

The Wnt co-receptor Arrow-LRP5/6 is required for Planar Cell Polarity establishment in Drosophila

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript shows both directly and through genetic interaction experiments that Arrow, the Drosophila homolog of LRP5/6, acts not only in canonical Wg/Wnt signaling but also in Fz/Vang PCP signaling in the Drosophila wing, eye, and abdomen. This is intriguing, as Arrow and LRP5/6 are usually framed as a Wg/Wnt co-receptor that acts along with Drosophila Fz and Fz2 in canonical beta-catenin signaling. A role for Arrow in PCP is thus novel and worth publishing, and the PCP phenotypes are convincing.

As Arrow and its homologs act as co-receptors, this manuscript also bears on a possible role of Wg/Wnt in Drosophila PCP. This issue has been long-debated, especially as the spatial cue for orienting core PCP in Drosophila is not known; many have suggested that gradients of diffusible Wg/Wnt might be one such cue. The authors have some genetic interaction experiments that suggest a role for Wg and Wnt4, in agreement with their earlier Wu et al. work from 2013. They also show genetic interactions with Fz and Dsh, and effects especially on Dsh levels.

The main weakness of the work, however, is that it does not have much that is definitive concerning the mechanisms connecting Arrow to the PCP pathway. The authors obviously prefer effects through Fz and Dsh, and state so in several places, but as they note there are some results in the eye that do not match this model. The data they show to support this connection are largely based on a change in Dsh protein levels, a possible effect of Fz and Fz2 on Arrow levels, and a few genetic interaction experiments. However, the genetic interactions shown are limited to a few candidates in the pathway, as are the effects on the levels and distribution of pathway proteins. Are there other canonical or PCP pathway components that do not genetically interact with Arrow in PCP, or that do not change their levels or polarity in arrow mutant clones? If there were, this would provide much stronger evidence for a specific role for Fz and Dsh.

There are a few other places where the work could be improved, largely in clarity and improved discussion and a couple bits of data.

1) "manipulating arr/LRP5/6 levels did tilt the dsh phenotypic balance, enhancing and suppressing the respective phenotypes when their levels were changed, very similar to effects seen with the Wnt ligands and Fz family receptors (Supplemental Figure S1A-C and A'-C', also Supplemental Table 1)."

1a) The wording here is a bit confusing. Does this mean that when the core wing margin phenotype gets worse, the PCP phenotype always gets better, and vice versa?

1b) The quantification in S1 is all canonical signaling (wing margin), but nothing on PCP. And while the picture shows a PCP improvement with arr/+, it is not clear if there is any change in PCP defects with Arrow overexpression.

2) Changes in Fmi levels?

The abstract states that in arrow clones "defects are manifest as reduced levels of Fmi/Celsr and Dsh along with loss of their asymmetric localization."

However, the Results say "reduced and uncoordinated asymmetry" of Fmi, which is different from levels, and there is no data quantifying Fmi levels in Fig. 2. Moreover, any change in Fmi levels in Fig. 2D seems to be mirrored by a similar change in

DEcad levels.

3) Dsh levels

The reduction of Dsh levels in arrow clones appears to be in the cytoplasm as well as the cell cortex. If this is real, is this typical of loss of Fz/Fz2 or other core components? Or is this something new?

4) Recruitment of Arrow by Fz and Fz2

4a) The authors state: "As imaginal disc cells are in a pseudostratified columnar arrangement and Arr localization is punctate throughout the apical cytoplasm and periphery of cells, we examined the flat cuboidal cells of the peripodial cell layer of discs." But since Fz and Fz2 are largely at apical junctions of the disc epithelial cells, wouldn't the authors still be able to detect increased Arrow at apical junctions? If they tried and didn't see an increase they should say so.

4b) The images do not show clone-autonomous increases in Arrow. There is equally heightened Arrow in wild type cells outside the Fz overexpressing clone (bottom of 4C') and the heightened Arrow is limited to one boundary of the Fz2-expressing clone (4D'). How do the authors explain this? It would also help to know a bit more about how the authors picked the wild type regions to include for the quantification.

5) The Discussion of studies on Wg and other Wnts

The discussion of this important issue is far too brief, and also a bit inaccurate. In fact many of the manipulations in references 88 and 89 did reduce growth. But it is true that the manipulations were largely designed to allow growth in early stage discs but greatly reduce or eliminate Wnts at later stages when PCP is thought to be established. The authors are correct that there may be residual Wg signaling; in fact the membrane-tethered Wg was used by one lab may allow precisely that. And it is possible that there are still low levels of Wnts after the inhibition of Wnt secretion done by both studies. But the studies argue strongly that PCP orientation is largely retained in situations where the gradient of diffusible Wg and other Wnts was eliminated, and there must be other ways of orienting PCP.

This still leaves open the possibility that Wg and Wnt4 help Fz/Fz2-based PCP activity, even if they do not provide a spatial cue, something that might become especially critical in a sensitized genetic test. That would also fit the polarity defects observed in Wg/Wnt4 mutants, which in Wu 2013 still orient distally but with less precision.

It would be valuable to include a full discussion of these points.

Minor notes, typos:

"Wnt/b-catenin"

"However, no specific pattern in developing photoreceptor cells, nor an asymmetric localization in developing wings, was detected with the available tools,"

Please describe the tools used. Did the authors look at boundaries between tagged and untagged Arrow?

"A first possibility could arise from the fact that Wnt/b-catenin signaling transcriptionally regulates the graded expression of four-jointed (fj) and dachsous (ds), two key components of the Fat/Ds PCP system (reviewed in 87)."

Reference 87 only describes this in the eye disc, not the wing.

"whereas fat or ds mutant clones show an upregulation of Fmi and its mispolarization with an increase in actual polarity (reviewed in 87)"

Does Reference 87 mention this? Has someone observed an upregulation of Fmi levels?

Reviewer #2

(Remarks to the Author)

In the study, "The Wnt co-receptor Arrow-LRP5/6 is required for Planar Cell Polarity establishment in Drosophila," Weber and colleagues show that arr loss-of-function (LOF) clones display key planar cell polarity (PCP) defects in both the wing and the eye, suggesting that Arr plays a positive role in Wnt/PCP signaling. This finding challenges current models that mainly assign Arr/LRP5/6 the role of a Wnt/ β -catenin-specific co-receptor, suggesting instead that Arr is also necessary for proper PCP signaling. The specificity of this requirement is highlighted by rescue experiments: coexpression of Arr within clonal patches restores wing PCP phenotypes, thereby confirming a PCP-specific function for Arr/LRP5/6.

In pupal wings, arr mutant clonal patches show a loss or misorientation of the characteristic asymmetric localization of Flamingo and Dishevelled. These polarity defects happen alongside a reduction in the levels of Fmi and Dsh proteins within the affected regions. Functionally, these molecular issues result in misoriented wing hairs, a phenotype similar to what is seen in loss-of-function mutants of core PCP components like frizzled, dsh, or Van Gogh. In the eye, arr clones interfere with the binary fate determination of the R3 and R4 photoreceptors and disrupt the chiral arrangement of ommatidia, again linking arr function to the core PCP machinery that controls tissue-wide polarity and mirror-symmetric patterning.

Genetic interaction and epistasis analyses place *arr* either downstream of or at a similar hierarchical level to *fz* and *dsh*, suggesting that these factors work closely together within the PCP signaling network. Consistent with this positioning, *Arr* appears to enhance PCP activities mediated by *Fz* and *Dsh*: increasing *Arr* dosage strengthens the PCP phenotypes caused by *Fz* or *Dsh* overexpression, while *arr* LOF or knockdown reduces these effects. Additionally, PCP-specific defects involving *arr* are seen in genetic combinations with *wg*, *Wnt4*, and *fz*, connecting *Arr* functionally to *Wg/Wnt4* inputs in the PCP pathway.

Overall, the evidence indicates that *Arr* functions similarly to a component of the *Fz*–*Dsh* complex in PCP signaling. By enhancing *Fz*- and *Dsh*-dependent activities and affecting the stability and asymmetric localization of core PCP proteins, *Arr* likely acts as a link that connects the *Fz*–*Dsh* module to *Wg/Wnt4* signals, thereby integrating ligand input with the development of planar polarity in wing and eye tissues.

The findings in this paper are of great interest for the general Wnt signaling field. They are surprising, but likely due to a lack of attention to Arrow phenotypes in previous studies. The genetics approach makes it difficult to argue with the findings, as the phenotypes are positive (very sound methodological approach).

Suggestions:

One issue that could be addressed to strengthen the paper would be to mention recent structural studies that define the interaction of Wnts with *Fzs* and LRP5/6. These structures show that it isn't easy to see how a simple Wnt-*Fz* interaction could activate signaling of any sort, as two domains of Wnt bring together *Fz* and a co-receptor. Wnt agonists also rely on nanobodies against *Fz* and LRP in combination to stimulate pathway activation.

Downstream, LRP and *Fz* coordinate *Dsh* activity, suggesting a function in both Wnt pathways.

Reviewer #3

(Remarks to the Author)

We review the paper by Weber et al whose title states that the Arrow receptor protein is required for PCP. There are a number of fundamental problems with this work:

1. There are several previous papers that look at the requirement for Arrow in polarised cells in *Drosophila*, they have all drawn the same general conclusion, that arrow has a function in PCP. But they have taken analysis in other directions than Weber et al. One earlier paper is not mentioned or included although the data therein are relevant (Lawrence et al, 2002). Clones lacking Arrow cause a tissue transformation as well as affecting polarity. They do not behave autonomously (as might be expected with simple loss of a receptor) but as in the eye in the opposite way to clones lacking *frizzled*, that is wildtype cells around the clone are affected so that their polarity points away from the clone. Interpretations in that paper are also confusing.

2. This manuscript is a mess, the attempts to analyse the data in a constructive way lack focus. It starts with a review, which is extensive but barely justified by the subsequent data. The results themselves are insufficiently analysed so the reader remains unclear of the phenotype of arrow clones, and how they compare with earlier results and then what to make of them. There are numerous illustrations that are confusing and all in all were any reader to try to understand this paper in the context of previous work with clones lacking Arrow we believe they would get lost.

One area that needs clarification is the wing, we are told that arrow- clones cause reversal of polarity outside the clone, but that the side of the clones that shows the reversal (i.e. if the hairs points away from or into the clone) is random. Do these opposite results depend on location of the clone within the wing? That conceivably might be a way of making sense of the findings. Their final conclusion, that "[arrow] non-autonomous PCP requirement, affecting wild-type cells adjacent to mutant *arr* LOF clones, could be caused by its effects on Wnt protein distribution" is intriguing: do they think that Wnt distribution may be also random?

I think it's up to the authors to do their best to present the results in as clear and logical way as possible, to extract some kind of storyline from any new data and to help the reader.

There may be publishable material in this work, but the paper itself is not nearly ready for publication.

Other points:

Fig1 Is the shape of *arr*- clones in the wing similar to wild type clones?

Fig2F Hair orientation is affected in *arr*- cells but since the authors pooled together data from different clones, it is not clear if hair orientation is consistent in each clone although the orientation may be still different from the wild type. Is the presence of *Arr* in a cell a requisite for polarity propagation?

Fig3D The authors claim that the phenotype of R3/R4 *arr*- mosaics is the same than *fmi*- ones: "When either R3 or R4 (or

both) are fmi-, the ommatidium selects chirality randomly or stays symmetrical.” (Das et al, 2002). Is chirality random in R3/R4 arr- mosaics? It doesn't seem to be the case. Between 74 and 88% of these mosaics have normal chirality.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript has been improved from the original, and the additions are valuable. The experiment the authors lead with, showing that arrow weakens the PCP effect of dsh knockdown, is still confounding and needs explaining. The PCP effect of Arrow in the wing is novel, and the effects on polarization of core PCP proteins are new and convincing. There remain some questions about whether the eye phenotypes might be caused by canonical pathways. The manuscript remains a bit weak in discussing mechanisms; there is little discussion of what is known about Arrow's interactions with intracellular members of the Wnt pathways.

1) PCP vs canonical- The most critical issue is whether arrow's effects are via the PCP or canonical Wg/bcat pathways. In the wing the effects on core protein polarization argue for the former, and the new data finds genetic interactions with several PCP pathway but not bcat (arm) heterozygotes. However, the authors themselves raise the possibility that some of the wing PCP effects may be mediated by changes in Dachshous or Fj levels, an effect that could influence PCP protein polarization through the Fat-Dachshous pathway but would be initiated by a change in canonical Wg/bcat signaling. Nonetheless, Wg regulation of Dachshous or Fj has never been demonstrated in this tissue and is unlikely (see below).

But this is a distinct possibility in the eye, where expression of Fj and Dachshous are both sensitive to canonical Wg signaling (see below), and so the question remains whether their eye phenotypes are via the PCP or canonical Wnt/bcat pathways. Since the authors raise this possibility in the wing, is there something that rules this out in the eye?

The issue of arrow acting via canonical signaling in the eye is also raised by ref 14, who showed in 1998 that both arrow and hypomorphic bcat/arm mutations affected eye PCP. The manuscript does not discuss ref 14 in any depth, saying only that these phenotypes are caused by “indirect effects of earlier requirements for Wnt/b-catenin signaling” and “in setting up the polar-equatorial eye field”. However, ref 14 dates from a time before the discovery of polarized PCP proteins or the role of Fat-Dachshous signaling, and did not rule out an effect for arrow via effects other than the induction of novel equators. To put it another way, do the authors have data showing that the PCP defects in their arrow clones are being caused by something different than the PCP defects in 14's arrow clones, based on this work or other studies? Is the location of the clone really sufficient to rule out an effect on equator formation, or an effect via Fj or Dachshous changes?

Ref 14 claimed that sev-gal4 effects would be late enough in the process to affect only PCP interpretation and not the equator field, but did not test arrow. If that is still viewed as a valid test, the authors could argue that arrow's ability to modify sev-gal4 phenotypes showed a similarly late role that was less likely to be via the canonical pathway. Or, if sev>arr-RNAi had an effect, that might also provide a counter-argument

2) Tilting the phenotypic balance- “In a pilot screen with most ligands and transmembrane proteins/receptors acting in the two main Wnt-signaling pathways, we discovered that manipulating arr/LRP5/6 levels did tilt the dsh phenotypic balance, enhancing and suppressing the respective phenotypes when their levels were changed, very similar to effects seen with the Wnt ligands and Fz family receptors (Supplemental Figure S1A-C and A'-C', also Supplemental Table 1).”

