Arf6 is necessary for senseless expression in response to Wingless signalling during Drosophila wing development

Julien Marcetteau¹, Tamás Matusek¹, Frédéric Luton²*, Pascal P. Thérond¹**#

¹ Université Côte d’Azur; UMR7277 CNRS; Inserm 1091; Institut de Biologie de Valrose (iBV); Parc Valrose; 06108 Nice cedex 2, France. ² Université Côte d’Azur; UMR7275 CNRS; Institut de Pharmacologie Moléculaire et Cellulaire (IPMC); 660 Route des Lucioles, Sophia Antipolis, 06560 Valbonne, France.

* Frédéric Luton and Pascal P. Thérond should be considered as joint senior authors.

# Corresponding authors: therond@unice.fr (+33)492076443

Running title: Arf6 requirement in wing patterning

Keywords: Drosophila, Signalling, Wnt, Wingless, Arf6, Armadillo, Pangolin.

Abstract

Wnt signalling is a core pathway involved in a wide range of developmental processes throughout the metazoa. In vitro studies have suggested that the small GTP binding protein Arf6 regulates upstream steps of Wnt transduction, by promoting the phosphorylation of the Wnt coreceptor, LRP6, and the release of β-catenin from the adherens junctions. To assess the relevance of these previous findings in vivo, we analysed the consequence of the absence of Arf6 activity on Drosophila wing patterning, a developmental model of Wnt/Wingless signalling. We observed a dominant loss of wing margin bristles and Senseless expression in Arf6 mutant flies, phenotypes characteristic of a defect in high level Wingless signalling. In contrast to previous findings, we show
that Arf6 is required downstream of Armadillo/β-catenin stabilisation in Wingless signal transduction. Our data suggest that Arf6 modulates the activity of a downstream nuclear regulator of Pangolin activity in order to control the induction of high level Wingless signalling. Our findings represent a novel regulatory role for Arf6 in Wingless signalling.

Introduction

The ADP-ribosylation factor (Arf) family of small GTP-binding proteins is remarkably well conserved throughout the eukaryotes (Donaldson and Jackson, 2011). Arf6 is the most divergent of the Arfs, and localises to the plasma membrane and endosomes where it regulates various steps of endosomal trafficking and recycling (D’Souza-Schorey and Chavrier, 2006; Donaldson and Jackson, 2011). Previous in vitro studies have implicated Arf6 in the upstream stages of Wnt signalling (Grossmann et al., 2013; Kim et al., 2013; Pellon-Cardenas et al., 2013). However, a potential physiological, in vivo, role of Arf6 in Wnt signalling is yet to be addressed (Kim et al., 2013).

Despite the evolutionary distance between humans and Drosophila, Arf6 shares 97% sequence identity conservation between the two species (figure S1A). Combined with the availability of powerful genetic tools, this makes Drosophila an ideal model in which to investigate the requirement for Arf6 in Wnt signalling in an in vivo context.

The Drosophila Wnt1 homologue, wingless (wg), is initially expressed throughout the wing primordium, and becomes progressively refined to a narrow strip of cells of the presumptive wing margin late in larval development (Ng et al., 1996; Williams et al., 1993). The Drosophila wing has classically served as a developmental model of Wg signalling and has played a fundamental role in our understanding of Wnt/Wg signalling (Bejsovec, 2018; Jenny and Basler, 2014; Langton et al., 2016; Wiese et al., 2018). Canonical Wg signalling is contingent upon the stability of cytoplasmic Armadillo (Arm, the Drosophila β-catenin homologue) in signal receiving cells. In the absence of the Wg ligand, Arm is constitutively phosphorylated by the β-catenin destruction complex, consisting of
the scaffold Axin, APC, and the kinases GSK3β and CK1 (Stamos and Weis, 2013), promoting Arm proteasomal degradation. The binding of Wg to the Frizzled 2 (Fz2) receptor and Arrow (Arr) co-receptor at the cell surface activates Dishevelled (Dsh), leading to the deactivation of the destruction complex and the stabilisation of cytoplasmic Arm (Swarup and Verheyen, 2012). Arm then translocates to the nucleus where it binds to Pangolin (Pan, a LEF/TCF homologue), converting it from a transcriptional repressor to an activator, and triggering the expression of Wg target genes (Mosimann et al., 2009; Schweizer et al., 2003).

High level Wg signalling is essential for the establishment and patterning of the wing margin (Couso et al., 1994; Jafar-Nejad et al., 2006; Phillips and Whittle, 1993). Cells flanking the wing margin respond to the local high levels of Wg protein by expressing the zinc finger transcription factor *senseless* (*sens*) which acts as the proneural factor for the anterior stout mechanosensory, and posterior non-innervated margin bristles (Jafar-Nejad et al., 2003; Jafar-Nejad et al., 2006; Nolo et al., 2000). Low level Wg signalling further into the wing blade induces the expression of more sensitive target genes such as *distal-less* (*dll*) which is more broadly expressed in the wing blade (Neumann and Cohen, 1997; Zecca et al., 1996).

