA5-FRT-PLEKHA4-GFP | This paper | N/A |
| pCDNA5-FRT | This paper | N/A |
| pcDNA3.1+zeo-VSV-KLHL12 | Addgene | Cat#16761 |
| pCMV10-3xFLAG | Sigma | Cat#E7658 |
| 3xFLAG-KLHL12 | This paper | N/A |
| GFP-KLHL12 | This paper | N/A |
| mCherry-KLHL12 | This paper | N/A |
| pCMV-HA-N | Clontech | Cat#635690 |
| CUL3 ORF | ORFeome8.1 library (Haiyuan Yu, Cornell University) | N/A |
| HA-CUL3 | This paper | N/A |
| HA-Ub | Pietro De Camilli, Yale | N/A |
| M1R-mCherry | Pietro De Camilli, Yale | N/A |
| iRFP-PLC31-PH | Pietro De Camilli, Yale | N/A |
| mCherry-CRY2(PHR) | (devall-Hagren et al., 2012) Pietro De Camilli, Yale | N/A |
| mCherry-CRY2-PLEKHA4<sup>ΔDR</sup> | This paper | N/A |
| mCherry-CRY2-PLEKHA4<sup>ΔCC-ΔDR</sup> | This paper | N/A |
| GFP-PLEKHA4<sup>ΔPH</sup> R75A | This paper | N/A |
| GFP-PLEKHA4<sup>ΔPH</sup> R129A | This paper | N/A |
| GST-PLEKHA4<sup>ΔPH</sup> R75A | This paper | N/A |
| GST-PLEKHA4<sup>ΔPH</sup> R129A | This paper | N/A |
| PLEKHA4<sup>ΔBP-PH-GFP</sup> | This paper | N/A |
| PLEKHA4<sup>ΔBP-PH-GFP-F40E</sup> | This paper | N/A |
| PLEKHA4<sup>ΔBP-PH-GFP-R75A</sup> | This paper | N/A |
| PLEKHA4<sup>ΔBP-PH-GFP-R129A</sup> | This paper | N/A |
| PLEKHA4<sup>ΔBP-PH-GFP-K42A/R43A/R48A/R49A (4A)</sup> | This paper | N/A |
| GST-PLEKHA4<sup>ΔBP-PH</sup> | This paper | N/A |
| GST-PLEKHA4<sup>ΔBP-PH-F40E</sup> | This paper | N/A |
| GST-PLEKHA4<sup>ΔBP-PH-R75A</sup> | This paper | N/A |
| GST-PLEKHA4<sup>ΔBP-PH-R129A</sup> | This paper | N/A |
| GST-PLEKHA4<sup>ΔBP-PH-K42A/R43A/R48A/R49A (4A)</sup> | This paper | N/A |
| pcDNA3.1+zeo-VSV-KLHL12: Q405X | This paper | N/A |
| GFP-PLEKHA4 S103, I106, R107, D109, and G110 silent (siRNA resistant) | This paper | N/A |
| PLEKHA4-GFP S103, I106, R107, D109, and G110 silent (siRNA resistant) | This paper | N/A |

**Software and Algorithms**

| Tool | Reference | Website |
|------|-----------|---------|
| Fiji (ImageJ) | Schindelin et al., 2012 | https://imagej.net/Fiji |
| Zen Blue 2.3 | Zeiss | N/A |
| Zen Black | Zeiss | N/A |
| BD Accuri C6 | BD Biosciences | N/A |
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeremy Baskin (jeremy.baskin@cornell.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture

Flp-In T-REx HeLa (Thermo Fisher), Flp-In HEK293 (Thermo Fisher), C57MG WntRGreen (Anthony Brown), L, L Wnt-3a, and L Wnt-5a cells (ATCC) and HEK293TN cells (Anthony Bretscher) were cultured in Dulbecco’s modified Eagle medium (DMEM, Corning) supplemented with 10% fetal bovine serum (FBS, Corning) and 1% penicillin/streptomycin (P/S, Corning) at 37°C in a 5% CO2 atmosphere. HEK293 cell lines were also supplemented with 1% sodium pyruvate (Corning) in the media. MV7-Rat2a-Wnt1 (Wnt1-secreting) and MV7-Rat2a (control) were cultured in above-mentioned conditions but in low glucose (1 g/L) DMEM. Stable expression of GFP, GFP-PLEKHA4 or PLEKHA4-GFP was achieved by transfecting Flp-In T-REx HeLa or Flp-In HEK293 cells (Thermo Fisher) with flippase (pOG44, Thermo Fisher) and above-mentioned plasmids cloned in pCDNA5-FRT vector following the manufacturer’s protocol (Thermo Fisher). Twenty-four h post transfection, cells were selected with 100 µg/mL hygromycin B (Sigma). Conditioned media (CM) from L, L Wnt-3a, and L Wnt-5a cells was harvested by collection of supernatant from cells grown for at least 48 h and that had achieved at least 80% confluence, followed by passage through a 0.2 µm filter and storage at 4°C until use. Cell lines were obtained and used without further authentication.

