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Report

Wnt/Frizzled Signaling Requires dPRR, the *Drosophila* Homolog of the Prorenin Receptor

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Summary

Wnt/Wg signaling pathways are of key importance during development and disease [1–4]. Canonical and noncanonical Wnt/Frizzled (Fz) pathways share a limited number of signaling components that are part of the membrane proximal signaling complex. In *Drosophila*, Fz [5–7] and Dishevelled (Dsh) [8, 9] are the only two components known to be involved in both Wnt/β-catenin and planar cell polarity (PCP) signaling. PCP signaling is required for the planar polarization of epithelial cells [10, 11], which occurs, for instance, during hair orientation and gastrulation in vertebrates [12]. Both pathways have been studied intensively in the past years. However, it still remains unresolved whether additional components are required at the receptor complex. Here we identify the *Drosophila* homolog of the mammalian prorenin receptor (dPRR) as a conserved modulator of canonical Wnt/β-cat and Fz/PCP signaling. We show that dPRR depletion affects Wg target genes in cultured cells and in vivo. PRR is required for epithelial planar polarity in *Drosophila* and for convergent extension movements in *Xenopus* gastrulae. Furthermore, dPRR binds to Fz and Fz2 receptors. In summary, our data suggest that dPRR has an evolutionarily conserved role at the receptor level for activation of canonical and noncanonical Wnt/Fz signaling pathways.

Results and Discussion

dPRR Is Required for Canonical Wg Signaling

In a large-scale RNA interference (RNAi) screen for regulators of Wg signaling [13], we have identified the uncharacterized transmembrane protein CG8444, which we named dPRR (*Drosophila* homolog of prorenin receptor), as a modulator of Wg signaling in *Drosophila* cells. Two independent nonoverlapping double-stranded RNAs (dPRR #1 and dPRR #2) led to a modest but significant reduction of Wg-induced reporter activity in Kc_{167} and to an efficient reduction of dPRR mRNA levels, whereas cell proliferation and viability were not affected (see Figures S1A–S1D available online). In addition, dPRR reduced the expression of nkd and CG6234 mRNA, two transcriptional targets of canonical Wg signaling in *Drosophila* [14], by more than 50% (Figures 1A and 1B). Taken together, these results indicate that dPRR is a modulator of Wg signaling in *Drosophila*.

Analysis of the dPRR protein sequence revealed a transmembrane protein with an extracellular (or luminal) domain, a transmembrane domain, and a short 20 amino acid intracellular tail. BLAST analysis indicates that it is homologous to human PRR/ATP6AP2, with 26% sequence identity and a higher degree of homology in a conserved renin receptor-like domain (IPR012493) (Figures 1C and 1D). Therefore, we tested whether PRR is similarly required for canonical Wnt signaling in human cells. Small interfering RNAs targeting hPRR significantly reduced Wnt-responsive TopFlash reporter activity in HEK293T cells activated by overexpression of mWnt1 and an EGFP-mWnt3A fusion protein (Figures S2A–S2C). We conclude that PRR has a conserved role in mediating Wnt signaling that is also consistent with its role in Wnt signaling in *Xenopus* [15].

PRR has previously been shown to bind to (pro)renin and to modulate the renin/angiotension system [16, 17]. Even though the renin receptor seems to be conserved in *Drosophila*, and renin-like enzymes have been identified in invertebrates [18], a renin/(pro)renin system has so far not been characterized in *Drosophila*.

dPRR Localizes Mainly to the Plasma Membrane and Acts Upstream of CK1α

To determine the subcellular localization of PRR, we generated C-terminal GFP-fusion proteins. In *Drosophila* ovaries, dPRR-EGFP mainly localized to the plasma membrane (Figures 1E–1G) and was enriched at the apical surface in the wing imaginal disc epithelium (data not shown). We found that hPRR-EGFP mainly localized to the endoplasmic reticulum (Figures S2D–S2F), consistent with previous localization studies [19]. Neither ubiquitous overexpression of dPRR (actGal4) nor expression in wing imaginal discs (nubGal4) caused Wg gain-of-function phenotypes (data not shown).