First, this is the only time that the authors claim that reducing arrow improves the PCP phenotype caused by reducing Fz/Dsh etc (in this case, a weak dsh RNAi). This result is exactly the opposite of their summary statement in the Discussion, that “Arr/LRP5/6 acts positively in the Fz/Dsh-PCP complex in a Wg/dWnt4 associated manner, and in many ways behaves like a core PCP factor.”, and conflicts with their double and triple heterozygote data. Do the authors have an explanation for this?

Second, to be sure of this result I asked for quantification. However, the authors have added quantification only for the canonical wing notching effect and only for the clonal analysis, but not the dsh RNAi experiment and, in either case, the more critical PCP effect. They show only one example of PCP improvement; even a simple statement that xx number of wings resembled the photo shown would help. The authors also claim in Table S1 that PCP defects are worse in Fig. S1C' than A', but this is not obvious in the photo and needs numbers to back it up.

Third, there is no data showing that this is “very similar to effects seen with the Wnt ligands and Fz family receptors (Supplemental Figure S1A-C and A'-C', also Supplemental Table 1).” or “arr levels affected the dsh phenotypic balance in both Wnt signaling pathways in a similar manner as the Wnts themselves and the fz, fz2 receptors (see above).” Unless there is data the authors are not showing, these statements are wrong and should be removed. Fig. S1A-C has no data on Wnts or Fz. Fig S1 has no data on dsh-RNAi with anything other than Arrow. And none of the other manipulations in Table S1 “tip the balance” between canonical and PCP phenotypes by having simultaneous improvement of one phenotype and worsening of the other.

Finally, the rebuttal letter said this description, which the authors admitted was confusing, had been clarified along with the legend. No changes have been made from the original. Please fix this.

3) Fz and Fz2 overexpression affecting Arrow levels- First, I did not find the original data convincing, as the change in Arrow was limited to only a part of the peripodial clone. The authors did not address this point. They have added additional data using dpp-gal4-driven overexpression, but the claimed effect is limited to the basal sides of cells and in a small portion of the dpp-gal4 region of expression. Given the extreme folding in this region, and thus difficulties with focal planes, I need a cross section of a Z series to trust this new data. And it is not clear why the maximum effect would be limited to this region, or in the basal focal plane shown instead of apically where Fz is concentrated.

This may be confounded by effects on signaling, as canonical signaling was shown to repress both arrow transcription and reduce post-transcriptional Arrow protein

Second, the authors do not fully explain why this data is important. Is there precedent for binding of Fz's to Arrow homologs, and some question whether they can also bind to Fz's that are less involved in the canonical pathway? Arrow's ICD also affects Dsh so there are other possible routes for regulation of the Dsh-based PCP pathway.

4) Discussion- This is improved. I still disagree a bit about the point of the other Wg/Wnt studies, as these were intended to test the effects of flattening gradients of diffusible Wg/Wnt in the wing, not completely remove them. Yu et al used conditional KO rather than inducible CRISPR for most of their studies and backed this up with inhibition of secretion and with ablation of the wing margin. Ewen-Campen used both inducible CRISPR and RNAi. This likely leaves room for Wg/Wnt4, but makes it unlikely to be required in any absolute sense as an instructional, polarizing cue.

The authors do not discuss the gradient idea. They do say earlier in the manuscript that "Wg/Wnt4 are thought to set up the asymmetric localization of core PCP factors early in pupal development" but might need to discuss the gradients in order for this to make sense to most readers.

The mechanistic discussion of Arrow/LRP5-6 is improved, but still largely focused on the extracellular role, rather than the intracellular role regulating Dsh etc. Separating the two is beyond the scope of this manuscript but would be an interesting avenue to explore.

5) Other comments

"with Fz2 inducing specifically Wnt/bcatenin phenotypes, whereas Fz causing exclusively PCP defects."
But loss of Fz2 alone has few phenotypes.

Fig. S1 (H,I) Wing schematics indicating position of clones for each genotype; note that clones in the central area of wings were analyzed.

What do the two different shades of grey mean?

Loss of cytoplasmic Dsh

Despite what the authors wrote in their rebuttal letter, there is no reduction in cytoplasmic Dsh in the PCP manipulation papers they referenced. The difference here is likely that arrow is also affecting the canonical pathway and thus stability of Dsh. The problem being that loss of cortical Dsh may not be a specific marker of the PCP pathway.

Polar clones were not considered in this study, because they can induce ectopic eye fields and anti-equators due to the Wnt catenin requirement in eye field definition^{71,72}.

Is there any clonal data in 71 or 72?

"nor an asymmetric localization in developing wings, was detected with the available tools,"

I requested a clarification on the tools used, but the text here is unchanged. Why not just say "was detected at the boundaries of arrow mutant or overexpression clones" and reference the figures that show this? It does the reader no good to add figures but not reference them in the text.

as it has been shown that Arr levels in the apical wing pouch cannot be manipulated⁸⁶ (Figure 4C,C').

What does this mean? Arr levels are regulated by Wg signaling in the pouch, so they can certainly be manipulated.

sep-gal4 vs sev-gal4- Do these drive similar expression patterns with similar timing?

While wg, Wnt4 knockdown or copy reduction, and fz, fz2 copy reduction enhanced the notching phenotype, no PCP effects were detected.

Are there in fact any experiments with wg, Wnt4 knockdown instead of copy reduction?

A first possibility could arise from the fact that Wnt/b-catenin signaling transcriptionally regulates the graded expression of four-jointed (fj) and dachsous (ds), two key components of the Fat/Ds PCP system^{97,98}

⁹⁸ has no data on this. ⁹⁷ and the unreferenced Zeidler et al. '99 show data from the eye, not the wing. Wu and Irvine '04 showed some data that wing expression depended in part on Vg, although in a complicated fashion further studied by Zecca and Struhl. However, removing Wg reception does little to anti-Vg in the wing unless the cells are dying. Moreover, there is no data showing that loss of wg signaling mimics Fat-Ds-Fj-specific phenotypes in the wing, such as the characteristic and very sensitive changes in spacing between the cross veins.

Grammar, awkward sentences, typos, etc

Explanations of some of the abbreviations or alternative terms (Dvl, trichome) are repeated. fw should be spelled out.

with defects manifest as loss of asymmetric PCP factor localization and reduced levels of Dsh with centrally forming wing hairs.

"localization, reduced levels of Dsh, and centrally forming wing hairs" would be better

classical PCP phenotypes with photoreceptors R3/R4 cell fate and chirality defects.

phenotypes with defects in chirality and the cell fates of photoreceptors R3 and R4.

we tested known Wg/Wnt signaling components, which showed arr/LRP5/6 to shift the dsh phenotypic balance and showed that arr/LRP5/6 shifted the dsh phenotypic balance

are observed around fz and Vang/stbm mutant tissue, and induced by Wnt4 misexpression clones and are induced

an insertion k0813 mutation

Makes it sound like a type. Are there other k0813 mutations? Or is arr[k] just a shorthand for arr[k0813]?

as has been described before, clones near the margin displayed loss of tissue or notches references?

To ascertain that arr mutations were responsible whether would be better than that, as the latter sounds like you have already decided.

can be rescued by expression of myristilated-Dsh (myrDsh), reference? myristoylated

past observations that Dsh constructs caused severe PCP defects by themselves references?

And mildly reduced Fmi levels for arrk (Figure 2C,C',D,D and quantified in E') as compared to neighboring wt control tissue. In contrast, E-cadherin (E-cad), a common component of adherens junctions serving as control, was not affected or mildly reduced in arrk (Figure 2C",D",E).

Serving as a control. This section also makes it difficult to tell that the authors are discussing two different arrow alleles, rather than just variability of arr[k]. Mention which result goes with arr[2] clones and which with arr[k] clones. And any idea why arr[k] had the more disruptive effect?

similar but weaker to effects seen in similar to but weaker than

with accumulation at arr-/arrwt membranes, before and during prehair formation (Supplemental Fig. S2A,A',B,B',C), respectively

Remove respectively. It is only used if A gives B and C gives D, and you say "A and C give B and D, respectively". If is not used if A and B both give C.

these experiments displayed

A similar strategy

used to test whether Wnts

Reviewer #2

(Remarks to the Author)

The authors have done an excellent job addressing the referees' comments, even the non-scientific ones. I recommend the paper for publication.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I thank the authors for their changes. I still think Figure S1 PCP needs quantification to support its claims. I do not think the altered Arr levels caused by Fz or Fz2 overexpression provide any compelling proof of recruitment into a complex. I have also added some discussion about points raised by the most recent changes, and some suggestions I raised previously that have not been addressed.

However, the main thrust and data of the paper remains valuable, and the new additions have improved many of the arguments. I leave any further changes up to the editor and authors.

1) Canonical pathways, Fat-Ds, and eye PCP- The authors now include reasonable arguments suggesting that not all of the eye PCP defects can be via canonical signaling that changes Ds or Fj expression. These are based on past studies on the types of PCP defects noted with Fat/Ds/Fj: inversion defects but not so much the rotation and symmetric outcomes common with Arr changes. I do worry a bit about the older Ft-Ds literature simply ignoring those other defects; Simon 2004, for instance, shows “incorrectly constructed ommatidia or ommatidia exhibiting severe misrotation” not noted in his earlier papers. However, the rates certainly seem lower than the Arr phenotypes, so the argument seems sound.

However, since Arr will affect canonical signaling, and canonical signaling regulates Ds and Fj expression in the eye, it seems likely that Arr will be causing changes in Ds and Fj expression. To my mind, if such changes occur, they are likely to contribute to some of the eye PCP phenotypes, especially at clone boundaries. Or is the argument that the changes will be too slight near the equator to have much effect?

I also brought up the use of the late driver *sev-gal4* as a way of separating early axis-forming from later PCP readout events. But I did not ask before (and I apologize for not knowing), can *sev-gal4*-driven changes in Wnt reception change Ds or Fj expression?

The changed terminology the authors now use for *sev-gal4* is a bit confusing, as the authors now refer to it as “PCP-specific” but then state that it causes both canonical and PCP defects (lines 363-4). So PCP-specific is the wrong term; late-expressed, perhaps? Also, the nature of the canonical defect, and the criteria by which it was determined to be canonical, are not described in this part of the Results, and it might be helpful to add a sentence that does that.

2) Figure S1. I had three complaints about this data. A) The PCP differences claimed in Fig. S1C' was not convincing and required quantification. Here I strongly disagree with the authors' most recent comment; I was very clear about this in comment 1B in my first review. The authors have also changed the “defect” areas shown for Fig. S1A' and C' despite showing the same photos; to my mind this just shows how subjective their criteria are, and how important quantification is for confirming their claims. This is even more important this time around, as the authors now include additional PCP data not present in the first or second versions of Fig. S1.

In their first rebuttal, the authors apparently misunderstood me and added PCP quantification for Fig. 1, not Fig. S1. In the most recent, they have followed my suggestion of stating how many wings they examined but have not said how they quantified PCP in those wings. This is odd because they quantified hair PCP in two other data sets. Pick a region and measure hair angles.

Instead they say “More detailed quantification will be published elsewhere” (Fig. S1 legend).

I will leave this up to the editor, but I do not think that is acceptable. The claim is being made in this manuscript, and the data supporting that claim should be here too.

If the data is too hard to get, a more radical alternative is to remove much of Fig. S1 from this paper, as it has little impact on the authors' conclusions. Begin the paper with the stronger and more straightforward data of Fig. 1.

2B) My second point was that the Arr data in Fig. S1 shows that reducing Arr actually improves PCP defects caused by *dsh*-RNAi, whereas all the other data in the manuscript says that reducing Arr worsens PCP. The authors now acknowledge this in the Discussion and, as in their rebuttal, ascribe this to complex epistasis. I appreciate the change, although I had hoped for a bit more than that, as I think the contrast between the *Dsh* RNAi and *Dsh* overexpression results may be telling; when *Dsh* is reduced, Arr acts as if it is competing some limiting factor (*Dsh*?) away from the PCP pathway and into the canonical complex.

2C) I had complained about the authors not previously supporting the claim that the Arr interactions with *dsh*-RNAi were “very similar to effects seen with Wnt ligands and Fz family receptors” with any data. I thank the authors for now including that data in Fig. S1.

However, I must point out, having seen the data, that the only similarity is that they each change the balance between canonical and PCP phenotypes. They are completely opposite in terms of the direction of that change. Reducing Arr dosage worsens canonical and improves PCP defects, while reducing Wg or Fz/Fz2 improves canonical and worsens PCP defects. Increasing Wnt4 improves both canonical and PCP. If the authors want to call this “very similar”, they should be explicit in the Results text about what they mean. This description of these results as “similar” is repeated in other parts of the manuscript.

3) Recruitment of Arr by Fz2 or Fz- I thank the authors for showing the cross-sections of the *dpp-gal4* UAS-Fz and UAS-Fz2 experiments, and the basal Arr increase in some of the *dpp-gal4* cells is as they said. However, in their second rebuttal they appear to have misunderstood my concerns with this experiment. Their claim is that this shows that Fz and Fz2 can “recruit” Arr, binding them into a complex. The alternative is that Fz and Fz2 are doing something indirect that changes Arr levels or localization without actually forming a complex.

My expectation is that, if a complex is being formed, Arr should concentrate in the same subcellular local as the overexpressed Fz and Fz2. As shown by Wu et al., 2004, even overexpressed Fz preferentially concentrates in the apical junctions, overlapping with DE-cadherin. So why does overexpressed Fz concentrate Arr only basally, far from the junctions? And why is the effect limited to a subset of the dpp-gal4 cells (it does not extend to the far right limit of the zone)? This looks much more like an indirect effect. Unless the authors can demonstrate co-localization of Arr and Fz their claim that a complex is being formed is greatly overstated and should be weakened.

I also asked why the authors think this effect is limited to the disc folds, rather than the pouch. They answered that “Schilling et al 2014 study Arr protein accumulation and try to manipulate it in the wing pouch with the conclusion it cannot be changed there...” However, this is wrong. Schilling et al. expressed Arr with a tubulin promoter and saw a strong increase in levels throughout the disc, including the pouch (their Fig. 1D-F). The pouch expression retained a proximal to distal gradient, but the overall levels were higher than normal. Please change line 328 in the text to reflect this

I also asked what point was being addressed by this experiment. The authors replied that that Fz family receptors and LRP coreceptors are largely complexed by simultaneous binding to Wnts. But given that Fz, Fz2 and Arr can all bind Drosophila Wnts, they should all form a complex, why is this a novel question? Has it been posited that Fz and Arr cannot be bridged by Wnts, even though they can both mediate canonical signaling?