Drosophila melanogaster husbandry

Information on individual fly strains is provided in the Key Resources Table. Flies were reared at room temperature in density-controlled vials (60-100 embryos/vial) on standard yeast-glucose medium, for experiments at L3 larval or adult stages. For experiments at 30 h after puparium stage, flies were reared in an incubate at 25°C and collected at the appropriate stage. Where possible, experiments were performed on both male and female flies to avoid sex-specific effects. Due to lethality of alleles on the X chromosome, involving flies with FM7a-balanced chromosomes, only female flies from such crosses were used for further analysis.

METHOD DETAILS

Plasmids and cloning

A PLEKHA4 cDNA (obtained from DNASU, corresponding to BC024157) was cloned into the pEGFP-C1 and -N1 vectors (Clontech) using EcoRI and SalI to generate GFP-PLEKHA4 and PLEKHA4-GFP, respectively. The full-length proteins were subcloned into mCherry-C1 and -N1 vectors using EcoRI and SalI. Fragments and deletions of PLEKHA4 were subsequently subcloning into these vectors using standard or overlap PCR-based methods, again with EcoRI and SalI. The amino acid sequences of the deletions/fragments are the following: PLEKHA4 PH (45–167), PLEKHA4 PRD (167–357), PLEKHA4 PRD-CC (167–495), PLEKHA4 PRD-CC- IDR (167–779), PLEKHA4 CC (357–495), PLEKHA4 CC- IDR (357–779), PLEKHA4 IDR (495–779), PLEKHA4H-BP-PH (28–167), PLEKHA4 D N+IDR (28–495), PLEKHA4 D CC+IDR (1–357), PLEKHA4 D N+H+BP (54–779), PLEKHA4 PH (1–45, 167–779), PLEKHA4 D H+BP+PH (1–27, 168–779), PLEKHA4 D PRD (1–167, 357–779), PLEKHA4 D IDR (1–495). For bacterial expression, N-terminal fusions to GST of PLEKHA4 PH and PLEKHA4H-BP-PH were generated by subcloning into the pGEX-6P-1 vector (GE Healthcare) using EcoRI and SalI.

For generation of stable HeLa or HEK293 cells, GFP-PLEKHA4, PLEKHA4-GFP, and GFP were subcloned into the pCDNA5-FRT vector (Thermo Fisher) using NheI and KpnI (GFP-PLEKHA4), NheI and NotI (PLEKHA4-GFP and GFP). mCherry-KLHL12, GFP-KLHL12 and 3xFLAG-KLHL12 were generated by subcloning pcDNA3.1+zeo-VSV-KLHL12 (Addgene # 16761) into mCherry-C1, pEGFP-C1 vector using KpnI and Apal, and into pCMV10-3xFLAG (Sigma) using HindIII and NotI. HA-CUL3 was cloned into pCMV-HA-N vector using XhoI and NotI by amplifying the CUL3 ORF from the ORFome8.1 library (corresponding to GenBank BC039598.1, a gift from Haiyuan Yu, Cornell University). HA-Ub, M1R-mCherry, and iRFP-PLCd1-PH was a gift from the De Camilli lab (Yale University). For optoDroplet experiments, mCherry-CRY2-PLEKHA4 IDR and mCherry-CRY2-PLEKHA4 CC-IDR were generated by cloning of the relevant PLEKHA4 fragment into mCherry-CRY2(PHR) (Idevall-Hagren et al., 2012) (a gift from Pietro De Camilli) using PvuI and KpnI.

The following mutations were introduced by Quikchange site-directed mutagenesis (Agilent) followed by DpnI digestion of the parental DNA strand. GFP-PLEKHA4 PH, GST-PLEKHA4 PH, PLEKHA4 H-BP-PH, GFP, GST-PLEKHA4 H-BP-PH, F40E, 4A (K42A/R43A/R48A/R49A), R75A, R129A; pcDNA3.1+zeo-VSV-KLHL12: Q405X. For rescue experiments, siRNA-resistant GFP-PLEKHA4 or PLEKHA4-GFP were generated by performing silent mutations at the following codons S103, I106, R107, D109, and G110, which is within the siRNA target region. All constructs were verified by Sanger sequencing (Cornell University Biotechnology Resource Center Genomics Facility).
Transfection of plasmids and siRNAs

Plasmid transfections were performed using Lipofectamine 2000 (Thermo Fisher) according to the manufacturer’s protocol but using Transfectagro (Corning) instead of Opti-MEM. Cells were incubated with transfection mix in Transfectagro supplemented with 10% FBS for 6–8 h, following by a change of media to regular growth media and analysis after 18–20 h.

siRNA duplexes were obtained from Integrated DNA Technologies. Transfections with siRNA were performed with the appropriate duplexes (see Key Resources Table) using Lipofectamine RNAiMAX (Thermo Fisher) following the manufacturer’s protocol except using Transfectagro in place of Opti-MEM. Cells were incubated with transfection mix in Transfectagro supplemented with 10% FBS for 12–16 h, followed by exchange with fresh media. NC1 (negative control 1, IDT) was used as the control siRNA duplex for all experiments. Forty-eight h post transfection, cells were subjected to analysis via western blot, microscopy or flow cytometry.