To epistatically map PRR function in the Wnt/Wg signaling pathway, we depleted dPRR in Kc_{167} cells and stimulated the pathway by addition of Wg-conditioned medium (Figure 2A). dPRR knockdown reduced Wg target gene expression in the receiving cell, similar to the depletion of armadillo (Arm). As a control, depletion of evi (wls/srt), which acts in the Wg-secreting cell, did not influence target gene induction (Figure 2A). Consistent results were obtained in HEK293T cells (Figure S2G). To map the function in the Wg-responding cell, we induced Wg signaling activity by depletion of CK1α (CG2028), which has been shown to phosphorylate Arm prior to degradation [20]. dPRR knockdown did not affect CG6234 expression induced by CK1α knockdown (Figure 2B), indicating that it acts upstream of the β-catenin/Arm degradation complex. Together with the plasma membrane localization of dPRR, our results place dPRR in the Wg-receiving cell at the receptor level, which is in accordance with studies about human PRR [15].

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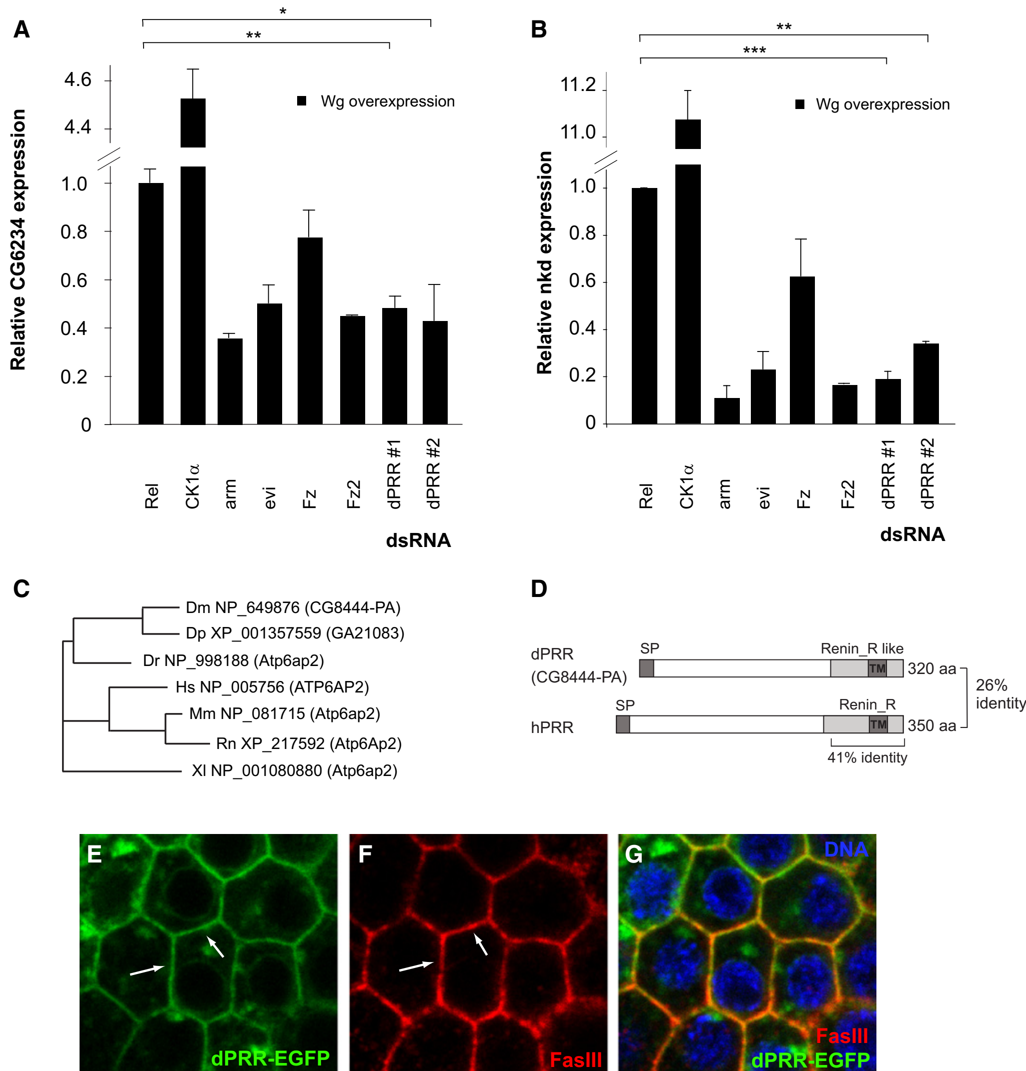