In this study we assessed the *in vivo*, developmental role of Arf6 in Wg signalling using a *Drosophila* model. *Arf6* mutants show a dominant loss of wing margin bristles and a concomitant loss of Wg-dependent *sens* expression in the wing imaginal disc, phenotypes indicative of a defect in high level Wg signalling. Arf6 has previously been suggested to act upstream in the transduction of Wnt signalling by promoting the phosphorylation of the Wnt co-receptor, LRP6, and the release of β-catenin from the adherens junction into the cytoplasm (Grossmann et al., 2013; Kim et al., 2013; Pellon-Cardenas et al., 2013). In contrast to these findings, our data indicate that in *Drosophila* Arf6 is necessary downstream of Arm stabilisation for the activation of high level Wg signalling. Moreover, we show that Arf6 acts genetically upstream, or at the level of Pan activity. These findings represent a novel function for Arf6 necessary for high level Wg target gene expression during wing
margin development, and is the first demonstration of an *in vivo* role for Arf6 in, or in parallel to, Wg/Wnt signalling.

**Materials and Methods**

**Fly genetics**

Flies were raised in standard conditions. Crosses were carried out at 22°C unless stated otherwise.

**Clone induction**

Clones were generated by crossing males of either FRT42B, Arf6KO/CyO, Tb::RFP or FRT42B, Arf61/CyO, Tb::RFP with virgins of y1, w1118, hsFLP; FRT42B, ubi-nlsGFP. Heat shock induction was carried out for 30 minutes in a water bath at 37°C, 48h after egg lay. Larvae carrying Arf61 or Arf6KO were selected based on the absence of Tb, then dissected and stained in wandering stage L3. Mutant clones were recognised based on the absence of a GFP signal.

**Fly stocks**

The following fly stocks were used during this study: w1118 (Bloomington #3605) served as a wild-type control and the source of wild-type chromosomes. Arf51fG16w− (Arf6KO) (Bloomington #60585 (Huang et al., 2009)), Arf61 (Dyer et al., 2007) (A kind gift from Marcos Gonzalez Gaitan) are both independently generated null alleles of Arf6 lacking the full coding region. Arf6KO was initially recessive lethal, so we introgressed both Arf6 null alleles into a w- background for 5 generations and reconfirmed the presence of the deletions by PCR. Arf6KO and Arf61 were maintained as a stock balanced over CyO, Tb::RFP (Bloomington #36336) to allow homozygous larvae to be recognised. ARF6::GFP (Bloomington #60586) is an endogenous, C-terminally tagged form of Arf6 generated in the Arf6KO background (Huang et al., 2009). High level Wg activation was induced using UAS-
dsh::myc (Bloomington #9453), UAS-sgg$^{A81T}$ (Bloomington #5360) (Bourouis, 2002), UAS-Arm$^{S10}$ (encoding Arm lacking amino acids 37-84 in the N-terminus, Bloomington #4782) (Pai et al., 1997), vgMQ-arm$^{Ndel}$ (expresses a form of Arm lacking amino acids 1 to 138 from the N terminus, Bloomington #8370) or UAS-axin-RNAi (Bloomington #31705). Wg signalling was induced downstream of Arm stabilisation was achieved using UAS-pan$^{VP16}$::HA (generated in this study, see methods below).

Wg signalling suppression was achieved with UAS-dsh-RNAi (KK330205, VDRC), UAS-arr-RNAi (GD6707 and GD6708, VDRC) or wg$^{Cxx}$ (Bloomington #2980). Wild-type sens was over-expressed with UAS-sens (Bloomington #42209). The following Gal4 drivers were used to drive expression in the wing: nubbin-Gal4 (expressed throughout the wing pouch) (Azpiazu and Morata, 2000) C96-Gal4 (expressed in a wide domain overlapping the D/V boundary) (Bloomington #43343). Mitotic clones were induced using y,w,hsFLP; FRT42B, ubi-GFP$^{NLS}$ (derived from Bloomington #5826), and Arf6$^{AQ}$; FRT42B/ CyO, Tb::RFP or Arf6$^{1}$; FRT42B/ CyO, Tb::RFP (derived from Bloomington stocks #1956 and #36336).

The following independently generated EMS-induced pav alleles were used: pav$^{B200}$ (Bloomington #4384) (Salzberg et al., 1994) and pav$^{G63}$ (Bloomington #23926) (Collins and Cohen, 2005).

Generating pan$^{VP16}$::HA

pan$^{VP16}$::HA was generated in order to allow the induction of Wg signalling downstream of Arm stabilisation. The construct is conceptually based on a construct previously shown to act independently of enhanceosome components Legless (Lgs) and Pygopus (Pygo) (Thompson, 2004). A sequence encoding full length Pan, excluding the stop codon, followed by 3xHA flanked by GGGGS linkers, and finally the $^{VP16}$ transcriptional activation domain was synthesised (GeneArt). The sequence was directionally subcloned into 5’ KpnI and 3’ Xbal into $^{pUAST attb L34}$ plasmid (Bischof
et al., 2007). Purified maxipreps were injected into the \(M(3xP3-RFP.attP')ZH-68E\) background (Bl# 24485) (Bischof et al., 2007) in order to generate third chromosome insertions.

**Antibodies**

The following primary antibodies were used: rabbit anti-GFP (1:400, Life Technologies A6455), Guinea pig anti-Sens (1:1000, a kind gift from Hugo Bellen), rat anti-Distalless (1:100, a kind gift from Marc Bourouis), mouse Anti-Wg (1:100, DSHB 4D4), mouse anti-Arm (1:10 DSHB N2 7A1). Rat anti-DE-cadherin (1:50, DSHB DCAD2).