Confocal microscopy

Prior to transfections (24 h), cells were seeded on 35 mm glass-bottom MatTek (#1.5 thickness, MatTek Corporation) imaging dishes for live cell imaging or on 12 mm cover glass (#1.5 thickness, Fisherbrand) for fixed cell imaging by immunofluorescence. Live cells were imaged 24–30 h post transfection. For immunofluorescence, cells were fixed in 4% paraformaldehyde in 100 mM sodium phosphate buffer (81 mM Na2HPO4-7H2O, 21 mM NaH 2PO4 pH 7.4) for 20 min, rinsed three times with PBS, blocked and permeabilized with blocking buffer (5% BSA and 0.1% Triton-X in 1X PBS) for 30 min. Cells were treated with primary antibody in blocking buffer for 1 h, rinsed with wash buffer (0.1% Triton-X, 1X PBS), incubated with secondary antibody in blocking buffer for 1 h at room temperature, rinsed with wash buffer and then PBS, mounted on slides in ProLong Diamond Antifade with DAPI (Thermo Fisher), and incubated overnight at room temperature in dark before imaging. For long-term storage, slides were stored at 4°C.

Images were acquired on a Zeiss LSM 800 confocal laser scanning microscope equipped with Plan Apochromat objectives (20x 0.8 NA or 40x 1.4 NA), and two GaAsP PMT detectors. Solid-state lasers (405, 488, 561, and 640 nm) were used to excite blue, green, red and far-red fluorescence respectively. Live-cell time-series movies were acquired using definite focus. For opto-Droplet experiments, a brief 488 nm pulse was used for photoactivation at the indicated frame in the time series. For colocalization-based analysis, multicolor images were acquired using line-scanning mode. Super-resolution structured illumination microscopy (SR-SIM) was performed on a Zeiss Elyra Super Resolution Inverted AxioObserver.Z1 microscope equipped with 405, 488, 561 and 640 nm lasers, definite focus and a Piezo-Z stage insert for fast focusing. Images were acquired using Zeiss Zen Blue 2.3 (confocal), Zeiss Zen Black (SR-SIM) and analyzed using FIJI (Schindelin et al., 2012).

Immunoprecipitation and western blots

Cells were harvested (500 x g, 3 min), lysed in lysis buffer (150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 5 mM EDTA, 50 mM Tris pH 7.5), sonicated for 3-5 pulses at 10% intensity, and centrifuged for 10 min at 13000 x g. A fraction of the supernatant was saved, quantified using the BCA assay (Thermo Fisher), and normalized as input, and the remainder was immunoprecipitated by rotation at 4°C overnight using either anti-GFP-nanobody Sepharose (Chromotek), EZview anti-FLAG-M2, or EZview anti-HA resins (Sigma). For immunoprecipitation with the soluble DVL3 antibody, the sample was incubated with primary antibody for 1 h at 4°C with rotation, followed by rotation overnight at 4°C with Protein G Sepharose (BioVision). The resin was then centrifuged for 10 min at 10000 x g, washed three times with lysis buffer and analyzed by SDS-PAGE and western blot, with detection by chemiluminescence (using SuperSignal West Pico (Thermo) or Clarity (Bio-Rad)) or, as described below in detail, mass spectrometry-based proteomics.

SILAC labeling and mass spectrometry-based proteomics analysis

For quantitative proteomics analysis, Flip-In HEK293 cells stably expressing GFP, GFP-PLEKHA4, or PLEKHA4-GFP were cultured in SILAC DMEM media (Thermo 89985) supplemented with 10% dialyzed FBS (JR Scientific) and 1% P/S for at least 5 passages (approximately 2 weeks) to allow full labeling of cells before analysis. “Light” SILAC media contained arginine 12C6, 14N2 and lysine 12C6, 14N4, while “heavy” SILAC media contained “heavy” lysine 13C6, 15N2 and “heavy” arginine 13C6, 15N4.

Cells were lysed and immunoprecipitated with anti-GFP-nanobody Sepharose as described above and processed for mass spectrometry as described (Bastos de Oliveira et al., 2015, 2018). Briefly, the resin was washed three times with lysis buffer before treatment with elution buffer (100 mM Tris pH 8.0, 1% SDS) by incubation at 65°C for 15 min with intermittent tapping. The samples were reduced (10 mM DTT for 15 min), alkylated (10 mM iodoacetamide, 50 mM Tris pH 8.0), and then the “heavy” and “light” solutions were mixed in a 1:1 ratio. The protein was then incubated on ice for 30 min, centrifuged (4700 x g, 10 min) and washed with a solution of 50% acetonitrile, 49.9% methanol, 0.1% acetic acid. The pellet was air-dried and reconstituted in 0.1% trifluoroacetic acid and analyzed using a Q-Exactive Orbitrap. Database search and quantification of heavy/light peptide isotope ratios were performed using Sorcerer as previously described (Bastos de Oliveira et al., 2015, 2018). A complete list of hits from these proteomics studies is provided in Table S1 and describes two different experiments. Experiment #1: PLEKHA4-GFP (Light), GFP (Heavy); Experiment #2: GFP-PLEKHA4 (Heavy), GFP (Light).
**Protein expression and purification in E. coli**