Figure 1. dPRR Modulates Canonical Wg/Wnt Signaling at the Plasma Membrane

(A and B) Relative expression of endogenous Wg target genes in Kc_{167} cells activated through Wg overexpression. mRNA expression levels of CG6234 and nkd (CG11614) are normalized to expression levels of control cells treated with double-stranded RNAs (dsRNAs) against relish (rel). Data are shown as mean ± standard deviation. A paired t test was performed to determine statistical significance (*$p < 0.003$, **$p < 0.0002$, ***$p < 0.00001$).

(C) Phylogenetic tree of sequences of different species homologous to *Drosophila* PRR (dPRR). GenBank accession numbers are indicated. The following abbreviations are used: Dm, *Drosophila melanogaster*; Dp, *Drosophila pseudoobscura*; Dr, *Danio rerio*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Xl, *Xenopus laevis*.

(D) Predicted domain architecture of dPRR indicates a C-terminal type 1a transmembrane and a renin receptor-like protein domain (IPR012493). Sequence identity with the human homolog hPRR or ATP6AP2 (ATPase H(+)-transporting lysosomal accessory protein 2, NP_005756) is shown in percent identity.

(E–G) dPRR-EGFP localizes to the plasma membrane in *Drosophila* ovarian follicle cells. dPRR-EGFP is driven by GR1-Gal4. Dissected ovaries were costained with FasIII (red) and Hoechst to label DNA (blue).

To address whether dPRR RNAi has a more generic effect on signaling, we assessed its effect on JAK/STAT, IMD, and Toll-specific reporter assays in *Drosophila* cells. Neither reporter gene activity was affected by dPRR depletion (Figures S3A–S3C).

dPRR Is Required for Wing Development and Target Gene Expression In Vivo

Wg signaling controls the development of *Drosophila* wings, including growth and patterning decisions in wing imaginal discs [21]. To analyze dPRR function in vivo, we expressed dPRR RNAi hairpins under the control of the wing pouch-specific *nubbin* (*nub*) promoter (nubGal4). Whereas ubiquitous depletion of dPRR was lethal, nubGal4>dPRR RNAi animals failed to develop normal adult wing structures and showed severe growth defects (Figure 2C). To confirm the specificity of the RNAi phenotype, we expressed a human PRR-EGFP transgene in the dPRR RNAi background, which rescued the growth defects in 70% of all flies (Figures 2D and 2E, $p < 0.0005$), in contrast to a control GFP transgene. These experiments underline an evolutionarily conserved function of PRR. By reducing the levels of dPRR RNAi hairpin expression at lower temperatures, we observed additional developmental defects (Figures 2F–2G′), including loss of wing margin bristles, a phenotype that is often observed for loss of function of Wg pathway components, and misorientation of wing hairs,