The following secondary antibodies were used: Goat anti-rabbit Alexa488 (1:500; Invitrogen A11034), goat anti-rabbit Alexa546 (1:500; Invitrogen A11035), donkey anti-mouse Alexa488 (1:500; InvitrogenA21202), donkey anti-mouse Alexa546 (1:500; Invitrogen A10036), donkey anti-rat Alexa488 (Invitrogen A21208), goat anti-rat Alexa546 (1:500; Invitrogen A11081) and TRITC-phalloidin (1:100; Sigma P1951-1MG)

**Wing imaginal disc preparation and imaging**

Wandering stage L3 larvae were washed then dissected in ice-cold 1xPBS. Fixation was carried out for 20 minutes at room temperature in 3.7% formaldehyde with constant agitation. Samples were washed and permeabilised for 30 minutes in PBT (0.3% Triton X-100, 1x PBS) then blocked for 1h in blocking buffer (0.1% Triton X-100, 1% BSA, 1x PBS) at room temperature. Primary antibody incubations were carried out over-night at 4ºC in 200µl of antibody diluted in blocking buffer. Samples were washed 3x 20minutes in PBT, then incubated for 1 hour at room temperature with secondary antibodies. Samples were washed in PBT then mounted in VECTASHIELD mounting medium (Vector Laboratories).
Images were acquired with a Leica TCS upright SP5 confocal microscope using a 40x objective (HCX PLAN APO; Numerical aperture of 1.3). The Leica LAS AF software package was used for image capture (v2.6.3.8173). Images were analysed using FIJI (Schindelin et al., 2012) and the data analysed and visualised in R (R Core Team, 2020). Data-points were overlayed on the boxplots to display data distribution. Larger points represent numerical outliers, defined as points that fall outside 1.5x the interquartile range, above the upper, and below the lower quartiles.

**PCR validation of Arf6 deficiencies**

Genomic DNA was extracted from individual flies. Flies were crushed in PCR tubes using a pipette tip containing 50μl of squashing buffer (10mM Tris-HCl, 1mM EDTA, 25mM NaCl and 200 μg/mL proteinase K). Samples were incubated at 37°C for 30 minutes then heat inactivated at 95°C for 2 minutes using a thermocycler. 1μl of the resulting extraction was used as the PCR template.

The deficiency described for Arf6<sup>1</sup> was validated using PCR ([figure S1B’](#)) and the primer combinations shown in ([figure S1B](#)). Arf6<sup>KO</sup> has previously been characterised in (Huang et al., 2009) Primer sequences used are provided in the table below. 2x GoTaq Green Master Mix (M7121, Promega) was used for the PCR reactions. The following primers were used to validate the Arf6<sup>1</sup> allele:

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arf6_A</td>
<td>GATCTGCGGGTCCACTGAAA</td>
</tr>
<tr>
<td>Arf6_D</td>
<td>TGTCTCGCAAATTGAGGCAGA</td>
</tr>
</tbody>
</table>
**Adult wing dissection**

Adult flies were collected in ethanol at least 12h following emergence to ensure their wings had fully expanded and dried. Wings were removed at the hinge in ethanol, dried on blotting paper, then mounted in a drop of Euparal (Carl Roth #7356.1) and left to cure over-night on a slide heating plate set at 60ºC. Wings were imaged using a Leica DM2000 with an attached Leica DFC7000T camera. Wings were excluded from quantifications if damage to the wing margin prevented bristle quantification.

**Quantification and statistical analysis**

The numbers of both ectopic and stout wing margin bristles ([figure S1C](#)) were quantified manually using the cell counter plugin in FIJI (Schindelin et al., 2012). Statistical analyses and plotting were carried out in R (version 3.6.3)(R Core Team, 2020). The counts of both stout bristles and ectopic bristles for multiple genotypes were analysed using the Kruskal-Wallis test. Post-hoc pairwise comparisons between the counts for individual genotypes were carried out using the Dunn test. The p values resulting from multiple comparisons were corrected for Type 1 error using the Benjamini–Hochberg procedure. Single comparisons were made using Mann-Whitney U tests. Plots were generated using the GGPlot2 package and exported using the egg package (Auguie, 2019; Wickham, 2009). Sample sizes are marked on the plots or provided in figure legends.

**Results**

**Arf6 is necessary for wing margin patterning**

We observed a dominant reduction in the number of bristles throughout the wing margins of adult flies heterozygous for the amorphic Arf6 alleles, *Arf6*¹ and *Arf6*KO (Dyer et al., 2007; Huang et al., 2009) ([figure 1A, A”](#)) (see [figure S1C](#) for an overview of wing margin bristle patterning). This
phenotype was strongly enhanced in homozygous Arf6 mutants (figure 1A, A’’). The trans-allelic combination of Arf6\(^{1}\) and Arf6\(^{KO}\) resulted in a comparable phenotype to the respective homozygotes (figure 1A, A’’), showing that the loss of the DNA region common to both deficiencies is responsible for the phenotype (figure 1A, figure S1B).

The patterning of the wing margin is coordinated by high level Wg signalling late in larval development (Couso et al., 1994; Jafar-Nejad et al., 2006). We therefore tested whether the Arf6 mutant phenotype is sensitive to the level of Wg. Although the null wg allele, wg\(^{CX4}\), does not induce a dominant wing margin phenotype (figure 1A’, A’’), when in combination with either heterozygous Arf6\(^{1}\) or Arf6\(^{KO}\), it strongly enhanced the Arf6 wing margin phenotype (figure 1A’, A’’). We did not observe notching of the wing margin, or morphological defects in the bristles in Arf6 mutants either alone or in combination with wg\(^{CX4}\) (figure 1A, A’, A’’).