A single colony of *E. coli* BL21-pRosetta2 transformed with wild-type or mutant GST-PLEKHA4PH or GST-PLEKHA4H-BP-PH was grown in terrific broth (TB) supplemented with potassium phosphate buffer (0.17 M monobasic potassium phosphate, 0.17 M dibasic potassium phosphate), ampicillin and chloramphenicol for 6–8 h at 37°C, 250 rpm until OD600 was between 2 and 3. The temperature was then shifted to 18°C for 1 h, expression was induced with 0.25 mM isopropyl-β-D-galactosidase (IPTG), and cells were grown overnight for at least 18–20 h at 18°C, 250 rpm. Cells were harvested at 2100 x g for 15 min, 4°C and stored at –80°C until use.

Frozen cell pellets were thawed in bacterial lysis buffer (20 mM Tris pH 8, 500 mM NaCl, 5% glycerol, 10 mM β-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at 16,500 x g for 30 min to clear the lysate. The supernatant was incubated with washed Glutathione Sepharose 4B resin (GE Healthcare), and at 4°C protecting from light. The final phosphoinositide concentration was 20–100 μM (2–10 mol%), and the total lipid concentration was 1 mM. Catalog numbers of the exact lipid species used are reported in the Key Resources Table.

**Liposome co-sedimentation assays**

Liposomes were prepared by mixing 5:94.1 mol% ratio of phosphoinositide species:dioleoylphosphatidylcholine (DOPC):DIR (to aid in visualization of liposomes following SDS-PAGE) in 18:1 chloroform:methanol. Control liposome were DOPC:DIR (99:1 ratio) and dioleoylphosphatidylserine (DOPS):DOPC:DIR (20:79:1 ratio). After mixing, lipids were vacuum-dried, rehydrated in 25 mM HEPES pH 7.4 and 125 mM potassium acetate and incubated overnight at 37°C. Liposomes were generated by extruding lipids through 400 nm membranes (Whatman) and stored at 4°C protecting from light. The final phosphoinositide concentration was 20–100 μM (2–10 mol%), and the total lipid concentration was 1 mM. Catalog numbers of the exact lipid species used are reported in the Key Resources Table.

Liposome pelleting assay was performed to assess the binding of purified PLEKHA4 constructs to varying concentrations of phosphoinositide species in vitro. To avoid using any aggregates, each aliquot of purified protein was centrifuged at 163,000 x g for 10 min at 4°C following thawing and then re-quantified prior to use. This protein (2 μg) was incubated with liposomes (500 μM), giving a total lipid:protein ratio of 150:1 (phosphoinositide species:protein of 15:1 for 10% phosphoinositide-containing liposomes) for 10 min at room temperature in the dark. The reaction mixture was centrifuged at 163,000 x g for 30 min at 4°C. Supernatant and pellet were separated, denatured in SDS sample buffer and analyzed via SDS-PAGE gel. Images were acquired and quantified using a ChemiDoc MP system (Bio-Rad).

**PI(4,5)P2 depletion assay**

HeLa cells were co-transfected with plasmids encoding M1R-mCherry, iRFP-PLCγ1PH, and either GFP- or PLEKHA4-GFP bearing silent mutations to render it resistant PLEKHA4H-BP-PH as described in Figures S1B and S1C. Twenty-four h after transfection, live-cell time-series with 5 s intervals between each frame, using definite focus as described above. During the time series, oxotremorine-M (10 μM) was added to induce PI(4,5)P2 depletion.

**Western blot analysis of DVL levels**

**Overexpression**

HeLa cells were co-transfected with 500 ng each of FLAG-hDVL3 and the combination of GFP, GFP- or pCDNA3-VSVG-KLHL12 plasmids as described in Figure 4A. The total DNA amount was normalized to 2.5 μg using pCDNA3 empty vector. After 36 h of cell growth post-transfection, the cells were lysed, quantified, normalized and analyzed by western blot. Experiments were performed in biological replicates, and chemiluminescence measured and quantified using a ChemiDoc MP system.

**Endogenous levels**

SiRNA duplexes (50 nM) against PLEKHA4 or KLHL12 was performed on either HeLa (human) or C57MG WntRGreen (mouse) cells on a 6-well plate. Forty-eight h post RNAi, cells were harvested, analyzed by western blot and levels of DVL1, DVL2 and DVL3 were quantified. Reported quantifications are from at least three biological replicates.

**Analysis of DVL3 ubiquitination**

**Overexpression**

HeLa cells were co-transfected with the combination of plasmids as described in Figures 4A and 4B. After 36 h of cell growth post-transfection, the cells were lysed, quantified, immunoprecipitated using EZview α-FLAG resin and analyzed by western blot.