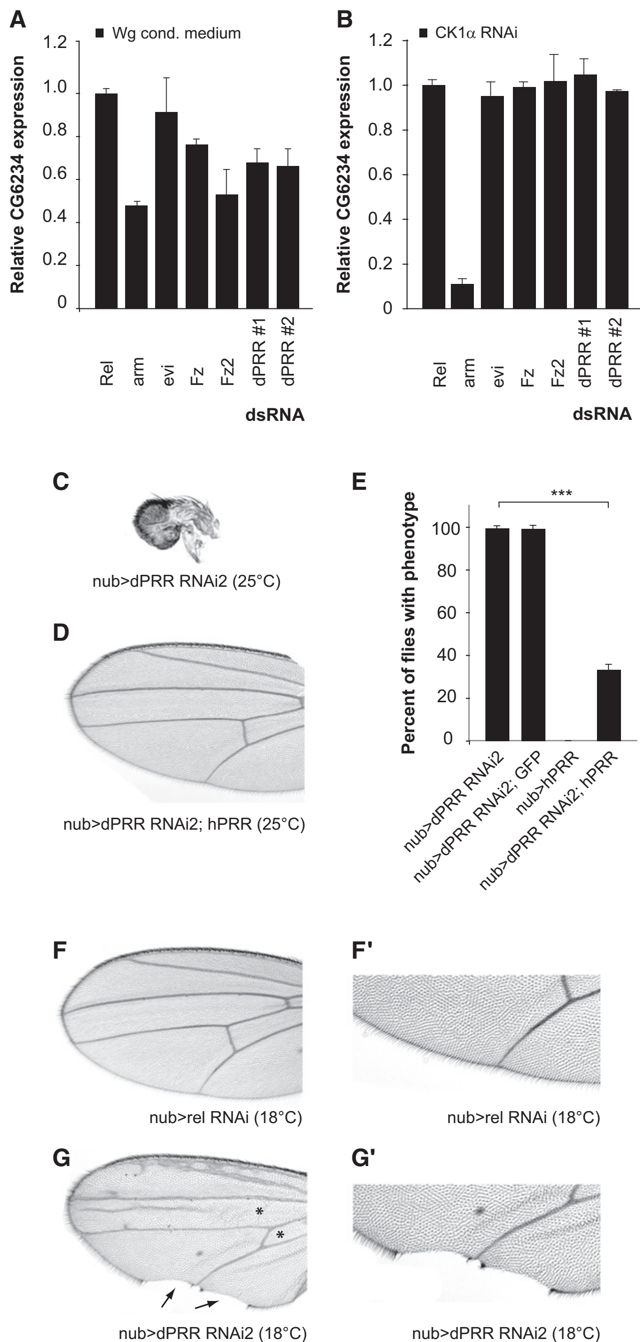

Figure 2. dPRR Is Required for Wing Development In Vivo

(A) Relative mRNA expression of CG6234 in Kc_{167} cells treated with the indicated dsRNAs and stimulated by addition of Wg-conditioned medium. Data are shown as mean ± standard deviation. Reductions in reporter activity, except for evi RNA interference (RNAi), are statistically significant, as determined by paired t test ($p < 0.05$).

(B) Relative expression of CG6234 in Kc_{167} cells stimulated by knockdown of the negative regulator CK1α.

(C–E) Overexpression of a full-length hPRR (UAS-hPRR) rescues the RNAi-induced phenotype (nubGal4/UAS-hPRR-EGFP; UAS-dPRR RNAi2/+), as compared to a control GFP construct (nubGal4/UAS-GFP;UAS-dPRR RNAi2/+). The rescue experiment was quantified by counting the percentage of adult flies showing a phenotype (25°C, n > 150 flies per genotype, ***$p < 0.0005$). Error bars represent standard deviation.

(F–G′) Adult wings of rel RNAi and dPRR RNAi2 expressed under the control of nubGal4 at 18°C. Note the wing margin defects in the posterior compartment in dPRR RNAi wings (G′) compared to wild-type (F′). The asterisks indicate veination defects in dPRR RNAi2 animals.

indicating a possible role in planar cell polarity (PCP) signaling. Furthermore, we also observed veination defects that are usually not seen in Wg/Fz mutants, indicating functions of dPRR in processes other than Wnt/Fz signaling.

We next assessed the effect of dPRR depletion during wing imaginal disc development. The proneural gene *senseless* (*sens*) is expressed along the dorsoventral boundary and is dependent on high levels of Wg signaling activity [22]. dPRR depletion in the developing wing disc led to a weak but reproducible reduction of *sens* expression (Figure S4). These observations suggest that dPRR modulates Wg signaling in vivo, consistent with the results obtained from cell culture experiments.