**Wg-dependent Senseless expression is suppressed in an Arf6 mutant**

The zinc finger transcription factor Sens acts as the proneural factor for many of the margin bristles, and is expressed in two narrow stripes flanking the wing margin in response to high level Wg signalling (Jafar-Nejad et al., 2006; Nolo et al., 2000) (figure 2A). Sens staining was strongly reduced throughout the wing margin in an Arf6 mutant context, but not in the sensory organ precursor in which the expression of Sens is independent of Wg (figure 2A’). The bristles induced by ectopically expressing sens were not dominantly suppressed in the Arf6 mutant, indicating that the loss of bristles was not due to a loss of Sens proneural activity (figure S2A, A’).

To test whether the reduction in Sens is due to a defect in wg expression, we analysed the pattern of Wg in Arf6\(^{1}\) mutant wing discs (figure 2A). The Wg stripe at the dorso-ventral (D/V) boundary was not disrupted by the loss of Arf6. Interestingly, the low-threshold Wg target Distal-less (Dll) was not reduced in Arf6 mutant conditions (figure S3A, A’, B) indicating that Arf6 is not necessary for low level Wg signalling.
In order to assess whether Arf6 is required cell autonomously in Wg signal transduction, we generated random mitotic Arf6\(^1\) clones which we then stained for Sens and Wg. Consistent with the dominant loss of bristles in Arf6 mutants, we observed a strong reduction in Sens staining in homozygous Arf6\(^1\) clones, an intermediate level in heterozygous tissue and the wild-type levels in the wild-type tissue (figure 2B, B'). Importantly, clones that overlapped with the sens expression domain, without entering the wg expressing margin cells, still induced a strong reduction in Sens staining (closed orange arrowheads, figure 2B'), demonstrating that removing Arf6 activity cell autonomously suppresses Sens in Wg receiving cells. Importantly, we did not observe ectopic Wg expression in Arf6 clones near the wing margin (figure 2B, 2B'), nor wing notching in the Arf6 mutant wing (figure 1) indicating that the integrity of the D/V boundary was not affected by the loss of Arf6 (Rulifson and Blair, 1995; Rulifson et al., 1996). Altogether, these data show that while Arf6 is not required for the integrity of the D/V boundary, its activity is required cell autonomously for the transduction of high level Wg signalling controlling the expression of sens necessary for wing margin bristle development.

Arf6 is necessary downstream of Armadillo stabilisation

In order to determine the level at which Arf6 is required in Wg signal transduction, we began by activating the Wg signalling pathway in an Arf6 mutant background. We suppressed the activity of the destruction complex by expressing a dominant-negative form of the Drosophila GSK3\(\beta\) homologue, shaggy (sgg\(^{A81T}\)) (Bourouis, 2002) or knocking-down axin. Both treatments induce high level Wg signalling and the formation of ectopic bristles in the wing blade (figure 3A, B). The number of ectopic bristles was dominantly suppressed in heterozygous Arf6 mutant backgrounds (figure 3A, A', B, B'). These data indicate that the loss of bristles and Sens expression in the Arf6 mutants is not
a result of the hyperactivation of the Arm destruction complex, and suggest that Arf6 acts downstream of Arm stabilisation.

We next confirmed that Arf6 acts downstream of the stabilisation of Arm by expressing two constitutively active forms of Arm: Arm$^{S10}$ and Arm$^{Ndel}$ (Pai et al., 1997). Importantly, these N-terminally truncated forms of Arm accumulate in the cytoplasm, triggering constitutive, high level Wg signalling in a ligand independent manner (Pai et al., 1997; Somorjai and Martinez-Arias, 2008). We expressed Arm$^{S10}$ in a broad domain overlapping the D/V boundary with the C96-Gal4 driver, while Arm$^{Ndel}$ expression is directly driven by the vestigial quadrant and margin enhancers (subsequently referred to as vgArm$^{Ndel}$). Both Arm variants induced ectopic bristles in the wing blade (figure 3C, C', D, D'). Importantly, the bristles induced by vgArm$^{Ndel}$ were not dependent on endogenous Wg signalling (figure S4A, A, B, B', B'') and vgArm$^{Ndel}$ is active in canonical Wg signalling (figure S4C). The ectopic bristles induced by both constructs were dominantly suppressed in the Arf6 mutant background (figure 3C, C', D, D'). Moreover, vgArm$^{Ndel}$ or Arm$^{S10}$ did not rescue the wing margin bristles lost in the wing margin of Arf6$^{KO}$ flies, and instead caused an enhancement of the Arf6 mutant phenotype (figure 3E, E', figure S5A, A'). Over-expressing wild-type dsh also induced ectopic bristles which were suppressed in a heterozygous Arf6$^{KO}$ background (closed orange arrow, figure S5B, B'). dsh over-expression also enhanced of the heterozygous Arf6$^{KO}$ phenotype (compare figure S5C, S5B, S5C'). This is unlikely to be due to a dominant negative effect of Arm$^{S10}$ or Dsh overexpression as expressing either of these constructs in a wild-type background did not induce wing margin defects. Moreover, we did not observe a change in the levels of endogenous Arm and Cadherin at the adherens junctions in Arf6$^{1}$ mutant clones (figure S6A, A'), suggesting that Arf6 does not regulate Wg signalling through the sequestration of Arm to the adherens junction in Drosophila (Grossmann et al., 2013; Pellon-Cardenas et al., 2013). Altogether, these data demonstrate that Arf6 is required genetically downstream of Arm stabilisation in order to activate high level Wg signalling.
To test whether stabilised Arm had a generally reduced signalling activity in the Arf6 mutants, we stained for both Sens and Dll in wing imaginal discs expressing vgArm\textsuperscript{N\text{Del}} in either a wild-type (figure 3F) or heterozygous Arf6\textsuperscript{KO} background (figure 3F'). Clusters of ectopic Sens positive nuclei were apparent far from the wing margin in control wings expressing vgArm\textsuperscript{N\text{Del}} (closed orange arrowheads figure 3F) accompanied by an upregulation of Dll (open orange arrowheads figure 3F). Removing a single copy of Arf6 led to an almost complete suppression of the ectopic Sens expression, including in the wing margin, but both the ectopic and endogenous Dll remained (closed blue arrowheads figure 3F'). These data indicate that although vgArm\textsuperscript{N\text{Del}} is still able to activate low level signalling in the Arf6 mutant background, its ability to activate Sens expression is strongly attenuated.