**Endogenous**

SiRNA-mediated knockdown was performed against PLEKHA4 or KLHL12 on 60 mm dishes. After 48 h of cell growth post-transfection, cells were lysed and immunoprecipitated using 1 μg DVL3 primary antibody per 800 μg of lysate following the immunoprecipitation protocol as described above. Samples were analyzed in biological replicates via western blot and quantified as described above.

**Rescue of DVL3 levels by PLEKHA4 transfection**

RNAi was performed as described in HeLa cells but with cells seeded on 35-mm MatTek imaging dishes. Thirty-six h after the RNAi transfection, NC1-treated cells were transfected with 2 μg of GFP plasmid, siPLEKHA4 treated cells were transfected, using Lipofectamine 2000, with 2 μg of a plasmid encoding either GFP or PLEKHA4-GFP bearing silent mutations to render it resistant.
to siRNA (Figure 4E). After 24 h of cell growth after the plasmid transfection, cells were treated with Hoechst 33342 (Thermo Fisher) and imaged via confocal microscopy to assess the transfection efficiency (which, for these experiments, was determined to be 50%–70%). After imaging, cells were harvested and analyzed via western blot.

**Western blot analysis of β-catenin dependent Axin2 levels**

**Endogenous levels**

Appropriate siRNA duplexes (50 nM) against PLEKHA4 or KLHL12 were transfected into HeLa (human) or C57MG WntRGreen (mouse) cells on a 6-well plate. Cells were stimulated with Wnt3a conditioned media 24 h post transfection. Forty-eight h post RNAi, cells were harvested, analyzed by western blot and quantified for Axin2 levels. Reported quantifications are from at least three biological replicates.

**Rescue of Axin2 levels by PLEKHA4 transfection**

RNAi was performed as described in HeLa cells. Thirty-six h after the RNAi transfection, NC1-treated cells were transfected with 2 µg of GFP plasmid, siPLEKHA4 treated cells were transfected with 2 µg of a plasmid encoding either GFP or PLEKHA4-GFP bearing silent mutations to render it resistant to siRNA (Figure 5E). Eight h after the plasmid transfection, cells were stimulated with Wnt3a conditioned media for 24 h, and analyzed by western blot. Reported quantifications are from at least three biological replicates.

**Western blot analysis of β-catenin independent p-JNK levels**

SiRNA duplexes (50 nM) against PLEKHA4 was transfected into C57MG WntRGreen cells on a 6-well plate. Cells were stimulated with conditioned media from L cells (control) or L Wnt-5a cells 24 h post transfection. Forty-eight h post RNAi, cells were harvested, analyzed by western blot and p-JNK levels were quantified. Reported quantifications are from at least three biological replicates.

**Fluorescent Wnt reporter assay**

RNAi (50 nM) was performed on C57MG WntRGreen as described above. After Thirty-six h after the siRNA treatment, MV7-Rat2a-Wnt1 (Wnt1-secreting) or MV7-Rat2a (control) cells were co-cultured with knockdown sample dishes in a 10:1 ratio of C57MG WntRGreen cells to MV7-Rat2a cells and incubated at 37 °C, 5% CO2 atmosphere for an additional 26–30 h. Cells were then analyzed in biological replicates via flow cytometry (BD Accuri C6) or treated with Hoechst 33342 and analyzed by confocal microscopy. Separate dishes treated exactly in the same way were harvested for western blot analysis.

**Generation of kramer (kmr) knockout flies**

Sequences encoding an sgRNA targeting a region near the beginning of kramer (FlyBase: CG34383) open reading frame were cloned into the vector pCFD3 (Addgene # 49410). The construct was integrated into an attP docking site on the Drosophila chromosome 2 by injection into the strain y¹ v¹ Pfly¹7.7\nos-phiC31\int.NLS; P{CaryP}attP40 (Bloomington stock center line 25709). Injected animals were mated to y v flies, and progeny with wild-type eyes were selected.

To make kmr knockout flies, males with the integrated sgRNA construct were mated with females of genotype y¹ M(w¹mc Act5C-Cas9.P)PY2H-2A w¹ (Bloomington stock center line 54590). Male progeny expressing Cas9 and the sgRNA were crossed with y v; TM3, Sb / TM6B, Tb Hu females. Single male progeny with the TM3 balancer were then crossed to the same double balancer stock. Male and female progeny of this latter cross with the TM6B, Tb Hu balancer were intercrossed, and generating animals homozygous for putative mutations in kmr. These lines were genotyped by sequencing of appropriate PCR products to verify the homozygous knockout of kmr gene. We isolated and sequenced two kmr alleles with frameshift mutations early in the coding sequence predicted to result in loss of function, kmr¹ and kmr². In kmr¹, a 1 bp deletion created a frameshift mutation starting at the 89th codon. In kmr², a 2 bp deletion created a frameshift mutation starting at the 88th codon. See Figure S4 for relevant genomic sequences around the deletions that were determined by Sanger sequencing. Both stocks behaved identically in all subsequent experiments, and the either of the kmr¹ and kmr² alleles may be referred to simply as kmr⁻.