4) Discussion. Again, the new material in the Discussion improves the manuscript, especially the discussion that incorporates a possible role for the Arr ICD in PCP. I would have preferred a bit more discussion of the extensive literature on the Arr-LRP5/6 ICD, but as this largely pertains to effects on canonical pathway components I suppose the authors are less interested.

As for the prior studies on Wnt gradients in the wing, I am still a bit puzzled by the statements about “mosaicism in in vivo CRISPR assays” and the lengthy speculation based on that in the Discussion. I assumed by this the authors meant first generation CRISPR phenotypes created by UAS-gRNA and cas9, as in the CRISPR therapy articles they reference. But Yu et al did not use CRISPR in this way. They used CRISPR only to generate permanent deletion alleles and insert integrase sites, and homozygotes with such alleles will not be subject to future mosaicism. The conditional alleles used by Yu did not rely on CRISPR to generate the mutant condition; they used FLPase to flipout FRT constructs inserted at attB sites, either replacing a wt Wg with membrane-tethered NRT-Wg or in one case removing Wnt4. I suppose one could argue that the FLPout created mosaicism, but then that should be obvious since the NRT-Wg is tagged. Have the authors looked?

To reconcile Yu et al with the authors’ work, my guess is that there is low-level NRT-Wg throughout the wing that supplies a non-graded stimulus to Fz that is needed for normal PCP. Such expression does certainly exist in earlier stage wing discs, and I’ve seen nothing that completely rules it out at later stages after high level concentration of Wg expression at the margin and the proximal rings.

The situation may be parallel to the role of Ds-Fj gradients in the wing. Yes, there are gradients of expression, and ectopic gradients or boundaries can polarize hairs. But in many situations those gradients, and even Fat itself and thus all polarized Fat-Ds binding, can be removed with only very slight disruptions to wing hair polarity.

4) Clarity-Statements made in the Results should be clear enough that the reader does not have to look at figure legends or the Methods to understand what is being said. I had previously asked for a couple minor changes, but the authors have made no changes in the Results that help, despite saying that they would, or that they have when in fact they have not.

4A) “In a pilot screen with most ligands and transmembrane proteins/receptors acting in the two main Wnt-signaling pathways, we discovered that manipulating arr/LRP5/6 levels did tilt the dsh phenotypic balance, enhancing and suppressing the respective phenotypes when their levels were changed,” This sentence about Fig. S1 is completely identical to the original, as is most of the rest of the paragraph. The problem is that that “changing balances” does not make it clear that a SINGLE genetic change usually improves one phenotype and worsens the other, rather than some genetic changes altering the phenotypes one way, and other change altering phenotypes the other. How about something like:

In a pilot screen assessing the canonical and PCP phenotypes we discovered that any single change in Arr levels tilted the dsh IR phenotypic balance, simultaneously enhancing one phenotype while suppressing the other.

4B) nor was asymmetric localization in developing wings was detected with available tools.

Knowing what they looked at is important, as many authors might content themselves with measurements of nematics which are often not sensitive enough. If what the authors did was look at anti-Arr at mutant clone boundaries or tagged Arrow at boundaries of misexpression, simply say in the Result text “was detected with anti-Arr at mutant clone boundaries or boundaries of tagged Arr misexpression”

Other notes:

Line 232- “but milder, to what was described for fz and fmi clones”- Milder in what sense? The reduction in Dsh looks stronger, and the “loss” of asymmetric membrane associated localization sounds equivalent.

Line 297-305. The new paragraph is appreciated, but for the naïve reader it might be best to start with a statement about the possible role of Ft/Ds and canonical signaling.

Line 315- (Supplemental Fig S4K,L etc.). Because new panels have been added, these now refer to the wrong panels

Line 392- While this experiment shows that Fz might titrate Arr away from canonical signaling, is there evidence that this is "to PCP signaling"? Does PCP improve?

Lines 421-422-First, Fig. S6F shows one bar where *arr/+*; *arm/+* increased PC defects, and another where it doesn't. Is this a mistake? It is opposite to the statement in the text,

Second, in S6E *arm/+* appears to decrease the PCP defects; if statistically significant, this might be worth pointing out.

Third, how are the PCP defects being quantified? Does % mean the % of hairs with abnormal angles, or the % of wings showing any defects at all, or something else?

Line 481- I have looked at reference 46 and seen no claims about asymmetric enrichment of CK1e protein. The authors should check.

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Point-by-point Response to Reviewer comments

(our comments are in blue)

Reviewer #1 (Remarks to the Author):

The manuscript shows both directly and through genetic interaction experiments that Arrow, the Drosophila homolog of LRP5/6, acts not only in canonical Wg/Wnt signaling but also in Fz/Vang PCP signaling in the Drosophila wing, eye, and abdomen. This is intriguing, as Arrow and LRP5/6 are usually framed as a Wg/Wnt co-receptor that acts along with Drosophila Fz and Fz2 in canonical beta-catenin signaling. A role for Arrow in PCP is thus novel and worth publishing, and the PCP phenotypes are convincing.

As Arrow and its homologs act as co-receptors, this manuscript also bears on a possible role of Wg/Wnt in Drosophila PCP. This issue has been long-debated, especially as the spatial cue for orienting core PCP in Drosophila is not known; many have suggested that gradients of diffusible Wg/Wnt might be one such cue. The authors have some genetic interaction experiments that suggest a role for Wg and Wnt4, in agreement with their earlier Wu et al. work from 2013. They also show genetic interactions with Fz and Dsh, and effects especially on Dsh levels.

The main weakness of the work, however, is that it does not have much that is definitive concerning the mechanisms connecting Arrow to the PCP pathway. The authors obviously prefer effects through Fz and Dsh, and state so in several places, but as they note there are some results in the eye that do not match this model.

The data they show to support this connection are largely based on a change in Dsh protein levels, a possible effect of Fz and Fz2 on Arrow levels, and a few genetic interaction experiments. However, the genetic interactions shown are limited to a few candidates in the pathway, as are the effects on the levels and distribution of pathway proteins. Are there other canonical or PCP pathway components that do not genetically interact with Arrow in PCP, or that do not change their levels or polarity in arrow mutant clones? If there were, this would provide much stronger evidence for a specific role for Fz and Dsh.

We thank the reviewer for their positive assessment of our work and their helpful and constructive criticism to further improve the paper.

We have addressed all comments and listed below individual points raised by the reviewer in quotation marks and the explanations and/or experiments that pertain to it.

“The main weakness of the work, however, is that it does not have much that is definitive concerning the mechanisms connecting Arrow to the PCP pathway. The authors obviously prefer effects through Fz and Dsh, and state so in several places,”

To get a firm conclusion on the issues raised above, we have performed 4 lines of experimentation to address this:

(1) Multi-heterozygous interactions (which have been used to define pathway components for PCP and Wg/ β -catenin signaling in the past):

In these genetic assays, Arrow behaves like known core PCP factors, showing interactions with Fz, Dsh (shown in the original submission) and also with Vang/stbm and Fmi (new data, Fig. 6I-L and 6E-H). This is now presented in the fully revised new Fig. 6, Supplemental Fig. S6 and Suppl Table S1A for summary of genetic experiments. The multiply heterozygous assay to check for Wnt-PCP phenotypes is now complete with several additional genotypes, we included *arm* as Wnt/ β -catenin pathway factor (Supplementary Fig. S6E,F). These multi-heterozygous interactions, including Vang and Fmi, place Arr in a similar “functional context” as Fz, like a core PCP factor. There is no such interaction effect with components of the canonical Wg/ β -catenin pathway or *wg* alone (Supplementary Fig. S6D,E,F, Supplementary Table S1A). Importantly, heterozygosity for the *wg*, *dWnt4* double mutant, which are in combination critical for PCP, as shown earlier (Wu et al Nature Cell Bio 2013) also interacts with *arrow*^{-/+}.

(2) Arrow “recruitment assay” to test which transmembrane factors can “stabilize/associate with” Arrow:

In addition to the original studies with Fz (PCP specific) and Fz2, which serves as a positive control, and is the defined Arrow receptor partner in Wg/ β -catenin signaling, Vang and Fmi are now included to monitor their effects on Arr protein levels/localization. Vang and Fmi do not affect Arrow levels; this is shown in the new Fig. 4C-G, Supplementary Fig. S4A-D), demonstrating a specific effect of Fz on Arrow (with Fz2 being the known positive control).

(3) Modifications of core PCP factor gain-of-function/GOF effects, which are the most suited functional tools in PCP establishment to link a gene to a particular PCP requirement:

Arrow expression levels specifically (i) modify Fz and Dsh associated phenotypes, promoting their activity, (ii) do not affect Fmi effects, and (iii) display a modification of Vang, in the opposite direction to that with Fz and Dsh (shown in Fig. 5, and revised Supplementary Fig. S5I-L). These data are consistent with Arr promoting Fz and Dsh activity, and possibly antagonizing Vang likely indirectly via Fz-Dsh.

(4) General interactions with *arr* knockdown defects:

arr knockdown with gene copy number reduction for *fmi* and *Vang* has been added (new Supplementary Table 1B).

Taken together, these new and expanded data sets provide firm, genetic evidence for Arrow acting as a core PCP factor and for it promoting Fz-Dsh PCP activity. It furthermore provides *in vivo* evidence for a Wnt-requirement in PCP. These conclusion are discussed in the Results and Discussion sections and represented schematically in the revised model figures (new Fig. 7).

“The authors obviously prefer effects through Fz and Dsh, and state so in several places, but as they note there are some results in the eye that do not match this model.”

PCP establishment in the eye is a complicated process which culminates in the asymmetric activation of Dl-Notch signaling (Dl upregulated by Fz in R3 to induce the R4 fate via Notch activation). The genetic *arrow* requirement in R3 overlaps with *fz* and *dsh*, consistent with

the notion that Arrow is functionally linked to the Fz-Dsh complex activity. Its requirement in R4 suggests that it has a secondary role there, possibly by affecting Wnt distribution, which should affect both cells, R3 and R4. It might also be needed in R4 for the recruitment of a kinase (a CK1 family member or Abl etc) as these kinases are known to act in both Fz/PCP and Notch signaling. Or it might have an effect on another PCP factor in R4. The observation that Arrow is also required in R4 does not negate its link to the Fz-Dsh complex in PCP signaling in R3. We have added an explanation to this end in the introduction to the eye R3/R4 specification (page 11) and Figure 3 description in the main text (page 12-13), in the figure legend, and in the Discussion (page 21).

“However, the genetic interactions shown are limited to a few candidates in the pathway, as are the effects on the levels and distribution of pathway proteins.”

It is important to clarify that all core PCP factors are interdependent, as for example Fmi is required for stable Fz and Vang membrane localization and their polarization, as is Fz for stable Fmi and Vang polarity, or Vang for stable Fz and Fmi polarity. These relationships have been well documented by early papers from the Uemura, Strutt and Axelrod labs (e.g. Shimada et al, 2001, Strutt 2001, Axelrod 2001, Das et al., 2002, Strutt et al 2016) and confirmed and refined by several labs (Strutt, Mlodzik, Axelrod etc) and in many genetic combinations. As such the loss of polarity as we show with Fmi staining is a reliable marker for all core PCP factors and similar to *dsh* LOF and comparable but milder to *fz* effects. The same applies to Dsh and the reduction in Dsh levels as seen in our study is comparable and milder to that seen in *fz* LOF clones.

“Are there other canonical or PCP pathway components that do not genetically interact with Arrow in PCP, or that do not change their levels or polarity in arrow mutant clones?”

Please see above for a list of functional, genetic interactions added/included and the explanation above, that all core PCP factor localizations are interdependent (Strutt et al., 2016). The canonical Wg/ β -catenin components do not interact with *arrow* in a PCP context.

There are a few other places where the work could be improved, largely in clarity and improved discussion and a couple bits of data.

1) “manipulating arr/LRP5/6 levels did tilt the dsh phenotypic balance, enhancing and suppressing the respective phenotypes when their levels were changed, very similar to effects seen with the Wnt ligands and Fz family receptors (Supplemental Figure S1A-C and A’-C’, also Supplemental Table 1).”

1a) The wording here is a bit confusing. Does this mean that when the core wing margin phenotype gets worse, the PCP phenotype always gets better, and vice versa?

Thank you for pointing out our confusing phrasing. Yes, you’re understanding our attempt at its description correctly. It is now clearer phrased, also in the text of the figure legend.

1b) The quantification in S1 is all canonical signaling (wing margin), but nothing on PCP. And while the picture shows a PCP improvement with *arr*+, it is not clear if there is any change in PCP defects with Arrow overexpression.

Thank you for this helpful comment. We have added quantifications of the PCP rescue in wing clones to the revised Figure 1 (new panels Fig. 1G-I). The rescue of the PCP defects is almost complete as well (similar to the wing margin rescue, see quantification in 1G). Overexpression of Arrow alone has no detectable phenotypes in an otherwise wild-type background.

2) Changes in Fmi levels?

The abstract states that in arrow clones “defects are manifest as reduced levels of Fmi/Celsr and Dsh along with loss of their asymmetric localization.”

However, the Results say “reduced and uncoordinated asymmetry” of Fmi, which is different from levels, and there is no data quantifying Fmi levels in Fig. 2. Moreover, any change in Fmi levels in Fig. 2D seems to be mirrored by a similar change in DEcad levels.

We apologize for the confusing statements (Abstract vs Results).

The protein levels of Fmi are not reduced. We have added quantifications of Fmi levels in *arr*² and *arr*^k clones to the revised Figure 2 (new panel 2E) to document that Fmi levels are not affected in mutant cells of *arr*² (null allele) and mildly reduced in *arr*^k, indicating that the observed changes are in polarized Fmi localization, mainly loss of polarity. E-cad levels are also not changed in *arr*² clones (null allele), while we see a mild reduction in *arr*^k clones (Fig. 2E). We have added comments in the Results how the change on Fmi localization compares to core PCP mutant effects described in the literature (pages 9/10).

We would like to clarify here that earlier studies with the other core PCP genes did not use detailed “Packing/Tissue Analyzer software”-based quantification analyses (relying on observable qualitative changes), and hence our data document the defects in *arr* LOF clones to a greater detail. As such, the term we use “uncoordinated asymmetry” is based on data from the nematic order studies with the Packing Analyzer software, which reveal a random (angle) and markedly reduced (almost lost) nematic order (polarity strength).