Planar Cell Polarity Requires dPRR

The above results mapped dPRR function close to the membrane-proximal complex. The *Drosophila* Fz receptor is required for PCP in *Drosophila* epithelia, which is manifested by the orientation of cuticular structures [23]. Because we observed misorientation of wing bristles in dPRR RNAi animals, we asked whether dPRR was required for PCP. In the adult wing, overexpression of Fz causes wing hairs to point laterally away from the zone of gain-of-function activity [24], whereas depletion of Dsh by RNAi shows the opposite phenotype (Figures S5A–S5C). Similarly, dPRR depletion in a stripe on the proximal-to-distal axis of the wing (dppGal4) appears to affect the normal wing-hair polarity (Figures S5D and S5E). Moreover, we also observed additional phenotypes, such as a growth defect in dPRR RNAi adult wings, which is consistent with a smaller dpp expression domain in wing imaginal discs (Figures S5F–S5H). The growth defect also indicates that dPRR function might not be solely restricted to signaling via Fz receptors.

PCP signaling is required for the orientation of notum bristles. Depletion of dPRR (apGal4) leads to an abnormal polarity of notum bristles that can be reverted by expression of an hPRR transgene, which itself has no effect on bristle orientation (Figures 3A–3D; Figures S6A–S6C). However, loss of dPRR causes additional phenotypes different from a typical PCP phenotype, as reported, for instance, for Dsh [25].

In the developing wing, PCP signaling is required for proper orientation of the wing hairs toward the distal edge. Loss of dPRR in a stripe on the proximal-to-distal axis of the wing (ptcGal4) causes a slight delay in trichome formation at 32 hr after pupal formation. This phenotype is also observed with other PCP proteins, such as Fz [26, 27], suggesting that the hair initiation is defective in dPRR-depleted cells (Figures 3E and 3F; Figure S5I). At later stages, when the wing hairs have emerged, PCP defects were observed in the dPRR RNAi expression domain, suggesting that dPRR indeed plays a role in PCP signaling (Figures 3G and 3H). However, we also observed a smaller ptcGal4 expression domain, which might be caused by dPRR RNAi-induced apoptosis during pupal development (Figures S5J–S5L). Taken together, our results indicate that dPRR is required for PCP in *Drosophila*.

To further investigate PRR function in PCP signaling, we carried out experiments in *Xenopus*, a well-characterized vertebrate model for noncanonical Wnt signaling [28]. *Xenopus* PRR is ubiquitously expressed until gastrulation, and its depletion interferes with canonical Wnt signaling [15]. In addition to its function in canonical Wnt signaling, PRR morpholinos induced short body axes and smaller heads and caused a shorter and broader expression domain of the notochord