**Generation of flies containing kmr⁻ and other alleles**

To examine possible phenotypes associated with heterozygosity, five simple heterozygote strains (kmr⁻/kmr¹, kmr⁻/kmr², dsh¹/dsh², dsh¹/dsh⁷⁷, and dsh¹/dsh⁷⁷) were generated by crossing either homozygous (kmr⁻, kmr², and dsh¹) or balanced (dsh¹/FM7a or dsh⁷⁷/FM7a) strains each separately with w¹¹¹¹. The six transheterozygote strains (kmr⁻/kmr¹;dsh¹/dsh¹, kmr⁻/kmr¹;kmr⁻/dsh¹, kmr⁻/kmr¹;kmr⁻/dsh², kmr⁻/kmr¹;kmr⁻/dsh⁷⁷, for both the kmr¹ and kmr² alleles) were generated by crossing the appropriate homozygous kmr strain with either dsh¹, dsh²/FM7a, or dsh⁷⁷/FM7a. To control for possible maternal effects, each cross involving kmr¹, kmr², or dsh¹ was carried out in two ways by switching the males and females and collecting the appropriate F1-generation flies for analysis; the results were identical. For crosses involving dsh²/FM7a and dsh⁷⁷/FM7a, only virgin female flies were used and non-FM7a F1-generation female flies were selected for further analysis.

To determine whether wing hair polarity effects are due to knockout of kmr as opposed to off-target effects, complementation testing was performed between kmr⁻ and the deletion fragment Df(3R)6170 (abbreviated as df), a chromosomal deletion encompassing 23 genes including kmr. kmr⁻ flies were crossed with Df(3R)Exel6170 / TM6B, Tb¹ (Bloomington stock 7649), and non-Tb flies (kmr¹/kmr⁻) were selected for analysis.
For assessing Dsh protein levels in wing imaginal discs and loss of proximal-distal asymmetry in pupal wings 30 h after puparium formation, we utilized dsh::Clover flies (a gift from Jeffrey Axelrod, Stanford University), which were generated by replacing the EGFP in pCasper4-Dsh-EGFP (Axelrod, 2001) with the fluorescent protein Clover2 followed by creation of transgenic lines with insertions on the second and third chromosomes (BestGene Inc.). First, dsh::Clover/dsh::Clover virgins (with dsh::Clover on chromosome 2 because kmr is on chromosome 3) were crossed with sp/CyoW (II); TM2/TM6B, Tb (III) males. In the F1 generation dsh::Clover/ CyoW; +/TM6B, Tb flies were collected and then crossed with kmr flies. Finally, the Tb progeny of the previous cross (dsh::Clover/+; kmr+/TM6B) were collected and mated with kmr flies, generating kmr flies with a single dsh::Clover allele (dsh::Clover; kmr). Control flies that were wild-type at the kmr locus and bearing a single dsh::Clover allele (dsh::Clover), were also generated.

Dissection and imaging of wing imaginal discs, pupal wings, and adult wings

Wing imaginal discs
Wing imaginal discs (WIDs) were dissected from individual L3 larvae as described (Spratford and Kumar, 2014), and WIDs were fixed in 4% paraformaldehyde for 30 min, washed three times with wash buffer (5% BSA, 0.1% Triton-X, 1X PBS) and twice with 1X PBS. Genomic DNA was extracted from the remaining unfixed tissues from each individual larva for genotyping. From each animal, one pair of fixed and washed WIDs were mounted with ProLong Diamond Antifade without DAPI (Thermo Fisher) on glass slides under 12 mm coverglass (Fisher Scientific), and the slides were incubated overnight at room temperature in the dark before imaging. For long-term storage, slides were stored at 4°C. For quantification of fluorescence from the Dsh-Clover protein fusion in WIDs, orthogonal view images were generated from the images of larvae that showed Clover signals post-genotyping. Dsh-Clover localizes in the epithelial folds of the WIDs (shown in Figure 7A with arrow). Three integrated density values (independent of the area) were generated from each image, and background was subtracted to obtain corrected integrated densities. Image analysis was performed in a manner blinded with respect to the animals’ genotypes.

Pupal wings
Pupal wings were dissected from individual 30 h post puparium formation (APF) pupa as described (Bolatto et al., 2017). From each animal, one pair of fixed and washed pupal wings was mounted with ProLong Diamond Antifade without DAPI (Thermo Fisher) on glass slides under 12 mm coverglass (Fisher Scientific), and the slides were incubated overnight at room temperature in the dark before imaging. For long-term storage, slides were stored at 4°C. Wings were imaged by confocal fluorescence microscopy, and maximum intensity z-projection images were generated and assessed for extent of proximal-distal symmetry.

Adult wings
Wings of appropriate genotypes were dissected and mounted on glass slides with mounting media (3:1 ratio of DPX:xylenes), and the slides were dried overnight at 50°C. Mounted wings were imaged by widefield brightfield microscopy.