Effects on E-cad are likely through Wnt/b-catenin signaling and indirect (e.g reviewed in Heuberger and Birchmeier 2010). However, as this is not consistent and the null allele, *arr*², does not show significant loss of E-cad we prefer to not distract from the main points of the paper

3) Dsh levels

The reduction of Dsh levels in arrow clones appears to be in the cytoplasm as well as the cell cortex. If this is real, is this typical of loss of Fz/Fz2 or other core components? Or is this something new?

Thank you for this careful consideration and yes, reduction is seen in both cytoplasm and cortex. Quantification of Dsh levels in *arr* mutant cells reveals that Dsh levels are reduced

generally and we also observed that the asymmetric accumulation at the cortex is lost. We have described in more detail how Dsh localization is changed in the figure legend and main text and that this effect compares to, but is milder, to what was published for *fz* clones, with LOF *fmi* clones having a more severe effect on Dsh (all shown by Shimada et al., 2001 and Axelrod 2001) (pages 9/10).

It is not possible to separate the effect on Dsh between PCP and β -catenin signaling with the Fz receptors, as Fz2 alone has no detectable phenotype and Fz/Fz2 double mutants affect both Wnt signaling pathways.

4) Recruitment of Arrow by Fz and Fz2

4a) The authors state: “As imaginal disc cells are in a pseudostratified columnar arrangement and Arr localization is punctate throughout the apical cytoplasm and periphery of cells, we examined the flat cuboidal cells of the peripodial cell layer of discs.” But since Fz and Fz2 are largely at apical junctions of the disc epithelial cells, wouldn’t the authors still be able to detect increased Arrow at apical junctions? If they tried and didn’t see an increase they should say so.

This is an important point, thanks for mentioning it.

Arr localizes in punctae throughout the cytoplasm in all disc cells tested (here and in previous studies) via Arr antibodies and *UAS-ArrHA*, it does not appear apically enriched in any *wild-type* cell type we tested here (new Supplementary Fig. S4L’ inset). It has very distinct regions of elevated or reduced levels throughout tissues, as it is regulated by Wg/ β -catenin signaling (Marois et al., 2005, Rives et al., 2006, Schilling et al., 2014). Only *UAS-ArrHA* was shown to be apically enriched (Culi and Mann 2003, and our study: Fig. 4A,B). To better document this we have added Arrow stainings in eye-antennal imaginal discs, which reflect different stages of development in the same tissue (e.g. anterior to the furrow in eye disc during disc growth and posterior to the furrow during photoreceptor induction and PCP-based patterning), now shown in the new Suppl. Fig. S4K-M. These additional stainings reflect again a punctate localization throughout all cells anterior and posterior to the furrow (Fig. S4K’). The staining is specific, as it is lost in *arr-2* mutant cells and reduced in *arr-k* hypomorphic clones, quantified in Supplementary Fig. S4M.

4b) The images do not show clone-autonomous increases in Arrow. There is equally heightened Arrow in wild type cells outside the Fz overexpressing clone (bottom of 4C’) and the heightened Arrow is limited to one boundary of the Fz2-expressing clone (4D’). How do the authors explain this? It would also help to know a bit more about how the authors picked the wild type regions to include for the quantification.

We have repeated these Arr recruitment/stabilization studies using the *dpp-GAL4* driver (as it allows comparison in identical expression domains, allowing protein levels to be more reliably assessed, in contrast to randomly occurring *actin*-FLP-on clones, which can sit close to areas with endogenously elevated Arr levels). As specificity controls, we included also the other transmembrane core PCP factors, Vang/Stbm and Fmi, for comparison to effects seen with Fz and Fz2 (new panels in Fig. 4D-G). The new control experiments with Vang and Fmi overexpression do not affect Arrow levels, unlike Fz and Fz2 (Fig. 4D-E”), quantified in Fig.

4G. We analyzed Arr protein levels/localization outside the wing pouch and at the basal side of the tissue (boxed area in Fig. 4C), to avoid regions where *arrow* expression is transcriptionally regulated by Wg/ β -catenin signaling, and expressed at stable levels (described by Schilling et al., 2014), comparing levels within the *dpp-Gal4* expression domain (Fig 4C) with wild-type control area just adjacent to it for best possible direct comparison. Here we could find specific Arr level changes upon overexpression of Fz or Fz2 (see description in Methods and figure legends). Fmi and Vang do not affect Arr levels, but interestingly they affect E-cad levels to small but reproducible extent (quantified in Fig. 4G). These data are consistent with the notion that both Fz (PCP) and Fz2 (β -catenin signaling control) specifically engage with Arr, which is not seen with other PCP core factors, providing support for a unique functional relationship of Fz and Arr in PCP signaling.

5) The Discussion of studies on Wg and other Wnts

The discussion of this important issue is far too brief, and also a bit inaccurate. In fact many of the manipulations in references 88 and 89 did reduce growth. But it is true that the manipulations were largely designed to allow growth in early stage discs but greatly reduce or eliminate Wnts at later stages when PCP is thought to be established. The authors are correct that there may be residual Wg signaling; in fact the membrane-tethered Wg was used by one lab may allow precisely that. And it is possible that there are still low levels of Wnts after the inhibition of Wnt secretion done by both studies. But the studies argue strongly that PCP orientation is largely retained in situations where the gradient of diffusible Wg and other Wnts was eliminated, and there must be other ways of orienting PCP.

This is an important subject, and we agree deserves more discussion, but it is tricky to find the right balance on how much to discuss this subject in this paper. There might be several reasons why the studies reported in references 88 and 89 did not see PCP defects. These could be due to experimental reasons, like for example incomplete CRISPR knockouts with cells still present that have no or partial CRISPR knockdown, a well documented fact (e.g. Becker and Yamazaki 2024, <https://doi.org/10.1016/j.exphem.2023.11.007>); see also for additional references in the Discussion (page 23). Moreover, the wing disc is rather plastic and often, when generating clones that cause cell and tissue loss, neighboring cells proliferate in excess to compensate for this loss (generally termed as “compensatory proliferation”, this has been well described by Giraldez and Cohen 2003 for the Wnt/ β -catenin pathway and *arr* clones. Such an event could lead to (at least temporal) retention of cells that still do express Wg/Wnt4. The effects of wing size reduction via RNAi as documented in detail and elegantly by Barrio and Milan 2020, which show a more severe tissue loss than those reported in refs 88 and 89, suggest that some Wnt activity might have been still present in the assays in the above two studies (refs 88 and 89). As such in the experiments in refs 88 and 89, a few “wt cells”, which would produce (normal amounts of) Wg and Wnt4 could take over in the knockout region/compartiment, or compensate out CRISPR knockout cells and be capable to pattern PCP in a smaller wing (without being sufficient to induce wing margin cells due to the high threshold requirement for Wg in margin cell induction). The experiments in references 88 and 89 could have been analyzed in

developing tissue, positively marked for knockdown cells, an absolute prerequisite for studies of the Wnt/ β -catenin pathway (see above Giraldez and Cohen 2003 for example). It is also possible that there are multiple levels of PCP regulation and Wg/Wnt4 might be more important in the central and distal wing region, while the proximal wing area is more sensitive to forces generated by hinge constriction, as seen in (hypomorphic) mutants of *Fat* and *dachsous*, which primarily affect PCP in the proximal regions of wings, an area suggested to be PCP-patterned by hinge constriction effects (Aigouy et al 2010, Cell). Irrespective of the reasons why refs 88 and 89 did not detect PCP, the strong effects of Wg and Wnt4 heterozygosity in Arr and Fz or Dsh heterozygous conditions is consistent with a critical role for Wnts in PCP in Drosophila. We have expanded the Discussion of this topic. Hopefully, we have found the right balance in tone and weight (pages 23-24).

This still leaves open the possibility that Wg and Wnt4 help Fz/Fz2-based PCP activity, even if they do not provide a spatial cue, something that might become especially critical in a sensitized genetic test. That would also fit the polarity defects observed in Wg/Wnt4 mutants, which in Wu 2013 still orient distally but with less precision.

For clarification, PCP activity is not Fz2 dependent. Fz is the sole PCP receptor as far as is known.

The defects of Wg/Wnt4 double mutants shown in Wu et al 2013, reflect a largely randomized PCP orientation in early pupal wings, prior to hinge constriction, indicating that the Wnts are critical. So it appears to be essential, not just for precision. Also at this early stage and in late stages, Wnt misexpression reorients cells towards the Wnt expression clone, which is also consistent with a critical and instructive role of Wnts in early and late PCP signaling in the pupal wing.

It would be valuable to include a full discussion of these points.

Yes, we agree. We have expanded the discussion of this (pages 23-24, see also above), in the hope that the right balance in tone and argument was found.

Minor notes, typos:

“Wnt/ β -catenin”

Thank you, this has been corrected.

“However, no specific pattern in developing photoreceptor cells, nor an asymmetric localization in developing wings, was detected with the available tools,”

Please describe the tools used. Did the authors look at boundaries between tagged and untagged Arrow?

We have added the description of the *arrow* tools used (anti-Arr and *UAS-ArrHA*) in Methods, figure legends (Fig. 4 and S4) and main text references. Boundaries exist in the *arr* clones stained with α Arr, which we show now in new panel Supplementary Fig. S4K,L and no effects

are seen there. The same is true for *ptc>UAS-ArrHA* in panels 4A,A',B'B', boundaries are at the red to non-red cells.

“A first possibility could arise from the fact that Wnt/b-catenin signaling transcriptionally regulates the graded expression of four-jointed (*fj*) and dachsous (*ds*), two key components of the Fat/Ds PCP system (reviewed in 87).” Reference 87 only describes this in the eye disc, not the wing.

Apologies for this confusion. This review article summarizes Ft-Ds function in eye, wing and abdomen, it does not address that Wnt/ β -catenin is upstream of Ft/Ds expression in the wing. We have added the respective original paper reference(s) to clarify the link of Wnt/ β -catenin signaling to graded Ds and Fj expression.

“whereas fat or ds mutant clones show an upregulation of Fmi and its mispolarization with an increase in actual polarity (reviewed in 87)”

Does Reference 87 mention this? Has someone observed an upregulation of Fmi levels?

We apologize for this mis-statement, it was unintended.

What we tried to say is that PCP polarization is present but randomized (mispolarized) in Fat and Ds mutant clones as seen with Fmi staining (a reliable and general marker for core Fz/PCP factor polarization, see above). Fmi polarization is maintained although at misoriented angles. We have added the relevant original references: Yang et al, Cell 2002; Strutt and Strutt, Dev Cell 2002, Ma et al, Cell 2003 (mentioned in Discussion on page 22).

We observe a loss of polarity with Fmi localization (reflected in markedly reduced nematic order of Fmi polarization), resembling but milder than that of *fz* and similar to *dsh* mutant effects (Shimada et al., 2001, Axelrod 2001). The loss of polarity of both Fmi and Dsh as we observe it in *arr* mutant clones is consistent with an *arr* function in core Fz/PCP signaling. This has been clarified in the text with the respective original references.

Reviewer #2 (Remarks to the Author):

In the study, “The Wnt co-receptor Arrow-LRP5/6 is required for Planar Cell Polarity establishment in *Drosophila*,” Weber and colleagues show that *arr* loss-of-function (LOF) clones display key planar cell polarity (PCP) defects in both the wing and the eye, suggesting that *Arr* plays a positive role in Wnt/PCP signaling. This finding challenges current models that mainly assign *Arr*/LRP5/6 the role of a Wnt/ β -catenin-specific co-receptor, suggesting instead that *Arr* is also necessary for proper PCP signaling. The specificity of this requirement is highlighted by rescue experiments: coexpression of *Arr* within clonal patches restores wing PCP phenotypes, thereby confirming a PCP-specific function for *Arr*/LRP5/6.

In pupal wings, *arr* mutant clonal patches show a loss or misorientation of the characteristic asymmetric localization of Flamingo and Dishevelled. These polarity defects happen alongside a reduction in the levels of Fmi and Dsh proteins within the affected

regions. Functionally, these molecular issues result in misoriented wing hairs, a phenotype similar to what is seen in loss-of-function mutants of core PCP components like *frizzled*, *dsh*, or *Van Gogh*. In the eye, *arr* clones interfere with the binary fate determination of the R3 and R4 photoreceptors and disrupt the chiral arrangement of ommatidia, again linking *arr* function to the core PCP machinery that controls tissue-wide polarity and mirror-symmetric patterning.

Genetic interaction and epistasis analyses place *arr* either downstream of or at a similar hierarchical level to *fz* and *dsh*, suggesting that these factors work closely together within the PCP signaling network. Consistent with this positioning, *Arr* appears to enhance PCP activities mediated by *Fz* and *Dsh*: increasing *Arr* dosage strengthens the PCP phenotypes caused by *Fz* or *Dsh* overexpression, while *arr* LOF or knockdown reduces these effects. Additionally, PCP-specific defects involving *arr* are seen in genetic combinations with *wg*, *Wnt4*, and *fz*, connecting *Arr* functionally to *Wg/Wnt4* inputs in the PCP pathway.

Overall, the evidence indicates that *Arr* functions similarly to a component of the *Fz-Dsh* complex in PCP signaling. By enhancing *Fz*- and *Dsh*-dependent activities and affecting the stability and asymmetric localization of core PCP proteins, *Arr* likely acts as a link that connects the *Fz-Dsh* module to *Wg/Wnt4* signals, thereby integrating ligand input with the development of planar polarity in wing and eye tissues.

The findings in this paper are of great interest for the general Wnt signaling field. They are surprising, but likely due to a lack of attention to *Arrow* phenotypes in previous studies. The genetics approach makes it difficult to argue with the findings, as the phenotypes are positive (very sound methodological approach).

We thank the reviewer for their very positive assessment of our work and their nicely phrased summary of the paper.

Suggestions:

One issue that could be addressed to strengthen the paper would be to mention recent structural studies that define the interaction of Wnts with Fzs and LRP5/6. These structures show that it isn't easy to see how a simple Wnt-Fz interaction could activate signaling of any sort, as two domains of Wnt bring together Fz and a co-receptor. Wnt agonists also rely on nanobodies against Fz and LRP in combination to stimulate pathway activation. Downstream, LRP and Fz coordinate *Dsh* activity, suggesting a function in both Wnt pathways.

Thank you for this suggestion.