Importantly, although the Arf6 margin phenotype was mildly enhanced in a heterozygous arf1 mutant background, the signalling activity of Arm\textsuperscript{N\text{Del}} was not suppressed in a heterozygous arf1 background (figure S7A, A', B, B'). This suggests that although Arf1 contributes to wing patterning, it likely does so in a distinct manner to Arf6 (Hemalatha et al., 2016).

Together, these results emphasise the specific requirement for Arf6 for the cell autonomous establishment of sens expression in response to high level Wg signalling. The loss of margin bristles in the Arf6 mutants is therefore likely to be due to a loss of the Sens-positive proneural clusters of the wing margin due to a suppression of high level Wg signalling.

**Arf6 is necessary at the level or upstream of Pangolin**

The dominant suppression of N-terminally truncated Arm activity in Arf6 mutants suggests that Arf6 could be involved in positively regulating canonical nuclear Wg signalling. Pavarotti (Pav), a MKLP1 homologue (Dyer et al., 2007; Makyio et al., 2012) has previously been shown to act in the nucleus as a negative regulator of Wg signalling during embryonic development (Jones et al., 2010). MKLP1 also recruits, and physically interact with Arf6 at the Flemming body during cytokinesis.
(Makyio et al., 2012). We therefore hypothesised that Pav could provide the functional link between Arf6 and Wg signalling.

We began by testing whether the Arf6 phenotype is sensitive to changes in the level of Pav. Pav is essential during cytokinesis (Adams et al., 1998), we therefore opted to use hypomorphic pav alleles (pav^{B200} and pav^{963}) to avoid strong pleiotropic effects. Heterozygous pav^{B200} and pav^{963} flies in a heterozygous Arf6 background provided a partial rescue of the number of wing margin bristles (figure 4A, A’) in the wing margin. These conditions did not induce cytokinesis defects or wing notching (figure 4A, figure S8), consistent with Arf6 being dispensable for somatic cytokinesis in Drosophila (Dyer et al., 2007). The genetic interaction between Arf6 and pav indicate that Arf6 could be regulating nuclear Wg signalling by modulating the non-canonical activity of Pav as a negative regulator of Pan activity (Jones et al., 2010).

Once in the nucleus, Arm forms a complex with Pan, a TCF/LEF homologue forming the core of the enhanceosome (Gammons and Bienz, 2018). To determine whether Arf6 acts upstream of the enhanceosome, we generated a constitutively active form of Pan (Pan-VP16::HA, see Materials and methods) (figure S9A, S9A’). Expressing pan-VP16::HA in a wild-type background only induced low levels of ectopic Sens expression (figure S9B, closed orange arrowheads S9B’), and was not sufficient to activate sens expression far from the wing margin (open orange arrowheads, figure S9B’), indicating that its activity still requires endogenous permissive signals. Expressing Arm^{S10} under the same conditions induced extensive ectopic Sens throughout the C96 expression domain (figure S9C, C’). Despite its greater ability to induce Sens expression, expressing Arm^{S10} with C96-Gal4 in a heterozygous Arf6^{ex0} background did not rescue Sens expression (figure S9D, D’), whilst expressing pan-VP16::HA in the same conditions resulted in a substantial rescue of Sens throughout the wing margin (figure 4C, C’). Taken together, these results indicate that Arf6 activity is required genetically downstream of the stabilisation of Arm, but upstream or at the level of Pan activity for the induction of sens expression.
Discussion

We have demonstrated a novel requirement for the small GTP binding protein Arf6 during Drosophila wing development. The Arf6 mutant phenotype is characterised by a dominant reduction in the number of bristles in the adult wing margin, accompanied by reduced sens expression in the wing margin PNCs in the wing imaginal discs. The patterning of the wing margin requires the expression and activity of Sens in the cells flanking D/V boundary in response to high level Wg signalling activity (Jafar-Nejad et al., 2006; Nolo et al., 2000). sens begins to be expressed in this compartment late in larval development, and reducing Wg signalling during this period is associated with similar phenotypes to those we observed in the Arf6 mutant background (Couso et al., 1994). We therefore focussed on understanding the Arf6 mutant phenotype in the context of Wg signalling. Based on epistatic interactions, we established that Arf6 acts genetically downstream of the stabilisation of Arm, but upstream or at the level of nuclear Pan activity for the expression of sens in response to Wg signalling. As Arf6 acts at the plasma membrane and endosomal membranes, it is unlikely to directly regulate nuclear Wg signalling (Donaldson and Jackson, 2011). We therefore suggest that Arf6 could regulate Wg signalling through the non-canonical activity of the MKLP1 orthologue, Pav, previously shown to directly interact with Arf6, and to act as a nuclear repressor of Wg signalling during Drosophila embryogenesis (Jones et al., 2010). This could be achieved through the sequestration of Pav to endosomal membranes by Arf6, preventing its access to the nucleus.