Scanning electron microscopy (SEM)
Adult flies of correct genotype were collected and fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer pH 7.4 for 2 h at 4°C. The samples were rinsed with 0.05 M cacodylate buffer three times for 5 min each and post-fixed in 1% OsO4 (osmium tetroxide, EMS) for 1 h at 4°C. The samples were rinsed again with 0.05 M cacodylate buffer three times for 5 min each. Serial dehydration was performed in 25%, 50%, 70%, 95%, 100% ethanol for 20 min each and 100% for 24–48 h at 4°C. Samples were critical point dried using a BAL-TEC CPD 030, mounted on stubs, and sputter coated with gold:palladium. Image analysis was performed using Tescan Mira3 FE-SEM microscope. At least 12 individual flies per genotype for eyes and thorax tissues each were analyzed, and four representative images per genotype are provided, one in Figure 6 and three in Figures S6 and S7.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics and reproducibility
All imaging figures show representative images from experiments performed in at least three biological replicates on different days. For all experiments involving quantification, significance was calculated using an unpaired two-tailed Student’s t test with unequal variance. Statistical significance of p < 0.05 or lower is reported. In figures containing bar graphs, the number of biological replicates analyzed has been stated in the legend, the height of the bar is the mean, the error bars represent standard deviation, and each overlaid dot represents an individual biological replicate. In the boxplot shown in Figure 7B, the boxes represent the middle quartiles, with the line in the middle representing the median, and the whiskers denote the maximum and minimum values. Image analysis was performed in a blinded manner.

DATA AND SOFTWARE AVAILABILITY

Data availability statement
The authors declare that all data supporting the findings of this study are available within the paper and its supporting information files.
Supplemental Information

PLEKHA4/kramer Attenuates Dishevelled Ubiquitination to Modulate Wnt and Planar Cell Polarity Signaling