We have included a thorough discussion of the known structural features of Wnt-receptor/co-receptor complexes in the signaling contexts as related to Wnt-binding and *Dsh/Dvl* "activity" in the Discussion section (pages 24/25). It does indeed strengthen the

notion deduced from our data that (i) Wnts require a receptor/co-receptor pair for their binding and (ii) downstream signaling via Dsh/Dvl requires Arr-LRP5/6 activity.

Reviewer #3 (Remarks to the Author):

We review the paper by Weber et al whose title states that the Arrow receptor protein is required for PCP. There are a number of fundamental problems with this work:

1. There are several previous papers that look at the requirement for Arrow in polarised cells in *Drosophila*, they have all drawn the same general conclusion, that arrow has a function in PCP. But they have taken analysis in other directions than Weber et al. One earlier paper is not mentioned or included although the data therein are relevant (Lawrence et al, 2002). Clones lacking Arrow cause a tissue transformation as well as affecting polarity. They do not behave autonomously (as might be expected with simple loss of a receptor) but as in the eye in the opposite way to clones lacking frizzled, that is wildtype cells around the clone are affected so that their polarity points away from the clone. Interpretations in that paper are also confusing.

With all due respect, we disagree with these remarks.

First, there is only one paper: Lawrence et al. (2002), who show adult abdominal *arr* clones with PCP defects. That paper is addressing the role of how Hedgehog and canonical Wg/b-catenin signaling affect PCP, via effects on an unknown factor X, in the abdomen. They have looked at it as a component of canonical Wg/b-catenin signaling regulating transcriptionally the presumed factor X. It is the only study that reports *arrow* clones associated with PCP defects. There was no evidence presented that *arrow* is functioning as a core Fz/PCP group factor/gene. We have added the reference in the Introduction when setting up this work. We think it is fair to say that there is consensus in the field for several years that there is no factor X. The PCP non-autonomy in the Lawrence et al paper was shown in *arm*/β-catenin clones and interpreted as caused by the (secreted/diffusible/long-range acting) factor X, which was never identified.

2. This manuscript is a mess, the attempts to analyse the data in a constructive way lack focus. It starts with a review, which is extensive but barely justified by the subsequent data. The results themselves are insufficiently analysed so the reader remains unclear of the phenotype of arrow clones, and how they compare with earlier results and then what to make of them.

Again, with all due respect, but this is a non-scientific and rather inappropriate statement. Why is it a mess? The other reviewers understood the premise and lauded scope of the study and data as presented.

To clarify the major difference in *arrow* function analyses between the two studies (ours and Lawrence et al 2002), we have added a short discussion in the context of the old model, including the Lawrence et al. 2002 reference in the Introduction (see above).

Furthermore, many aspects related to Arrow acting in Wg/ β -catenin signaling and growth control have been nicely covered in the past. Giraldez and Cohen 2003 have extensively looked at *arr* clones in the context of proliferation and cell competition, so have Spencer et al., 2023 at cell divisions, a topic that is not the focus of this paper, and has been well covered in the past.

There are numerous illustrations that are confusing and all in all were any reader to try to understand this paper in the context of previous work with clones lacking Arrow we believe they would get lost.

This comment is confusing at best, we have used illustrations from earlier publications or reviews from our lab and edited them according to the new data. Again reviewers #1 and #2 liked and understood the premise and scope of the analyses shown in this paper, and the associated conclusion(s).

With respect to previous work, other canonical Wg-signaling associated aspects have been well covered in the past (e.g. Giraldez and Cohen 2003). We have attempted to clarify any possible reason for confusion to the best of our knowledge.

One area that needs clarification is the wing, we are told that *arrow*- clones cause reversal of polarity outside the clone, but that the side of the clones that shows the reversal (i.e. if the hairs points away from or into the clone) is random. Do these opposite results depend on location of the clone within the wing? That conceivably might be a way of making sense of the findings. Their final conclusion, that “[*arrow*] non-autonomous PCP requirement, affecting wild-type cells adjacent to mutant *arr* LOF clones, could be caused by its effects on Wnt protein distribution” is intriguing: do they think that Wnt distribution may be also random?

The position of the clones does not matter for the non -autonomy, as was summarized and represented in Figure 1F, and we clearly state that these non-autonomous effects do not resemble (or duplicate) any previously described PCP induced non-autonomous effects; they are novel. Nor do they show a “polarity reversal” – we do not say that. We have added some explanations of these effects to clarify and refine this, both in the Results and the Discussion.

We thank the reviewer for acknowledging that we discuss a possible involvement of Wnts: Data from Piddini et al., 2005, Rives et al 2006 show that Wg accumulates over *arrow* loss-of-function clones and therefore the Wg distribution and its gradient, thought to be emanating from the margin, could be interrupted/disturbed/increased by *arrow* clones. Similarly, Schilling et al 2014 document that the Wg gradient was affected by changes in Arrow levels (we have added this to the Discussion, pages 22/23).

This is an important point and in support of a Wg/Wnt-gradient contributing to both PCP and to PCP via Arrow. We have expanded the discussion of this, as was also suggested by reviewer #1.

I think it's up to the authors to do their best to present the results in as clear and logical way as possible, to extract some kind of storyline from any new data and to help the reader.

There may be publishable material in this work, but the paper itself is not nearly ready for publication.

With all due respect, this is a non-scientific statement and rather derogatory.

We also trust that the revised paper has further improved on several points that might have been confusing.

Other points:

Fig1 Is the shape of *arr*- clones in the wing similar to wild type clones?

Wg/ β -catenin regulation of growth is not the focus of this paper, we do not study or analyze growth related requirements of *arrow*. Please see Giraldez and Cohen 2003, Barrio and Milan, 2020, Zecca and Struhl, 2021 for growth related studies to canonical Wg-signaling. It is nicely documented in Giraldez and Cohen 2003, that *arr* clones are not similar to *wt* clones at all, cell proliferation, cell competition, affecting growth in general and apoptosis are major effects documented by them. Of course we also see this in our assays and have documented it, see Figure 2C-C", or new Supplementary panel S2F.

Fig2F Hair orientation is affected in *arr*- cells but since the authors pooled together data from different clones, it is not clear if hair orientation is consistent in each clone although the orientation may be still different from the wild type. Is the presence of Arr in a cell a requisite for polarity propagation?

Thank you for this comment. We have newly documented trichome orientation in *arr* internal wing clones and *arr* clones rescued by *UAS-Arr* via MARCM mediated co-overexpression. We have selected clones, that do not affect the margin and therefore the possible wg and wg, Wnt4 signal for both genotypes. There is no specific/unifying pattern to such internal *arr* LOF clones, except general trichome misorientation, often referred to as whorls, and random non-autonomy, see Figure 1B",D-F and new panels 1G-I for clonal rescue quantification and clone localization in the rescue experiments.

With regard to polarity propagation: Arrow is required for normal Fmi and Dsh polarization and hence it does affect "propagation" from cell to cell. The loss of Fmi polarization/asymmetry looks similar to *dsh* and milder than *fz* LOF effects on Fmi asymmetry (Strutt D. 2001, Das et al., 2002, Strutt and Strutt 2007), see Figure 2C',D',E and new comments in main text concerning Figure 2C,D,E. We also added new comments on Dsh localization

changes in *arr* clones (Supplementary Fig. S2A-C) and added how they compare to *dsh* and *fz* clones in the Results and Discussion (Shimada et al., 2001, Axelrod 2001).

Fig3D The authors claim that the phenotype of R3/R4 *arr*- mosaics is the same than *fmi*-ones: “When either R3 or R4 (or both) are *fmi*–, the ommatidium selects chirality randomly or stays symmetrical.” (Das et al, 2002). Is chirality random in R3/R4 *arr*- mosaics? It doesn’t seem to be the case. Between 74 and 88% of these mosaics have normal chirality. Correction, we see 64 and 84% normal chirality.

And yes, the *arr* phenotype is robust albeit a bit weaker than *fz*, *dsh* or *fmi* null clones.

We compared genetic requirements for *arr* and *fmi*, not phenotypic strength. The genetic requirement shows that both genes are required in individual R3 and R4 cells for normal ommatidial arrangement. While the R3 requirement aligns with Fz and Dsh, the additional R4 requirement could be due to several reasons. This is discussed in the Results (pages 12/13) and Discussion (page 21).

REVIEWER COMMENTS

(our response in blue)

Reviewer #1 (Remarks to the Author):

The manuscript has been improved from the original, and the additions are valuable. The experiment the authors lead with, showing that arrow weakens the PCP effect of dsh knockdown, is still confounding and needs explaining. The PCP effect of Arrow in the wing is novel, and the effects on polarization of core PCP proteins are new and convincing. There remain some questions about whether the eye phenotypes might be caused by canonical pathways. The manuscript remains a bit weak in discussing mechanisms; there is little discussion of what is known about Arrow's interactions with intracellular members of the Wnt pathways.

We thank the reviewer for acknowledging that the manuscript “has been improved with valuable additions” and for stating that the “PCP effect of Arrow-LRP5/6 and its effects on core PCP protein polarization are novel and convincing”.

The reviewer raises three general comments:

- 1) The experiment the authors lead with, showing that arrow weakens the PCP effect of dsh knockdown, is still confounding and needs explaining.
- 2) There remain some questions about whether the eye phenotypes might be caused by canonical pathways.
- 3) The manuscript remains a bit weak in discussing mechanisms; there is little discussion of what is known about Arrow's interactions with intracellular members of the Wnt pathways

Re (1): The initial experiment and observation in the pilot screen with *dsh-RNAi* led us to look at Arrow-LRP5/6 in PCP. The effect seen in the pilot screen shows an interaction with both pathways, PCP and canonical. The ‘direction’/type of interaction cannot be interpreted conclusively from the (pilot) genetic screen, as other known components (while interacting) also showed unexpected directionality of interaction. We have added comments along this line in the Results (first section) and the Discussion, and we have added figure panels and a table in the Suppl Information document (Suppl. Fig. S1D-H). Importantly, this paper is not about the genetic screen, which we plan to publish in detail in due time, this is outside the scope of this paper. Here we have focused on *arrow* to establish its role in Wnt/PCP signaling.

Re (2): The statement that “there remain questions whether the eye phenotype might be caused by canonical pathways” is incorrect. To make sure that there is no ambiguity, we have added explanations and new figure panels to clarify this. Please see below in our detailed responses to the reviewers “Point 1”.

Re (3): We have added more Discussion of potential/predicted intracellular effects of Arrow impacting members of the Wnt pathways (see below). For example, we have added discussion of a recent paper of how LRP5/6 affects Dishevelled “activity” via CK1 δ/ϵ phosphorylation, which is likely relevant for all Dsh functions in Wnt-signaling. The Discussion is already long due to previous requests and so we trust it is good to maintain this concise.

For the record, we would like to emphasize that several of the comments put forward in this second round of review are new and were not brought up in the first round of review. Some of these requests are based on very old data, from work going back >25 years, and work that has not been followed up on. Those old models, for example the presence of a “factor X”, have become obsolete in the meantime.

1) PCP vs canonical- The most critical issue is whether *arrow*'s effects are via the PCP or canonical Wg/ β cat pathways. In the wing the effects on core protein polarization argue for the former, and the new data finds genetic interactions with several PCP pathway but not *bcat* (arm) heterozygotes. However, the authors themselves raise the possibility that some of the wing PCP effects may be mediated by changes in *Dachsous* or *Fj* levels, an effect that could influence PCP protein polarization through the Fat-*Dachsous* pathway but would be initiated by a change in canonical Wg/*bcat* signaling. Nonetheless, Wg regulation of *Dachsous* or *Fj* has never been demonstrated in this tissue and is unlikely (see below).

We can exclude that it could be mediated via Fat/*Ft* and *Dachsous*/*Ds*, because of the phenotypes of *arrow* clones. Both cell-autonomous and non-autonomous effects of *arrow* mutant clones are distinct from *ft* or *ds* mutant phenotypes in the eye and in the wing. We have added information to make this clearer, for example by directly comparing the phenotypic effects of *arr* and *dsh* with *ft/ds* mutants in the eye (new quantification panel in Figure 3K).

Generally, we only included the comparison initially, as we tried to cover all possibilities, even those unlikely. We thank the reviewer for pointing out/reinforcing that Wg regulation of *Ds* and *Fj* has not been demonstrated in the wing, and hence the discussion of such a potential role distracts the reader. We added this to keep it brief in the Discussion.

Overall, we have better highlighted that the specific features of the *arrow* clones link them to core PCP phenotypic features, and not *ft* and *ds*.

We trust that not expanding this section in the paper, focusing on our novel data and demonstrating that phenotypes seen in *arrow* clones are classical core PCP defects and contrasting them to *ft* and *ds*, is a better solution for the reader. The below detailed responses are to clarify this in this point-by-point response, but not all of it is included in the paper as it would distract the interested, neutral reader.

But this is a distinct possibility in the eye, where expression of *Fj* and *Dachsous* are both sensitive to canonical Wg signaling (see below), and so the question remains whether their eye phenotypes are via the PCP or canonical Wnt/*bcat* pathways. Since the authors raise this possibility in the wing, is there something that rules this out in the eye?

Yes, we can rule out that the *arrow* defects we observe in the eye are due to *ft* and *ds* regulation. The PCP phenotypes of *arrow* mutant clones include R3/R4 cell fate specification defects, often resulting in symmetric arrangements of photoreceptors R3 and R4 (which we confirmed with molecular markers) and ommatidial rotation defects. These phenotypic features are representative of classical core PCP features. And exclude the possibility of being caused by *ft* and *ds* defects. In contrast, *ft* and *ds* clones

induce dorso-ventral chirality inversions, where chirality is maintained and R3 and R4 cells are specified accordingly, albeit randomly. The new figure panel Fig. 3K presents these phenotypic features in a quantitative manner.

In brief, again, our detailed phenotypic analyses in the eye indicate that *arrow* clones show core PCP type defects, resembling those of *fz* (e.g. Zheng et al 1995), *dsh* (e.g. Theisen et al 1995 or Boutros et al 1998), *Vang/stbm* (Wolff and Rubin 1998) or *fmi* (Das et al 2002). The observation that *arrow* mutant clones regularly display symmetric clusters is not compatible with phenotypes seen in *fat* and *ds* mutants (e.g. Yang et al, 2002; Rawls et al 2002; or Fanto et al 2003).