Our findings complement the results of previous in vitro studies in which Arf6 was shown to act upstream in Wnt signalling at the level of signalosome activity, or through reallocation of junctional β-catenin to the cytoplasm (Grossmann et al., 2013; Kim et al., 2013; Pellon-Cardenas et al., 2013). These findings are not mutually exclusive, as it is not yet clear whether the downstream role of Arf6 is conserved in Wnt signalling, as Wnt conditioned medium was used as a source of Wnts, meaning
that a role for Arf6 in upstream signalling steps would likely mask a potential downstream role. A
downstream role of Arf6 in Wnt signalling would be of particular relevance to pathologies such as
colorectal and breast cancers induced by hyperactivation of Wnt signalling (Zhan et al., 2017). This is
most commonly a result of mutations in components of the \( \beta \)-catenin destruction complex, or more
occasionally \( \beta \)-catenin itself, leading to \( \beta \)-catenin stabilisation (Clevers and Nusse, 2012). Wnt
signalling in these contexts is ligand-independent, making downstream regulators of Wnt
transduction potentially valuable therapeutic targets. Small molecule inhibitors of Arf6 have already
been identified, and Arf6 inhibition in adults has not been associated with secondary effects
(Grossmann et al., 2019; Macia et al., 2021).

The *Drosophila* Arf6 phenotype is particularly striking due to it being dominant, while specifically
impacting a high threshold Wg signalling target, *sens*, without affecting the low threshold target *dll*.
These observations can be interpreted as Arf6 specifically acting in the transduction of high
threshold Wg signalling, as *sens* has previously been shown to be much more sensitive to
perturbations in Wg signalling than other Wg targets such as *dll* or *vestigial* (*vg*) (Baena-Lopez et al.,
2009; Song et al., 2009). However, we cannot exclude the possibility that Arf6 is required for a
process acting in parallel to Wg signalling, specifically necessary for the induction of *sens*
expression in response to high level Wg signalling. Although *sens* expression is frequently used as a readout of
Wg signalling, little is known about the regulatory logic and temporal dynamics underlying its
regulation by Wg signalling. Furthermore, the wing margin PNCs represent one of the few known
contexts in which *sens* expression is regulated by Wg signalling rather than by the bHLH proneural
proteins, Achaete (Ac) and Scute (Sc) (Jafar-Nejad et al., 2006; Nolo et al., 2000; Vincent, 2014). In
contrast to Arf6 mutants, flies lacking both *ac* and *sc* lose the majority of sensory organs throughout
the body, while the stout mechanosensory organs, and non-innervated bristles of the wing margin
remain (García-Bellido and De Celis, 2009; Jack et al., 1991; Jafar-Nejad et al., 2006). This, combined
with lack of a more general defect in bristle development in the Arf6 mutant indicates that the Arf6
mutant affects the Wg-dependent regulation of sens. This is particularly pertinent in the posterior wing compartment, in which the bHLH proneural factors are not expressed. Understanding the mechanism underlying the Arf6 mutant phenotype could provide insights into the cellular response to different levels of Wg signal transduction, and into the regulation of sens expression during wing margin development.

The high level of conservation of Arf6 and the Wg signalling pathway makes the molecular mechanism underlying the Arf6 phenotype more likely to be relevant beyond Drosophila wing development. Identifying the Arf6 regulators and effectors relevant to wing margin development, and in turn whether Arf6 activity is regulated by Wg signalling will not only help to understand the Arf6 phenotype, but could also provide more general insights into the mechanisms governing Arf6 activity in patho-physiological conditions.

**Acknowledgements**

We thank all the members of the iBV “fly” community, Roland Le Borgne, Bruno Antonny, Jean Paul Vincent and Sarah Bray for discussion.

**Author contributions**

J. M. conducted all experiments except for Fig. 4BC and S9 B-D performed by T. M. Experiments were design and discussed by J.M., T.M., F.L. and P.P.T. J.M. wrote the paper. All authors commented on the manuscript versions.
Competing financial interests

The authors declare no competing interests.

Funding

This work is supported by the Agence Nationale de la Recherche (ANR) (grant number: ANR-18-CE13-0003) to P.P.T. J.M. was supported by the French Government (National Research Agency, ANR) through the "Investments for the Future" programs LABEX SIGNALIFE ANR-11-LABX-0028 and IDEX UCAJedi ANR-15-IDEX-01 and by Fondation pour la Recherche Médicale (FDT201904007978).

References


R Core Team (2020). R: A Language and Environment for Statistical Computing.


Somorjai, I. M. L. and Martinez-Arias, A. (2008). Wingless signalling alters the levels, subcellular
distribution and dynamics of Armadillo and E-Cadherin in third instar larval wing imaginal discs. 
*PLoS One* **3**,.