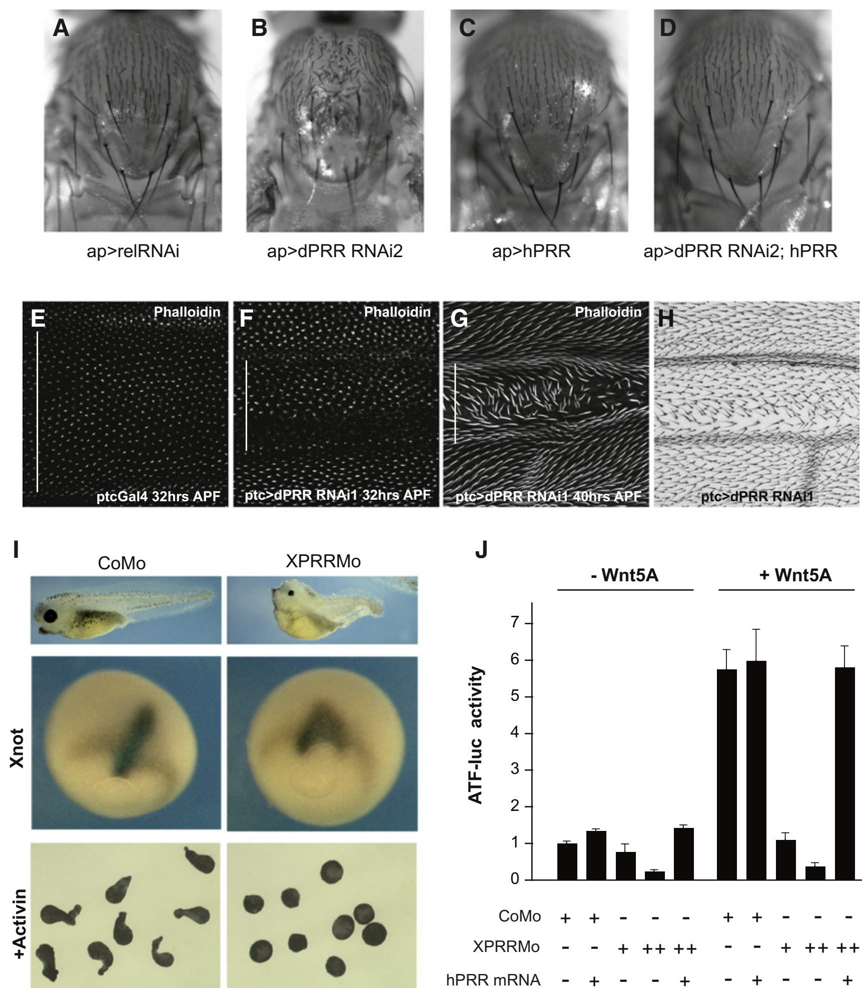

Figure 3. PRR Is Required for Planar Cell Polarity

(A–D) Dorsal view of adult thoraces of the indicated genotypes. dsRNA hairpins were expressed under the control of apGal4 at 25°C. RNAi of dPRR causes planar cell polarity-like phenotypes (B) that can be rescued by overexpression of hPRR (D).

(E and F) Phalloidin staining of pupal wings of the indicated genotypes aged for ~32 hr after pupal formation (APF), distal side to the left. Line indicates the approximate expression domain of ptcGal4. Note the affected ptc expression domain in dPRR RNAi animals.

(G) Phalloidin staining of a pupal wing of the genotype ptcGal4/UAS-dPRR RNAi1 at 40 hr APF.

(H) Adult wing of the genotype ptcGal4/UAS-dPRR RNAi1 at 25°C, anterior to the left.

(I) Loss of PRR function causes gastrulation defects in *Xenopus* embryos. Top: four-cell stage embryos were microinjected equatorially into two dorsal blastomeres with PRR morpholinos (Mo). Note the stunted axis in embryos injected with PRR Mo (75%, n = 32), but not control Mo (0%, n = 30), embryos. Middle: in situ hybridization for *Xnot* at gastrula stage (stage 12). Bottom: PRR Mo inhibits elongation of activin-treated animal caps.

(J) ATF2-luciferase reporter assay in *Xenopus* embryos. Four-cell stage embryos were injected equatorially with ATF2-Luc reporter plasmid, the indicated antisense morpholinos, and mRNAs. Luciferase reporter assays were carried out from whole embryos harvested at gastrula stage (stage 12). Data are shown as mean ± standard deviation.

marker *Xnot* in gastrulae embryos, a hallmark of impaired convergent extension movements (Figure 3I). Furthermore, PRR morpholinos also blocked activin-induced animal cap elongation (Figure 3I) without significantly changing mesodermal marker expressions (Figure S6D), confirming that PRR is required for convergent extension movements.