The issue of *arrow* acting via canonical signaling in the eye is also raised by ref 14, who showed in 1998 that both *arrow* and hypomorphic *bcat/arm* mutations affected eye PCP. The manuscript does not discuss ref 14 in any depth, saying only that these phenotypes are caused by “indirect effects of earlier requirements for Wnt/b-catenin signaling” and “in setting up the polar-equatorial eye field”. However, ref 14 dates from a time before the discovery of polarized PCP proteins or the role of Fat-Dachsous signaling, and did not rule out an effect for *arrow* via effects other than the induction of novel equators. To put it another way, do the authors have data showing that the PCP defects in their *arrow* clones are being caused by something different than the PCP defects in 14’s *arrow* clones, based on this work or other studies? Is the location of the clone really sufficient to rule out an effect on equator formation, or an effect via Fj or Dachsous changes?

Ref 14 is Wehrli and Tomlinson, 1998. They did not show that *arrow* affected eye PCP establishment, they state that it affected equator formation and polarity inversions (Fig 5 in ref 14) and factor X expression (their underlying model). Surprisingly, Wehrli and Tomlinson (1998) specifically show that equatorial *arrow* clones manifest no such phenotype (Fig 4C in ref 14). They also look at additional canonical Wg-pathway components (shaggy/GSK3 β and arm/ β -catenin) in this context, again to indicate that the canonical Wg-pathway regulates factor X expression and equator formation. Although one could discuss this paper in detail, it is not relevant and it distracts from our novel data. The points brought up in Ref 14 have been well described and published by others (e.g. Treisman and Rubin 1995, Ma and Moses 1995, Heberlein et al 1998; also reviewed by Treisman and Heberlein 1998, Legent and Treisman 2008).

Our statements regarding Ref 14, “indirect effects of earlier requirements for Wnt/b-catenin signaling” and “in setting up the polar-equatorial eye field”, are quite precisely what Wehrli and Tomlinson (ref 14) are proposing. We prefer to leave it at that to not confuse the interested, neutral reader of our paper.

In summary, ref 14 did not address PCP in detail, they made specific statements that (i) *arrow* clones do not have PCP phenotypes (to quote “not involved in read-out step”, their Fig. 4C) and (ii) that they are behaving like other canonical Wg/beta-catenin pathway components.

The fact that as stated by the reviewer: “However, ref 14 dates from a time before the discovery of polarized PCP proteins or the role of Fat-Dachsous signaling, and did not

rule out an effect for arrow via effects other than the induction of novel equators” is not correct, as ref 14 does say that no PCP defects are associated with *arrow* clones.

The reviewer also asks if “is location of the clone really sufficient to rule out an effect on equator formation, or an effect via Fj or Dachshaus changes?”.

The location of the clones helps to distinguish between effects on equator formation and PCP, as in the position of the endogenous equator an ectopic equator cannot form.

As mentioned above, there are several papers describing the effects of canonical Wg-signaling on equator formation and dorso-ventral eye field definition (e.g. Treisman and Rubin 1995, Ma and Moses 1995, Heberlein et al 1998; also reviewed by Treisman and Heberlein 1998, Legent and Treisman 2008) in addition to Wehrli and Tomlinson 1998. We acknowledge these effects and show them in Suppl. Fig. S3. We have added a cartoon there, describing these observations, and how canonical Wg-signaling shapes the eye field to avoid confusion. Importantly, these early effects of canonical Wg-signaling have been documented by several labs (see references above) and they do not take anything away from the work presented in this paper.

We trust that expanding these explanations would be repetitive and distract from the novel data, documenting an unexpected PCP role of Arrow.

For the record: these points were not brought up by this reviewer previously, but we have addressed them in detail as answered above.

Ref 14 claimed that *sev-gal4* effects would be late enough in the process to affect only PCP interpretation and not the equator field, but did not test *arrow*. If that is still viewed as a valid test, the authors could argue that *arrow*'s ability to modify *sev-gal4* phenotypes showed a similarly late role that was less likely to be via the canonical pathway. Or, if *sev>arr-RNAi* had an effect, that might also provide a counter-argument. The *sevenless*-enhancer (*sev-gal4*) driven effects are in post-mitotic cells posterior to the morphogenetic furrow in photoreceptors R3 and R4, and as such they are specific to core Fz-PCP signaling. This is and has been used routinely by several labs in eye PCP specific contexts. Rev #1 is correct to suggest that interactions where *sev*-driven expression is used causes PCP specific effects and can serve to separate function from canonical Wg-signaling. We have added a cartoon and description to explain this in Suppl. Fig. S3G. We also describe the *sev-GAL4* tool in the main text and Methods and highlight its late onset of expression (Suppl. Fig. S3F), which is PCP specific in photoreceptors R3/R4 (references e.g. Domingos et al., 2005, fig 1A). *Sep-GAL4* is derived from this driver (replacing the *hs*-promoter of *sev-Gal4* with the *sev*-promoter) and has an even tighter expression in R3/R4 and is mentioned in Methods, and shown in Suppl. Fig. S3F.

2) Tilting the phenotypic balance- “In a pilot screen with most ligands and transmembrane proteins/receptors acting in the two main Wnt-signaling pathways, we discovered that manipulating *arr*/LRP5/6 levels did tilt the *dsh* phenotypic balance,

enhancing and suppressing the respective phenotypes when their levels were changed, very similar to effects seen with the Wnt ligands and Fz family receptors (Supplemental Figure S1A-C and A'-C', also Supplemental Table 1)."

Thank you for bringing this up. As mentioned in the "first response" paragraph above, the initial experiment and observation in the pilot screen with *dsh-RNAi* led us to look at Arrow-LRP5/6 in PCP, because *arrow* interacted with both pathway phenotypes (PCP and canonical), unlike *bona fide* components of canonical Wg-signaling (e.g. *axin*, *sgg* or *APC*, which only interacted with canonical signaling phenotypes).

The *arr* effect seen in the pilot screen shows an interaction with both PCP and canonical. The balance (and phenotypic effect) between mild loss-of-function scenarios of Dsh, Fz, Fz2 and Arr is complex due to feedback loops and complicated (circular) epistasis relationships, and so the 'direction'/type of interaction cannot be always predicted. The fact that *arrow* modified PCP led us to look at its potential role in detail, which revealed a positive role in promoting core Fz-PCP function.

We plan to publish details of the screen and associated effects and results in a genetics paper focused on the screen. This is outside the scope of this paper.

First, this is the only time that the authors claim that reducing *arrow* improves the PCP phenotype caused by reducing Fz/Dsh etc (in this case, a weak *dsh RNAi*). This result is exactly the opposite of their summary statement in the Discussion, that "Arr/LRP5/6 acts positively in the Fz/Dsh-PCP complex in a Wg/dWnt4 associated manner, and in many ways behaves like a core PCP factor.", and conflicts with their double and triple heterozygote data. Do the authors have an explanation for this?

Based on the pilot screen we did not claim that reducing *arrow* improves PCP, and we also do not say that the result is opposite to other phenotypes seen with *arrow*. The pilot screen suggested that, due to its interaction with the PCP phenotype, it is worth to analyze it functionally in detail in the PCP context.

Second, to be sure of this result I asked for quantification. However, the authors have added quantification only for the canonical wing notching effect and only for the clonal analysis, but not the *dsh RNAi* experiment and, in either case, the more critical PCP effect. They show only one example of PCP improvement; even a simple statement that xx number of wings resembled the photo shown would help. The authors also claim in Table S1 that PCP defects are worse in Fig. S1C' than A', but this is not obvious in the photo and needs numbers to back it up.

This quantification was not specifically requested in the 1st set of reviewer comments. It was mentioned for several figures of specific experiments, where we added quantifications.

In general, the screen was performed on the basis of phenotypic observation, it is a qualitative visual screen not a quantitative screen. We trust that it makes little sense to quantify it, we have summarized it in the Supplemental Information and mentioned that the images are representative from 5-7 samples. Again, it only served the purpose to find genes worth to analyze based on interactions with one or the other or both pathway specific phenotypes.

Third, there is no data showing that this is "very similar to effects seen with the Wnt

ligands and Fz family receptors (Supplemental Figure S1A-C and A'-C', also Supplemental Table 1)." or "arr levels affected the dsh phenotypic balance in both Wnt signaling pathways in a similar manner as the Wnts themselves and the fz, fz2 receptors (see above)." Unless there is data the authors are not showing, these statements are wrong and should be removed. Fig. S1A-C has no data on Wnts or Fz. Fig S1 has no data on dsh-RNAi with anything other than Arrow. And none of the other manipulations in Table S1 "tip the balance" between canonical and PCP phenotypes by having simultaneous improvement of one phenotype and worsening of the other.

We apologize that this was potentially unclear. We have added a short description to justify why we chose to look at Arrow in detail and in which way its interaction was considered related to the other receptors etc as mentioned above (see Results and Discussion).

Finally, the rebuttal letter said this description, which the authors admitted was confusing, had been clarified along with the legend. No changes have been made from the original. Please fix this.

It should be different. We have addressed this as outlined above.

3) Fz and Fz2 overexpression affecting Arrow levels- First, I did not find the original data convincing, as the change in Arrow was limited to only a part of the peripodial clone. The authors did not address this point. They have added additional data using dpp-gal4-driven overexpression, but the claimed effect is limited to the basal sides of cells and in a small portion of the dpp-gal4 region of expression. Given the extreme folding in this region, and thus difficulties with focal planes, I need a cross section of a Z series to trust this new data. And it is not clear why the maximum effect would be limited to this region, or in the basal focal plane shown instead of apically where Fz is concentrated.

GAL4>UAS expression is very mosaic, as seen in many assays with most driver combinations, and we see the same here, with single cell overexpression levels frequently varying 2-10x (seen with GFP for example). We described in more detail, how the data was collected and also that Arr expression is very dynamic to make it clearer.

It has been documented that Arrow (like Fz2) is transcriptionally repressed by canonical Wg/Wnt-signaling and often targeted for degradation as part of a ligand-receptor complex. Schilling et al (2014) describe that Arr levels can 'barely be modulated' within the wing pouch area because of such regulatory feedback loops. Similarly, it was shown that Wg protein accumulates over areas of discontinuous Wg/ β -catenin signaling (e.g. Rives et al, 2006), as shown for *fz*-, *fz2*- double mutant clones and *arr*- clones. Therefore, we studied Arr protein localization/levels and potential effects of overexpression of other receptors/trans-membrane proteins only in cells of areas just outside the wing pouch region. Also, Wg has been shown to be secreted basally, originally by Strigini and Cohen (2000) describing basal accumulation of Wg upon GPI-Fz2 expression, and hence we have looked at Arrow levels in this region.

We have added a new data set of Z-sections for the Arr staining to document Arrow levels accordingly (new panels, Supplemental Fig S4E-H''').

This may be confounded by effects on signaling, as canonical signaling was shown to repress both arrow transcription and reduce post-transcriptional Arrow protein

Yes, the regulation of Arrow is complex in the wing pouch and affected by Wg/ β -catenin signaling transcriptionally and also by ligand-receptor complex formation through targeting such complexes for degradation (Wehrli et al 2000, Piddini et al 2005, Rives et al 2006, Schilling et al 2014 etc). The post-transcriptional regulation of Arr protein is largely through ligand-receptor complex degradation, but sufficient to eliminate ectopically expressed Arrow (e.g. via a Tubulin-driver, Schilling et al 2014). Wehrli et al., 2000 suggest that *arrow* transcription might be regulated by Wnt/ β -catenin signaling, but do not show data. Strigini and Cohen 2000 overexpress GPI anchored Fz2 and observe increased basal extracellular Wg and in turn ectopic, extracellular Wg ligand has been shown to accumulate Arrow (Piddini et al 2005, Rives et al 2006). Schilling et al., 2014 study Arr protein accumulation and try to manipulate it in the wing pouch, with the conclusion, that it cannot be changed there, due to the degradation dependent feedback control loops, with its levels within the pouch kept very low by these mechanisms.

Hence, we performed our assay in the basal region outside the wing pouch where Arrow seems to be more generally detected and much less under the strict feedback controls seen in the wing pouch. This was clearly evident during confocal image acquisition.

Second, the authors do not fully explain why this data is important. Is there precedent for binding of Fz's to Arrow homologs, and some question whether they can also bind to Fz's that are less involved in the canonical pathway? Arrow's ICD also affects Dsh so there are other possible routes for regulation of the Dsh-based PCP pathway.

These data allow a comparison between Fz (and Fz2) and the other core PCP TM-proteins Vang and Fmi.

It is documented and confirmed in structural biology studies – which we added to the discussion as suggested by rev #2 - that LRP5/6 and Fz-family receptors are brought together by the Wnt ligands. We use the assay to document that Fz, like Fz2, can be brought in proximity of Arrow by the ligand(s). And because the new double heterozygous results of *arr* with *fmi* or *stbm/Vang*, which also showed wing PCP defects, like the quadruple heterozygotes of *wg, Wnt4/+*, *arr/+*, *fz/+* originally, we decided to include Fmi and Vang overexpression in this assay as well.

The statement that “Arrow's ICD also affects Dsh so there are other possible routes for regulation of the Dsh-based PCP pathway” is a new request since the first round of comments.

We have added to the Discussion a paper that was just published, which might provide mechanistic insight into how Arrow-LRP5/6 affects Dishevelled protein activity in general: Micka et al (2026), Science Advances (published in May 2026). We have added a discussion of their data as it provides a potential universal mechanism for Dishevelled regulation in any Wnt-pathway.

4) Discussion- This is improved. I still disagree a bit about the point of the other Wg/Wnt studies, as these were intended to test the effects of flattening gradients of diffusible Wg/Wnt in the wing, not completely remove them. Yu et al used conditional KO rather than inducible CRISPR for most of their studies and backed this up with inhibition of secretion and with ablation of the wing margin. Ewen-Campen used both inducible CRISPR and RNAi. This likely leaves room for Wg/Wnt4, but makes it unlikely to be required in any absolute sense as an instructional, polarizing cue.

With all due respect, we disagree, the statement above is incorrect.

The two papers in question did not intend to flatten the gradients, they intended to remove Wg/Wnt from the margin to suggest that Wg/Wnt secreted from the margin is not required.

Importantly, Yu et al use clonal/conditional CRISPR as their only assay, and so it does apply that cells with less Wg/Wnt β -catenin signaling could be competed out by *wt* cells, which maintain Wg expression, as generally occurs with mosaicism of in *in-vivo* CRISPR assays, where “wt” cells or cells with reduced but some remaining signaling capacity will outcompete the mutant cells.