Biol.* **5**, 1–16.


hierarchy of regulatory genes subdivides the developing Drosophila wing disc into discrete 

Zeeva, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a wingless morphogen 

Fig. 1. Dominant loss of wing margin bristles in Arf6 mutants.

(A-A’) Representative wing blades and wing margins of control (w), Arf6\textsuperscript{KO}, Arf6\textsuperscript{1} and wg\textsuperscript{Cox} mutants and their genetic interactions. Zooms of the anterior (A) and posterior (P) wing margins are separated by a dashed black line. Slender chemosensory bristles are still present in the homozygous Arf6 mutants (solid blue arrows) while stout mechanosensory bristles (SM) are almost all absent. The solid orange arrows indicate the loss of distal costa bristles in Arf6 mutants. The number of SM is quantified in (A’’). SM counts were analysed using a Kruskal-Wallis test. Significance values for pairwise comparisons between genotypes were calculated using a post-hoc Dunn test and reported using the following abbreviations: N.S. = p > 0.05, * = p \leq 0.05, ** = p \leq 0.001, *** = p \leq 0.001.
Fig. 2. The level of Sens expression is strongly reduced in the absence of Arf6.

(A) Wg and Sens staining in control (w') and Arf6<sup>1</sup> mutant. Anterior wing margin is to the left, posterior is to the right. (A') Sens is almost completely absent in the posterior wing margin while Sens-positive cells are occasionally observed in the anterior wing margin (closed orange arrows) of the homozygous Arf6<sup>1</sup> mutant. Sens is also observed in the prospective ventral radius and campaniform sensilla (open orange arrows). Wild-type (WT) n=10, Arf6<sup>1</sup> n=10. (B) Sens and Wg staining in Arf6<sup>1</sup> mutant clones is marked by the absence of GFP (-/-). Heterozygous and homozygous wild-type tissues are marked by (+/-) and (+/+) respectively. In the merges, Sens is in magenta, Wg in blue, GFP in green (B-B') and actin in green (A-A'). n=18 (B') a strong reduction in Sens staining is observed in clones that do not enter the Wg expression domain. All scale-bars represent 20µm. n=19
Fig. 3. Epistatic analysis shows that Arf6 acts downstream of Arm stabilisation.

(A) Dominant negative Sgg (sgg^{A81T}) overexpressed with nub-Gal4 induces ectopic bristles (closed orange arrowheads), which are dominantly suppressed in the Arf6 mutant background (quantification in A'). (B) Knock-down of axin induces ectopic bristles (B') which are dominantly suppressed in the Arf6 mutant background. (C) Arm^{S10} (expressed with C96-Gal4) and (D) vgArm^{NDel} (expressed under vestigial margin and quadrant enhancers) introduce ectopic bristles that are dominantly suppressed in the Arf6 mutant background (quantified in C' and D'). (E) Arm^{S10} expression with C96-Gal4 at 25ºC enhances Arf6^{KO} margin phenotype, but introduces ectopic margin bristles in a wild-type background (solid orange arrowheads). (E') Quantification of stout mechanosensory bristles. Bristle counts were analysed using a Kruskal-Wallis test. Significance
values for pairwise comparisons between genotypes were calculated using a post-hoc Dunn test and reported using the following abbreviations: N.S. = $p > 0.05$, $^* = p \leq 0.05$, $^{**} = p \leq 0.001$, $^{***} = p \leq 0.001$ (F) vgArm$^{NDel}$ expression induces ectopic Sens (closed orange arrowheads) and Dll (open orange arrowheads) in a wild-type background. (F') Ectopic Sens, but not Dll (closed blue arrows) is suppressed in a heterozygous Arf6$^{KO}$ background. In the merges, Sens is in magenta, Dll in green.
Fig. 4. Removal of one copy of pav, as well as Pan-VP16::HA overexpression rescue the Arf6 phenotype.

(A) The Arf6 mutant phenotype is partially rescued in a hypomorphic pav background (stout mechanosensory bristles quantified in A’). (B) Wing imaginal discs showing Sens expression in Arf6^{KO}/+ and in (C) Arf6^{KO}/+ with Pan-VP16::HA expressed with C96-Gal4. Anterior zooms of control and rescue discs are presented in B’ and C’ (in the merge, HA is magenta and Sens is green).
Fig. S1. The deficiencies of the Arf6^1 and Arf6^KO alleles delete the complete Arf6 coding region.

(A) An alignment of the primary protein sequences of Drosophila and human Arf6. There is a 97% identity conservation between the two proteins. Non-conserved residues are highlighted in pink. (B) the break points and deficiencies of the two null Arf6 alleles used in this study (Arf6^1 and Arf6^KO). Both deficiencies delete the complete Arf6 coding region (shown in orange) of all predicted Arf6 isoforms (RC, RD, RB, RA and RE). Primers are represented by half arrows above (see Table S1 for primer sequences). Thin horizontal grey lines represent intronic regions, while the grey and orange blocks represent exons. (B’) the PCR products resulting from the PCR testing the location of the Arf6^1 deletions. A 2000bp amplicon is present in control (w^-) samples whereas no signal is present in Arf6^KO due to loss of the region to which primer D binds. A 500bp band is present in Arf6^1 due to amplification of regions flanking Arf6^1 deletion. Three biological replicates are shown for each genotype. The deficiency in Arf6^KO was analysed in details in (Huang et al., 2009) (C) An outline of the main morphological features of the adult wing: AWM = anterior wing margin, PWM = posterior wing margin, ACV = Anterior crossvein, PCV = posterior crossvein. Details of the AWM and PWM in (C’). C = chemosensory bristle, SM = stout mechanosensory bristle, FM = fine mechanosensory bristle, NI = non-innervated bristle. The stout mechanosensory and non-innervated bristles are collectively referred to as the margin bristles in the text.
Fig. S2. Sens proneural activity is not impaired in an Arf6 mutant