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Figure S1, related to Figure 1. Minimal motif for PLEKHA4 plasma membrane localization via interaction with PI(4,5)P₂ comprises a putative amphipathic helix, basic peptide, and PH domain. (A) Shown at left is a helical wheel projection of residues 28–42 of human PLEKHA4, with dotted line separating hydrophobic (bottom) from hydrophilic (top) faces of the helix. The primary amino acid sequence of helix and adjacent basic peptide are provided below, with residues that were mutated for structure–function studies bolded. Shown at right is an x-ray crystal structure of the PLEKHA4 PH domain in complex with inositol-1,3,4,5-tetrakisphosphate (PDB ID: 1UPR), with the two key Arg residues indicated that form contacts with the PIP head group. (B) PLEKHA4<sub>H-BP-PH</sub> (28–167) interacts with PI(4,5)P₂ at the plasma membrane. HeLa cells co-transfected with a PI(4,5)P₂ marker (iRFP-PLCδ1<sub>PH</sub>), an mCherry-tagged muscarinic M1 receptor (M1R-mCherry), and either GFP-PLEKHA4<sub>H-BP-PH</sub> or GFP-PLEKHA4<sub>FL</sub> were imaged before and after PI(4,5)P₂ depletion induced by treatment with oxtremorine M (Oxo-M). Note that Oxo-M treatment causes a decrease in plasma membrane localization and increase in cytosolic localization of PLEKHA4<sub>H-BP-PH</sub> but not of PLEKHA4<sub>FL</sub>. Scale bar: 10 µm.
Figure S2, related to Figure 2. PLEKHA4 does not localize to known assemblies at the plasma membrane or endolysosomal compartments. Confocal microscopy images of HeLa cells showing that PLEKHA4 puncta do not colocalize or minimally colocalize with markers of established assemblies such as clathrin-coated pits (A), caveolae (B), endoplasmic reticulum–plasma membrane contact sites (C), or endosomes and lysosomes (D). Cells were transfected with the indicated GFP, mCherry (mCh), or mRFP-tagged plasmids. For clarity, in the merged image, PLEKHA4 (PLEKHA4FL, full-length) is shown in green and organelle markers are shown in magenta. For images where the organelle marker is a transfected plasmid (AP2, CLC (clathrin light chain), ORP5, E-syt2, E-syt3, Rab5, Rab7, and LAMP1), confocal microscopy imaging was performed on live cells. For images where the organelle marker is the endogenous protein (Cav1, EEA1, Rab3, Rab8, and Rab11), fixation and immunofluorescence labeling was performed, followed by confocal microscopy imaging. Scale bars: 10 µm; 1 µm (insets).
Figure S3, related to Figure 4. KLHL12 does not modulate ubiquitination of PLEKHA4. Western blot analysis of anti-GFP immunoprecipitates from HeLa cells transfected with plasmids for either 3xFLAG-KLHL12 or 3xFLAG only (empty vector) in combination with GFP-PLEKHA4 and HA-ubiquitin (HA-Ub).
Figure S4, related to Figure 6. (A) Transcript alignment showing the guide RNA target site, early in the coding sequence, for generation of knockout strains of kramer (kmr), the PLEKHA4 homolog in Drosophila melanogaster. Exons of kmr (CG34383) are shown in rectangles, with the open reading frame in black. (B) Alignment of partial genomic sequence after genotyping PCR reaction of the mutant alleles kmr<sup>1</sup> and kmr<sup>2</sup>, compared to the wildtype, with predicted translation shown above. The guide RNA target sequences are colored in red. Note that for both the kmr<sup>1</sup> and kmr<sup>2</sup> alleles, the deletion of 1 and 2 bases, respectively, leads to a frameshift that causes an early stop codon in the Kramer protein. (C) Sanger sequencing traces verify the knockout in kmr<sup>1</sup> and kmr<sup>2</sup>. Red arrows indicate the start of frameshift in the knockout clones.
Figure S5, related to Figure 6. Knockout of the fly PLEKHA4 homolog, kramer (kmr), results in defects in orientation of hair in the adult wing. Shown is brightfield imaging analysis of Drosophila melanogaster adult wings oriented proximal to distal (left to right) showing defects in PCP signaling. The panels here provide three additional examples of the identical genotypes present in Figure 6A–D: wild-type and homozygotic kmr or dsh strains (A), complementation with a deletion fragment strain (B), simple heterozygotic kmr or dsh strains (C), and transheterozygotic strains for kmr/dsh (D). Scale bars: 50 µm.
Figure S6, related to Figure 6. Knockout of the fly PLEKHA4 homolog, kramer (kmr), results in defects in polarization of adult eye bristles. Shown are scanning electron microscopy (SEM) images of the eyes of adult flies. The top row of each part shows the complete eye, and the bottom two rows show additional zoomed in examples. The panels here correspond to and provide additional examples of the identical genotypes shown in Figure 6E–G: wild-type and homozygotic kmr or dsh strains (A), simple heterozygotic kmr or dsh strains (B), and transheterozygotic strains for kmr/dsh (C). Arrowheads indicate examples of hairs with altered orientations due to defective PCP signaling. Note loss of polarized eye bristle patterning in homozygotic strains and modest loss of patterning in transheterozygotes, compared to wild-type and simple heterozygote controls. Scale bars: 100 µm (top rows of each part; full eyes) and 50 µm (middle and bottom rows of each part; zoomed-in views).
Figure S7, related to Figure 6. Knockout of the fly PLEKHA4 homolog, kramer (kmr), results in defects in polarization of thoracic bristles. Shown are scanning electron microscopy (SEM) images of the thoraxes of adult flies. The panels here correspond to and provide additional examples of the identical genotypes shown in Figure 6H–J: wild-type and homozygotic kmr or dsh strains (A), simple heterozygotic kmr or dsh strains (B), and transheterozygotic strains for kmr/dsh (C). Arrowheads indicate examples of hairs with altered orientations due to defective PCP signaling. Note loss of polarized thoracic hair patterning in homozygotic strains and modest loss of patterning in transheterozygotes, compared to wild-type and simple heterozygote controls. Scale bars: 50 µm.

Table S2, related to STAR Methods. List of oligonucleotides used in this study.

| Oligonucleotides | REAGENT | SOURCE | IDENTIFIER |
|------------------|----------|--------|------------|
| REAGENT          |          |        |            |
| Negative Control dsiRNA Sense: | rCrGrUrArUrArCrGrCrGrUrArUrArArCrGrCrGrUAT | IDT | N/A |
| Negative Control dsiRNA Antisense: | rArUrArCrGrCrGrUrArUrArArCrGrCrGrUrArC | IDT | N/A |
| siRNA hPLEKHA4 Sense: | rArGrCrUrArCrArUrArUrArGrArCrGrArUrGrGGC | IDT | N/A |
| siRNA hPLEKHA4 Antisense: | rGrCrCrCrArUrCrUrGrGrUrCrUrArArUrArUrUrGrUrArGrCrUrGrG | IDT | N/A |
| siRNA mPLEKHA4 Sense: | rArGrCrUrGrGrArGrCrGrArUrArCrGrUrUrUGA | IDT | N/A |
| siRNA mPLEKHA4 Antisense: | rUrCrArArArCrGrUrCrUrCrArArGrCrUrCrU | IDT | N/A |
| siRNA hKLHL12 Sense: | rArArCrUrArUrGrUrArGrCrArCrArGrUrGrUTT | IDT | N/A |
| siRNA hKLHL12 Antisense: | rArArCrUrArUrGrGrArGrCrArCrArGrUrGrUrUrC | IDT | N/A |
| siRNA mKLHL12 Sense: | rArCrGrArGrUrArUrCrGrArGrUrUrUrArGrArUrAgA | IDT | N/A |
| siRNA mKLHL12 Antisense: | rUrCrUrArCrGrArUrArCrCrCrUrCrGrUrCrG | IDT | N/A |
| CG34383 gRNA: | GTCGGCCTACCAGACGAATCTCG | IDT | N/A |