Wnt/PCP signaling leads to the activation of JNK [9] and the transcription factor ATF2 [29, 30]. In order to confirm the requirement of PRR for *Xenopus* PCP signaling, we used a JNK/ATF2-responsive luciferase reporter [31] that specifically monitors Wnt/PCP signaling in *Xenopus* embryos (B.O. and C.N., unpublished data). In brief, this reporter is activated by *Wnt5a* and *Fzd7*, a receptor-ligand combination that mediates Wnt/PCP signaling in *Xenopus*; its activity is inhibited by morpholinos targeting *Wnt5a* but not *LRP6.* In PRR morphants, both endogenous and a Wnt5A-induced reporter activity are blocked, a phenotype that could fully be rescued by hPRR mRNA coinjection (Figure 3J). Taken together, these experiments demonstrate that PRR is required for PCP signaling in vertebrates. Like Fz and Dsh, PRR seems to be an evolutionarily conserved component of canonical and noncanonical Wnt/Fz pathways that acts at the receptor complex or immediately downstream of it.

dPRR Interacts with Frizzled Receptors

Because dPRR mainly localized to the plasma membrane and colocalized with Fz when overexpressed in tissue culture cells (Figures S7A–S7C), we asked whether it may directly interact with Fz receptors. To this end, we tested whether dPRR can suppress a Fz gain-of-function phenotype. Overexpression of Fz in the dpp expression pattern causes a nonautonomous misorientation of trichomes pointing away from the expression domain [24]. This effect was reduced by two independent dPRR *P* element insertions, dPRR-1890P and dPRR-EY03616 (Figures 4A–4F). Both *P* elements cause first instar larval lethality and show a significantly reduced expression of dPRR mRNA (Figures S7D and S7E). In contrast, the Fz gain-of-function phenotype was not suppressed by a homozygous viable revertant of dPRR-EY03616 (exc.$\Delta^{dPRR\text{-}EY03616}$), which showed restored dPRR mRNA levels (Figures S7F–S7H), indicating that the genetic interaction is specific to the dPRR locus.

During establishment of PCP, Flamingo (Fmi) is the first among the core PCP proteins to be recruited to the membrane [32] and is required for proper localization of other core PCP proteins such as Fz [33]. Thus, loss of *fz* does not cause a significant change in the apicolateral localization of Fmi [33]. In pupal wings depleted for dPRR within the ptc expression domain, the asymmetrical localization of Fz protein was lost and less Fz was detected at the plasma membrane, whereas the apicolateral localization of Fmi appeared to be unaffected (Figures 4G–4N). This suggests that dPRR affects membrane localization of Fz after Fmi has reached the apicolateral junctions. In addition, dPRR RNAi affected the