Ewen-Campen et al use both CRISPR and RNAi. However, and importantly, they do not see an effect with RNAi, as compared to Barrio and Milan, 2020. This indicates that their RNAi assay was not an effective knock-down.

The authors do not discuss the gradient idea. They do say earlier in the manuscript that “Wg/Wnt4 are thought to set up the asymmetric localization of core PCP factors early in pupal development” but might need to discuss the gradients in order for this to make sense to most readers.

We do discuss the gradient data (page 22). We trust it is discussed adequately, also in the context of the *arrow* clones, this is a quite expansive section within the Discussion part of the paper.

The mechanistic discussion of Arrow/LRP5-6 is improved, but still largely focused on the extracellular role, rather than the intracellular role regulating Dsh etc. Separating the two is beyond the scope of this manuscript but would be an interesting avenue to explore.

We have added discussion of the most recent paper (published in May) by Micka et al (2026), Science Advances. This study provides insight on a potential universal mechanism of how Dishevelled activity is regulated downstream of Fz-receptors complexed with LRP5/6-members in any Wnt-pathway.

“Separating potential differences between the two pathways is beyond the scope of this manuscript but will be an interesting avenue to explore in the future”: Yes, we agree.

With the additional discussion of Micka et al 2026, potential insight and direction of research is provided in the Discussion.

5) Other comments

“with Fz2 inducing specifically Wnt/bcatenin phenotypes, whereas Fz causing

exclusively PCP defects.”

But loss of Fz2 alone has few phenotypes.

We are sorry if this was unclear, we were referring to gain-of-function (GOF) effects, which are more suited to address signaling specificity by Fz and Fz2. The GOF effects as shown here and in several previous publications indicate that Fz2 is the primary, ‘dominant’ Fz-family member for canonical β -catenin signaling. The redundancy of fz and fz2 LOF mutants in canonical Wg/ β -catenin masks this in the LOF scenario.

Fig. S1 (H,I) Wing schematics indicating position of clones for each genotype; note that clones in the central area of wings were analyzed.

What do the two different shades of grey mean?

We have added a note in the figure legend to explain the 2 shades.

Loss of cytoplasmic Dsh – We apologize for this erroneous statement in the rebuttal document, we did not intend to say that.

Despite what the authors wrote in their rebuttal letter, there is no reduction in cytoplasmic Dsh in the PCP manipulation papers they referenced. The difference here is likely that arrow is also affecting the canonical pathway and thus stability of Dsh. The problem being that loss of cortical Dsh may not be a specific marker of the PCP pathway.

Loss of cortical Dsh is a very reliable marker for defects in the core PCP pathway, *fz* and *fmi* clones show exactly that.

Dsh is off the membrane and detected primarily cytoplasmically and at +/- clone borders (*fmi* and *fz* clones, stained with anti-dsh and dsh-DshGFP always show this as well). This was first documented by Shimada et al., 2001, and Axelrod, 2001, and used in many papers since.

Polar clones were not considered in this study, because they can induce ectopic eye fields and anti-equators due to the Wnt catenin requirement in eye field definition^{71,72}. Is there any clonal data in 71 or 72?

Yes, there is such data.

Treisman and Rubin (1995) show >Wg (GOF) and sgg/GSK3 (LOF) clones (ref 71 in the 1st revised version). Ma and Moses (1995) used wg-ts to document comparable effects. Treisman and Rubin (1995) also show *ptc* clones that induce ectopic equators with ommatidial chirality analysis.

“nor an asymmetric localization in developing wings, was detected with the available tools,”

I requested a clarification on the tools used, but the text here is unchanged. Why not just say “was detected at the boundaries of arrow mutant or overexpression clones” and reference the figures that show this? It does the reader no good to add figures but not reference them in the text.

In the revised paper, we did add a description of all tools used. The reviewer must have missed it, apologies if it was not clear.

We do not detect effects at boundaries of *arrow* mutant clones, we are not sure what this statement refers to.

as it has been shown that Arr levels in the apical wing pouch cannot be manipulated 86 (Figure 4C,C').

What does this mean? Arr levels are regulated by Wg signaling in the pouch, so they can certainly be manipulated.

Endogenous Arrow expression levels are tightly regulated within the wing pouch area, see above for references. Schilling et al (2014) show that levels of Arrow are repressed in Wg/ β -catenin signaling by targeting ligand-receptor complexes for degradation and thus ectopic expression levels of Arrow are thus kept very low within the pouch. Hence, for the assay we tried to apply, it was necessary to look at protein levels outside the area of the wing pouch.

sep-gal4 vs sev-gal4- Do these drive similar expression patterns with similar timing?

We apologize that this was not clear. Both GAL4 drivers have been discussed and shown in several papers previously. There are many references that describe this, e.g. Wehrli and Tomlinson 1998, Fanto and Mlodzik 1999, Fanto et al 2000, Domingos et al 2005, Djiane et al 2005, Weber et al 2008 etc. We have added a panel documenting expression (Suppl. Fig S3F) and a cartoon (Supplemental Fig. S3G) to specifically highlight the expression pattern in eye discs.

While wg, Wnt4 knockdown or copy reduction, and fz, fz2 copy reduction enhanced the notching phenotype, no PCP effects were detected.

Are there in fact any experiments with wg, Wnt4 knockdown instead of copy reduction?

There are individual wg or Wnt4 knockdown experiments described. It has been adjusted in the paper text.

A first possibility could arise from the fact that Wnt/ β -catenin signaling transcriptionally regulates the graded expression of four-jointed (fj) and dachsous (ds), two key components of the Fat/Ds PCP system 97,98

98 has no data on this. 97 and the unreferenced Zeidler et al. '99 show data from the eye, not the wing. Wu and Irvine '04 showed some data that wing expression depended in part on Vg, although in a complicated fashion further studied by Zecca and Struhl. However, removing Wg reception does little to anti-Vg in the wing unless the cells are dying. Moreover, there is no data showing that loss of wg signaling mimics Fat-Ds-Fj-specific phenotypes in the wing, such as the characteristic and very sensitive changes in spacing between the cross veins.

This refers to the reviewers main "point 1)" above. We trust that it is discussed there adequately.

Grammar, awkward sentences, typos, etc

Explanations of some of the abbreviations or alternative terms (Dvl, trichome) are repeated. fw should be spelled out.

We have checked this and when appropriate corrected it.

with defects manifest as loss of asymmetric PCP factor localization and reduced levels of Dsh with centrally forming wing hairs.

“localization, reduced levels of Dsh, and centrally forming wing hairs” would be better
[The suggested change has a different meaning. We have not changed this text to maintain what we tried to say.](#)

classical PCP phenotypes with photoreceptors R3/R4 cell fate and chirality defects.
phenotypes with defects in chirality and the cell fates of photoreceptors R3 and R4.
[This was changed accordingly.](#)

we tested known Wg/Wnt signaling components, which showed arr/LRP5/6 to shift the dsh phenotypic balance
and showed that arr/LRP5/6 shifted the dsh phenotypic balance
[This was adjusted accordingly in the main text.](#)

are observed around fz and Vang/stbm mutant tissue, and induced by Wnt4
misexpression clones
and are induced
[This was changed in main text.](#)

an insertion k0813 mutation
Makes it sound like a type. Are there other k0813 mutations? Or is arr[k] just a shorthand for arr[k0813]?
[arr\[k\] is just a shorthand for arr\[k0813\]. This was adjusted in the text.](#)

as has been described before, clones near the margin displayed loss of tissue or notches
references?
[Wehrli et al., 2000, was added to the main text.](#)

To ascertain that arr mutations were responsible
whether would be better than that, as the latter sounds like you have already decided.
[This was adjusted in the main text.](#)

can be rescued by expression of myristilated-Dsh (myrDsh),
reference? myristoylated
[This was corrected in the main text and referenced \(Kaur et al 2017\).](#)

past observations that Dsh constructs caused severe PCP defects by themselves
references?
[There are several references that can be added, for example we added Boutros et al 1998 \(several figures\) and Penton et al., 2002 \(here Fig 3E\) to main text as representative references.](#)

And mildly reduced Fmi levels for arrk (Figure 2C,C',D,D and quantified in E') as

compared to neighboring wt control tissue. In contrast, E-cadherin (E-cad), a common component of adherens junctions serving as control, was not affected or mildly reduced in arrk (Figure 2C",D",E).

Serving as a control. This section also makes it difficult to tell that the authors are discussing two different arrow alleles, rather than just variability of arr[k]. Mention which result goes with arr[2] clones and which with arr[k] clones. ‘

[This was added as appropriate in main text.](#)

And any idea why arr[k] had the more disruptive effect?

[No, we don't. It is an allele used by others in several previous publications. The chromosomes were outcrossed to remove potential secondary mutations and tested via complementation.](#)

similar but weaker to effects seen in
similar to but weaker than

[This was adjusted in the text.](#)

with accumulation at arr-/arrwt membranes, before and during prehair formation
(Supplemental Fig. S2A,A',B,B',C), respectively

Remove respectively. It is only used if A gives B and C gives D, and you say "A and C give B and D, respectively". If is not used if A and B both give C.

[This was adjusted in the main text.](#)

these experiments displayed

[Apologies for this typo. This was corrected.](#)

A similar strategy

[Apologies and thank you for pointing this out. This was corrected.](#)

used to test whether Wnts

[This was also corrected.](#)

Reviewer #2 (Remarks to the Author):

The authors have done an excellent job addressing the referees' comments, even the non-scientific ones. I recommend the paper for publication.

[Thank you for this very positive assessment of our revisions.](#)

REVIEWERS' COMMENTS

Point-by-point response

Reviewer #1 (Remarks to the Author):

I thank the authors for their changes. I still think Figure S1 PCP needs quantification to support its claims. I do not think the altered Arr levels caused by Fz or Fz2 overexpression provide any compelling proof of recruitment into a complex. I have also added some discussion about points raised by the most recent changes, and some suggestions I raised previously that have not been addressed.

However, the main thrust and data of the paper remains valuable, and the new additions have improved many of the arguments. I leave any further changes up to the editor and authors.

1) Canonical pathways, Fat-Ds, and eye PCP- The authors now include reasonable arguments suggesting that not all of the eye PCP defects can be via canonical signaling that changes Ds or Fj expression. These are based on past studies on the types of PCP defects noted with Fat/Ds/Fj: inversion defects but not so much the rotation and symmetric outcomes common with Arr changes. I do worry a bit about the older Ft-Ds literature simply ignoring those other defects; Simon 2004, for instance, shows “incorrectly constructed ommatidia or ommatidia exhibiting severe misrotation” not noted in his earlier papers. However, the rates certainly seem lower than the Arr phenotypes, so the argument seems sound.

We have added data in the previous revision to demonstrate that the phenotypes have different quality and hence it can be excluded that the PCP defects detected in arr- mutant clones are due to the Fat-Ds PCP system. The reviewer agrees that the argument as presented is sound.

However, since Arr will affect canonical signaling, and canonical signaling regulates Ds and Fj expression in the eye, it seems likely that Arr will be causing changes in Ds and Fj expression. To my mind, if such changes occur, they are likely to contribute to some of the eye PCP phenotypes, especially at clone boundaries. Or is the argument that the changes will be too slight near the equator to have much effect?

Yes, this is largely the same points as made above. In brief, arr- mutant clones show no non-autonomy in the equatorial eye regions, where we analyzed them. 2 labs published clones close to or on the equator for *fj* and *ds*, which generate massive chirality inversions and anti-equators (Zeidler et al., 1999, Rawls et al., 2002). Whereas in comparable arr- clones, no non-autonomous effects are seen. We analyzed for arr[k] – 11 eyes, and for arr[2] – 13 eyes, from 4 experiments each. Only polar arr- clones, close to the disc margins, show such effects. Description of these differences and observations have been added to the legend of the Suppl. Figure S3, where arr- phenotypes linked to Wg/b-catenin signaling are shown.

I also brought up the use of the late driver sev-gal4 as a way of separating early axis-forming from later PCP readout events. But I did not ask before (and I apologize for not knowing), can sev-gal4-driven changes in Wnt reception change Ds or Fj expression?

This is a novel request, as the reviewer admits themselves.

There is no evidence that this could be causing effects on Ds or Fj expression, and hence it is best to leave this out.

The changed terminology the authors now use for sev-gal4 is a bit confusing, as the authors now refer to it as “PCP-specific” but then state that it causes both canonical and PCP defects (lines 363-4). So PCP-specific is the wrong term; late-expressed, perhaps? Also, the nature of

the canonical defect, and the criteria by which it was determined to be canonical, are not described in this part of the Results, and it might be helpful to add a sentence that does that. We have changed the statement to “photoreceptor R3-R4 specific” in the text. We have added text that describes Wg/b-catenin signaling GOF effects using this driver.

2) Figure S1. I had three complaints about this data. A) The PCP differences claimed in Fig. S1C’ was not convincing and required quantification. Here I strongly disagree with the authors’ most recent comment; I was very clear about this in comment 1B in my first review. The authors have also changed the “defect” areas shown for Fig. S1A’ and C’ despite showing the same photos; to my mind this just shows how subjective their criteria are, and how important quantification is for confirming their claims. This is even more important this time around, as the authors now include additional PCP data not present in the first or second versions of Fig. S1.

In their first rebuttal, the authors apparently misunderstood me and added PCP quantification for Fig. 1, not Fig. S1. In the most recent, they have followed my suggestion of stating how many wings they examined but have not said how they quantified PCP in those wings. This is odd because they quantified hair PCP in two other data sets. Pick a region and measure hair angles.

Instead they say “More detailed quantification will be published elsewhere” (Fig. S1 legend).

I will leave this up to the editor, but I do not think that is acceptable. The claim is being made in this manuscript, and the data supporting that claim should be here too.

If the data is too hard to get, a more radical alternative is to remove much of Fig. S1 from this paper, as it has little impact on the authors’ conclusions. Begin the paper with the stronger and more straightforward data of Fig. 1.

There has been a misunderstanding with the reviewer, likely due to the fact that they often bunch several comments into one sentence.

There is also a contradiction in the reviewer’s assessment of Fig S1: while they complain about aspects of it here, below in their point 2C) they thank us for including the respective data in Figure S1.

Based on their earlier request, we have included additional data in Fig S1, and we have marked the regions that show PCP defects more extensively for clarity (it is not an arbitrary decision what to mark, we have added larger marked areas to make the defect clearer, as it might not be obvious to all readers). Within the genetic screen interactions all different genotypes were assessed blindly and independently by three people, visually at the microscope. If deemed that the interaction was obvious and reproducible the wings were imaged. The interactions reported were seen in largely all wings of a given genotype. It is not feasible to quantify them as the screen is/was a “visual screen”, based on yes/no decisions for an interaction of any given genotype.