(A) Over-expressing wild-type Sens induces ectopic bristles (closed orange arrowheads) and wing margin notching (closed blue arrowheads) in both a wild-type and Arf6 KO background, resembling the previously described hypermorphic sensLir allele (Nolo et al., 2001). (A') the number of ectopic bristles induced by Sens over-expression is not reduced in an Arf6 mutant background, indicating that the proneural capacity of Sens was not suppressed in a heterozygous Arf6 mutant background. Ectopic bristle numbers were analysed using a Kruskal-Wallis test. Significance values for pairwise comparisons between genotypes were calculated using a post-hoc Dunn test and reported using the following abbreviations: N.S. = p > 0.05, * = p ≤ 0.05, ** = p ≤ 0.001, *** = p ≤ 0.001.
**Fig. S3. distalless expression is not affected in the absence of Arf6.**

(A) Dll staining in Arf6<sup>1</sup> clones (labelled by the absence of GFP) with zoom shown in (A’) n=23. (B) Dll staining in both WT and homozygous Arf6<sup>1</sup> mutant discs (B’). In the merges, Dll is in magenta, GFP in green (B-B’) and actin in green (B’’). All scale bars represent 20µm. WT n=5, Arf6<sup>1</sup> n = 8
Fig. S4. $vg_{MQ}$-Arm$^{NDel}$ acts independently of endogenous Wg signalling

(A) The ectopic bristles induced by $vg_{MQ}$-Arm$^{NDel}$ ($vg_{Arm}^{NDel}$) are not dominantly reduced in a heterozygous $wg$ background ($wg^{CX4}/+$) indicating that $vg_{Arm}^{NDel}$ activity is independent of endogenous Wg ligand and signalosome activity. (A’) The quantification of the number of ectopic bristles. (B) Knocking down the Wg co-receptor $arr$ with nub-gal4 also does not suppress the formation of ectopic bristles induced by $vg_{Arm}^{NDEL}$, again suggesting that the signalling activity of $vg_{Arm}^{NDEL}$ does not depend on endogenous Wg signalling (quantified in B’). (B”) Knock-down of dsh caused strong wing defects, but does not suppress the activity of $vg_{Arm}^{NDEL}$. No quantification is shown due to the difficulty of reliably discerning ectopic bristles in this context. (C) $vg_{Arm}^{NDEL}$ is able to rescue the loss of the wing margin loss induced by knocking down $arr$ with nub-gal4. This shows that $vg_{Arm}^{NDEL}$ can recapitulate WT canonical wingless signalling activity and is sufficient to rescue a moderate Wg wing margin phenotype induced by the $arr$ knock-down.
Fig. S5. Wg activation b. vgArm^{Neel} and dsh over-expression enhance the Arf6 phenotype

(A) The loss of stout wing margin bristles in a heterozygous Arf6 mutant background is enhanced by the expression of vgArm^{Neel} (quantified in A'). (B) WT dsh over-expression triggers high level Wg signalling and the formation of ectopic bristles (closed orange arrowhead). (B') These bristles are dominantly suppressed in the Arf6^{KO} background. (C) Similar to the effect of Arm^{S10} and vgArm^{Neel}, the number of stout mechanosensory bristles is reduced by the over-expression of dsh in an Arf6^{KO} background (quantified in C').
**Fig. S6. Arm and DE-Cadherin localisation is not affected in Arf6 mutants**

(A) Staining for adherens junction components Arm and DE-Cadherin in Arf6KO clones. (A’) A zoom of the Arf6KO mutant clone is shown. In the merges, GFP is in green, Arm is in blue and DE-Cad is in magenta. n = 28.
Fig. S7. Arf6 acts independently of Arf1 in Wingless signalling

(A) Consistent with an upstream role of Arf1 in Wg signal transduction (Hemalatha et al., 2016), removing a copy of arf1 (arf1^{182-1} (West et al., 2017)) in a heterozygous Arf6^{KO} mutant background mildly enhances the loss of anterior stout mechanosensory bristles (quantified in A'). (B) Unlike Arf6 mutants, arf1^{182-1} does not reduce the number of ectopic bristles induced by vgArm^{NDel} (quantified in B'). This suggests that Arf1 and Arf6 play distinct roles in wing margin patterning.
Fig. S8. The genetic interaction between Arf6 and pav does not induce cytokinesis defects

(A) anterior and posterior zooms of wings of the genotypes shown in figure 4A showing details of trichomes. No multiple wing hair phenotype is present.
**Fig. S9. Structure and activity of Pan-VP16**

(A) schematic showing the structure of the pan-VP16 construct. The full-length pan coding sequence (blue) was synthesised with a sequence encoding the activation domain of herpes simplex VP16 (purple). 3xHA tags (orange) were introduced between the two sequences, with sequences encoding GGGGS linkers at either end (pink). The sequence was flanked by 5’ KpnI and 3’ XbaI. (A’) Abbreviated primary sequence of the Pan-VP16 construct is presented. The sequence colours