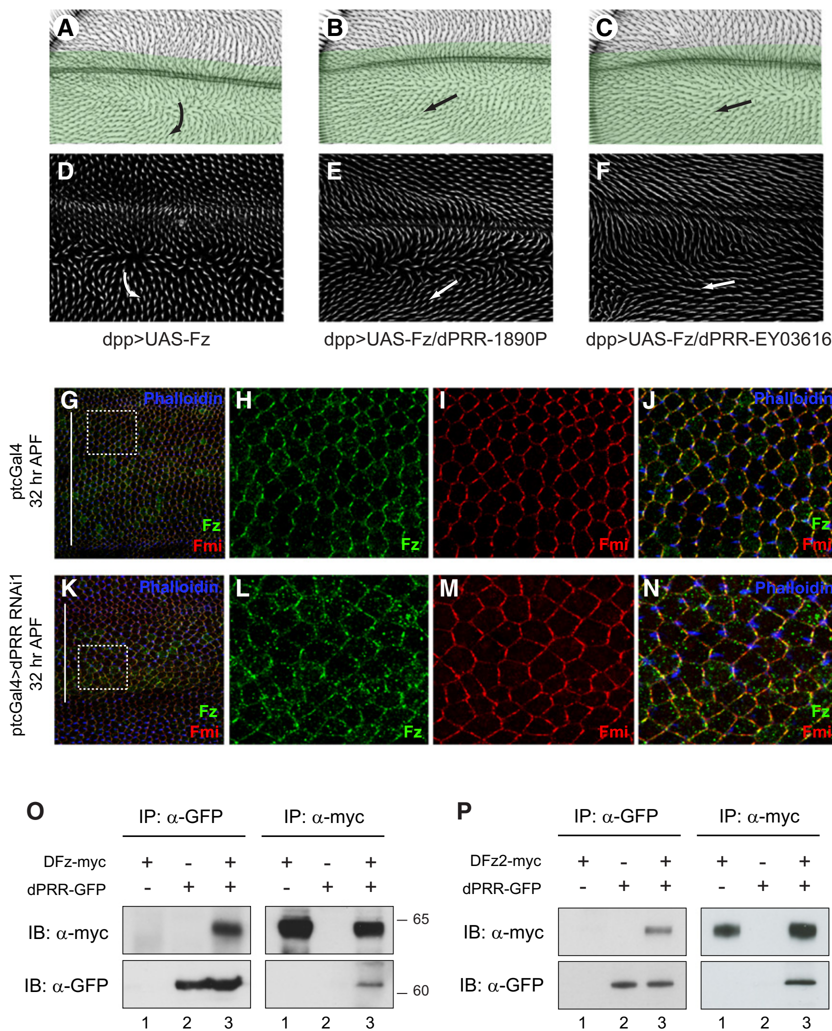

Figure 4. dPRR Interacts with Fz Receptors

(A–C) Adult wings of the genotypes dppGal4, UAS-Fz/+, dppGal4,UAS-Fz/ P{RS5}dPRR$^{5\text{-}HA\text{-}1890}$ and dppGal4,UAS-Fz/P{EPgy2}dPRR^{EY03616} at 25°C, anterior to the left. The approximate dppGal4 expression domain is marked in green. Arrows indicate wing hair orientation.

(D–F) Phalloidin staining of pupal wings of the indicated genotypes, aged for ~34 hr APF, distal side to the left. Arrows indicate trichome orientation.

(G–J) Pupal wing of ptcGal4 at 32 hr APF stained for phalloidin, Fz, and Fmi. Line indicates the approximate expression domain of ptcGal4. Dashed rectangle indicates the magnified region in (H)–(J).

(K–N) Pupal wing of the genotype ptcGal4/UAS-dPRR RNAi1 at 32 hr APF stained for phalloidin, Fz, and Fmi. Line indicates the approximate expression domain of ptcGal4. Dashed rectangle indicates the magnified region in (L)–(N).

(O and P) DFz-myc or DFz2-myc and dPRR-EGFP expression vectors were transfected as indicated into HEK293T cells. Cell lysates were immunoprecipitated and analyzed by western blotting with the indicated antibodies. The following abbreviations are used: IP, immunoprecipitation; IB, immunoblot.

hexagonal shape of cells in pupal wings. Loss of hexagonal cell packing has previously been reported for mutations in components of the PCP pathway [34].

Next, we tested whether dPRR and Fz receptors interact biochemically. In coimmunoprecipitation experiments, overexpressed dPRR interacted with Fz and Fz2, but not with a control plasma membrane-resident protein (Figures 4O and 4P; Figures S7I–S7J). These results support a model whereby dPRR mediates Fz/PCP signaling through interaction with Fz and Wnt/β-cat signaling through interaction with Fz/Fz2 and LRP6/Arr [15] (Figure S8).

PRR has been annotated as an accessory protein of the V-ATPase complex [35, 36] and was found to bind to several V-ATPase subunits [15]. In a related paper in this issue of *Current Biology*, Hermle et al. refer to this gene as VhaPRR [37]. Recently, loss-of-function analysis of several V-ATPase subunits revealed their requirement for trafficking of the Notch receptor complex [38]. In addition, Nhe2, a Na(+)H(+) exchanger, has been implicated in Fz/PCP signaling [39], indicating that regulated acidification influences Wnt/Fz signaling activity. Together with these studies, our findings therefore support a model in which dPRR might play a role in bridging noncanonical and canonical Frizzled receptor complexes with V-ATPases, providing a mechanism by which membrane potential could regulate Wnt/Fz signaling.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and one table and can be found with this article online at doi:10.1016/j.cub.2010.05.028.

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