While we could remove parts of Figure S1, the reviewer thanks for adding these data to it in their point 2C below. On balance, we trust that it is better to keep the data as present in the revised Fig S1, and adding a bit more explanation to the figure legend.

2B) My second point was that the Arr data in Fig. S1 shows that reducing Arr actually improves PCP defects caused by dsh-RNAi, whereas all the other data in the manuscript says that reducing Arr worsens PCP. The authors now acknowledge this in the Discussion and, as in their rebuttal, ascribe this to complex epistasis. I appreciate the change, although I had hoped for a bit more than that, as I think the contrast between the Dsh RNAi and Dsh overexpression results

may be telling; when Dsh is reduced, Arr acts as if it is competing some limiting factor (Dsh?) away from the PCP pathway and into the canonical complex.

We are not sure what the reviewer wants us to address. They acknowledge that we discuss this seemingly inconsistent result. We also discuss that Arrow might be segregating factors from one Wnt-signaling branch to the other, we call it “titrating away” and “competing”. We trust that our text is clear in this regard.

2C) I had complained about the authors not previously supporting the claim that the Arr interactions with dsh-RNAi were “very similar to effects seen with Wnt ligands and Fz family receptors” with any data. I thank the authors for now including that data in Fig. S1.

We appreciate that the reviewer thanks us for including the data (in contrast to their statement above).

However, I must point out, having seen the data, that the only similarity is that they each change the balance between canonical and PCP phenotypes. They are completely opposite in terms of the direction of that change. Reducing Arr dosage worsens canonical and improves PCP defects, while reducing Wg or Fz/Fz2 improves canonical and worsens PCP defects. Increasing Wnt4 improves both canonical and PCP. If the authors want to call this “very similar”, they should be explicit in the Results text about what they mean. This description of these results as “similar” is repeated in other parts of the manuscript.

We have adjusted the text to a more specific description (removing “similar”) when addressing the interactions (page 7) and removed it in later sections of the paper.

Along these lines, the direction of the interactions with Fz2 GOF and fz, fz2 double mutant LOF is also not what one would naively expect: adding more Fz2 would be expected to rescue the notching defects (Wnt/beta-catenin defects), but it does the opposite (it enhances notching defects and rescues PCP), while removing a copy of both Fz-receptors suppresses would be expected to enhance notching, it causes the opposite effect.

We have added a note along these lines to the figure legend in S1 to clarify this.

3) Recruitment of Arr by Fz2 or Fz- I thank the authors for showing the cross-sections of the dpp-gal4 UAS-Fz and UAS-Fz2 experiments, and the basal Arr increase in some of the dpp-gal4 cells is as they said. However, in their second rebuttal they appear to have misunderstood my concerns with this experiment. Their claim is that this shows that Fz and Fz2 can “recruit” Arr, binding them into a complex. The alternative is that Fz and Fz2 are doing something indirect that changes Arr levels or localization without actually forming a complex.

My expectation is that, if a complex is being formed, Arr should concentrate in the same subcellular local as the overexpressed Fz and Fz2. As shown by Wu et al., 2004, even overexpressed Fz preferentially concentrates in the apical junctions, overlapping with DE-cadherin. So why does overexpressed Fz concentrate Arr only basally, far from the junctions? And why is the effect limited to a subset of the dpp-gal4 cells (it does not extend to the far right limit of the zone)? This looks much more like an indirect effect. Unless the authors can demonstrate co-localization of Arr and Fz their claim that a complex is being formed is greatly overstated and should be weakened.

We appreciate this issue. We have removed the phrase “complex” when describing increased Arr staining in the Fz overexpression experiment (page 14 and later).

The key point of this experiment is that Fz overexpression only affects PCP (as compared to Fz2 overexpression, which only causes Wnt/beta-catenin signaling effects), and as such detecting increased levels of Arr protein in a Fz overexpression background argues for Fz engaging Arr in PCP, which is consistent with a PCP role of Arr.

The reason why we used ddp-Gal4 and analyzed Arr levels within that domain outside the wing pouch is to demonstrate increased Arr levels, independent of feedback loops that are known for Wnt/beta-catenin signaling acting negatively on Arr expression and protein levels. Also, cells with the highest expression level (as seen by GFP co-expression) within Wnt/beta-catenin signaling contexts are likely too sick.

I also asked why the authors think this effect is limited to the disc folds, rather than the pouch. They answered that “Schilling et al 2014 study Arr protein accumulation and try to manipulate it in the wing pouch with the conclusion it cannot be changed there...” However, this is wrong. Schilling et al. expressed Arr with a tubulin promoter and saw a strong increase in levels throughout the disc, including the pouch (their Fig. 1D-F). The pouch expression retained a proximal to distal gradient, but the overall levels were higher than normal. Please change line 328 in the text to reflect this

Schilling et al show a 2-fold increase in Arr protein in the wing pouch in a tub-Gal4, UAS-Arr background, but this solid overexpression cannot override the feedback loop based expression control/pattern of graded Arr levels within the wing pouch. Adding Fz2 in their assay did not show an effect either (Fig. 3. In Schilling et al), with Wg and Sens levels only very mildly increased at the expression compartment boundary in this assay. We have adjusted this in the text for clarity.

I also asked what point was being addressed by this experiment. The authors replied that that Fz family receptors and LRP coreceptors are largely complexed by simultaneous binding to Wnts. But given that Fz, Fz2 and Arr can all bind Drosophila Wnts, they should all form a complex, why is this a novel question? Has it been posited that Fz and Arr cannot be bridged by Wnts, even though they can both mediate canonical signaling?

The reviewer refers to data from Wnt/beta-catenin signaling, with the resulting complex(es) being the only signaling complexes analyzed and published thus far. It is a novel and specific question to ask whether Fz PCP function (as seen in overexpression assays where Fz is specifically acting in PCP signaling) can also cause an increase in Arr levels, directly or indirectly. Hence, we use this assay, to go in parallel to functional interaction studies, to ask whether a Fz-PCP scenario can stabilize/increase Arr levels as well.

4) Discussion. Again, the new material in the Discussion improves the manuscript, especially the discussion that incorporates a possible role for the Arr ICD in PCP. I would have preferred a bit more discussion of the extensive literature on the Arr-LRP5/6 ICD, but as this largely pertains to effects on canonical pathway components I suppose the authors are less interested.

Thank you for acknowledging that the Discussion is improved.

We are discussing the potential role of the ICD to the extent possible, based on current publications, adding more would be wildly speculative.

As for the prior studies on Wnt gradients in the wing, I am still a bit puzzled by the statements about “mosaicism in in vivo CRISPR assays” and the lengthy speculation based on that in the Discussion. I assumed by this the authors meant first generation CRISPR phenotypes created by UAS-gRNA and cas9, as in the CRISPR therapy articles they reference. But Yu et al did not use CRISPR in this way. They used CRISPR only to generate permanent deletion alleles and insert integrase sites, and homozygotes with such alleles will not be subject to future mosaicism. The conditional alleles used by Yu did not rely on CRISPR to generate the mutant condition; they used FLPase to flipout FRT constructs inserted at attB sites, either replacing a wt Wg with membrane-tethered NRT-Wg or in one case removing Wnt4. I suppose one could argue that the FLPout created mosaicism, but then that should be obvious since the NRT-Wg is tagged. Have the authors looked?

Yes, we have missed the generation of KO alleles for the various Wnts in Yu JJ et al., apologies. We acknowledge their multiple KOs combined with the Wg-cNRT transgene. Nonetheless, the assay employed for generating the “mutant wings”, relying on Gal4-FLP and FRT sites, also creates mosaic tissue with the mosaic effects allowing certain cells, the healthier cells, to take over (outcompete mutant cells). Therefore some Wnt expression very likely remains, which is consistent with the wing size in these assays being larger than the RNAi-based KDs as shown in Barrio and Milan (2020). The reviewer acknowledges that mosaicism occurs in the FRT/FLP-out technique. Their question “have the authors looked?” is beyond the scope of this study, and no “the authors”, Yu JJ et al (2020) have not looked, and we haven’t either as this question is outside the scope of this paper. But yes, it should certainly be looked at.

To reconcile Yu et al with the authors’ work, my guess is that there is low-level NRT-Wg throughout the wing that supplies a non-graded stimulus to Fz that is needed for normal PCP. Such expression does certainly exist in earlier stage wing discs, and I’ve seen nothing that completely rules it out at later stages after high level concentration of Wg expression at the margin and the proximal rings.

Yes, thank you for this comment. We certainly suspect so.

These observations will require a careful, completely new study to address the role of Wnt genes in PCP in the near future.

The situation may be parallel to the role of Ds-Fz gradients in the wing. Yes, there are gradients of expression, and ectopic gradients or boundaries can polarize hairs. But in many situations those gradients, and even Fat itself and thus all polarized Fat-Ds binding, can be removed with only very slight disruptions to wing hair polarity.

This is an interesting statement, but unrelated to this work as it would address a different signaling pathway/system.

4) Clarity-Statements made in the Results should be clear enough that the reader does not have to look at figure legends or the Methods to understand what is being said. I had previously asked for a couple minor changes, but the authors have made no changes in the Results that help, despite saying that they would, or that they have when in fact they have not.

We have made several specific changes and adjustments to the Results text in response to previous comments of the reviewer, so we are not sure what they are now referring to. We trust that our text is clear based on comments from colleagues and other reviewers.

4A) “In a pilot screen with most ligands and transmembrane proteins/receptors acting in the two main Wnt-signaling pathways, we discovered that manipulating arr/LRP5/6 levels did tilt the dsh phenotypic balance, enhancing and suppressing the respective phenotypes when their levels were changed,” This sentence about Fig. S1 is completely identical to the original, as is most of the rest of the paragraph.

We are confused by this comment.

Overall, we have revised this section quite a bit in response to the rev’s comments. We have added information and description to the Suppl. Fig S1 as requested, and the reviewer thanks us for adding this information and description in their point 2C) above.

The problem is that that “changing balances” does not make it clear that a SINGLE genetic change usually improves one phenotype and worsens the other, rather than some genetic changes altering the phenotypes one way, and other change altering phenotypes the other. How about something like:

In a pilot screen assessing the canonical and PCP phenotypes we discovered that any single change in Arr levels tilted the dsh IR phenotypic balance, simultaneously enhancing one phenotype while suppressing the other.

The suggested textual phrasing is fine with us, and we have adjusted the text accordingly in the newly revised manuscript (page 7).

4B) nor was asymmetric localization in developing wings was detected with available tools.

What is the reviewer referring to? All tools used (antibodies or tagged protein versions) are described in the main text and Methods adequately.

Knowing what they looked at is important, as many authors might content themselves with measurements of nematics which are often not sensitive enough. If what the authors did was look at anti-Arr at mutant clone boundaries or tagged Arrow at boundaries of misexpression, simply say in the Result text “was detected with anti-Arr at mutant clone boundaries or boundaries of tagged Arr misexpression”

This comment by the reviewer is confusing at best, sorry.

First, to say that “...measurements of nematics are often not sensitive enough” is wrong.

Generally, nematics analyses are very sensitive, I trust that all PCP researchers would agree.

Second, the statement is bunching together (i) nematics, (ii) Arrow localization, and (iii) clone boundaries. Nematics were used to analyze the arr LOF clones in detail (with Fmi and Dsh localization/staining as read-out). Arr protein localization was analyzed with anti-Arr and anti-HA (in a Arr-HA background) and described in quite some detail following the previous revision ‘cycle’, and neither of these two data sets has much to do with clone boundaries.

This comment cannot be addressed in a specific or productive way.

Other notes:

Line 232- “but milder, to what was described for fz and fmi clones”- Milder in what sense? The reduction in Dsh looks stronger, and the “loss” of asymmetric membrane associated localization sounds equivalent.

We tried to be as careful as possible, as we think it is difficult compare images from different publications. Nonetheless, we have removed “milder” from the sentence.

Line 297-305. The new paragraph is appreciated, but for the naïve reader it might be best to start with a statement about the possible role of Ft/Ds and canonical signaling.

Thank you for appreciating this paragraph. We have also added phenotypic quantifications to allow direct comparison (Fig 3K). To rewrite the paragraph with focus on Ft-Ds phenotypes would distract any reader from the focus of this study.

Line 315- (Supplemental Fig S4K,L etc.). Because new panels have been added, these now refer to the wrong panels

Apologies for this mishap. This has been corrected.

Line 392- While this experiment shows that Fz might titrate Arr away from canonical signaling, is there evidence that this is “to PCP signaling”? Does PCP improve?

The mild knock-down of arr (via this RNAi approach) does not affect PCP, and only shows mild wing margin loss (so PCP cannot get “better”). Changing the level of Fz (up or down) in this background affects the “margin loss/notching” defects, indicating that Fz and Fz2 compete for the available Arr protein and as only Fz2 normally signals via Wnt/beta-catenin pathway, levels of Fz have an inverse effect on that. Based on feedback from our colleagues, we trust that the phrasing is clear.

Lines 421-422-First, Fig. S6F shows one bar where *arr/+*; *arm/+* increased PC defects, and another where it doesn't. Is this a mistake? It is opposite to the statement in the text, Second, in S6E *arm/+* appears to decrease the PCP defects; if statistically significant, this might be worth pointing out.

It is not a mistake. In the genetic backgrounds the reviewer refers to the phenotypic effects are much weaker than in the triple heterozygotes (the genetic backgrounds the reviewer refers to were added upon their requests as controls). We have added a note to mention this observation and, with respect to Fig S6E, we have also added complete statistical analyses to document these effects.

Third, how are the PCP defects being quantified? Does % mean the % of hairs with abnormal angles, or the % of wings showing any defects at all, or something else?

The PCP defects are being quantified by their penetrance (how many wings show defects in a given genetic combination, resulting in % values). The phenotype being assessed is the one shown in the picture panels, focusing on a specific region of the wing that is rather sensitive to gene dosage and where dominant effects have been reported with *Vang* for example. We trust that this is adequately described in the text.

Line 481- I have looked at reference 46 and seen no claims about asymmetric enrichment of CK1e protein. The authors should check.

Apologies for this erroneous reference.

This comment was based on a presentation at a Wnt-signaling conference in 2022 (and our assumption that it was published then as well). The data has not yet been published. Hence the sentence was removed.