The role of the small GTP-binding protein Arf6 during Drosophila development

Julien Marcetteau

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Le rôle de la petite protéine G Arf6 dans le développement de la *Drosophile*

Julien MARCETTEAU

Institut de Biologie Valrose

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Dirigée par : Dr. Pascal Thérond
Co-dirigée par : Dr. Frédéric Luton
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Devant le jury, composé de :
Roland le Borgne, DR, IGDR, Université de Rennes, Président
Sarah Bray, Professeur, PDN, University of Cambridge, Rapporteur
Jean-Paul Vincent, DR, The Francis Crick Institute, Rapporteur
Pascal Thérond, DR, iBV, Université Côte d'Azur directeur de thèse
Frédéric Luton, DR, IPMC, Université Côte d'Azur co-directeur de thèse

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Le rôle de la petite protéine G Arf6 dans le développement de la *Drosophile*

Jury :

Président du jury*

Roland le Borgne, DR, Institut Génétique et Développment de Rennes (IGDR), Université de Rennes

Rapporteurs

Sarah Bray, Professeur, Department of Physiology, Development and Neuroscience (PDN), University of Cambridge, Rapporteur

Jean-Paul Vincent, DR, The Francis Crick Institute, Rapporteur

Thesis summary

The Wnt and Notch signalling pathways represent two of the core pathways critical to the determination of cell fates during the development of all metazoa. Many conserved components and mechanisms of these pathways have been described through work carried out using the *Drosophila* wing model. The precise patterning of the wing margin is achieved through the concerted and sequential activity of the Notch (N) and Wingless (Wg) signalling, and defects in either pathway leads to characteristic phenotypes. The small GTP binding protein Arf6 has previously been suggested to act in upstream steps in Wnt signalling through *in vitro* studies, but the developmental, *in vivo* relevance of these findings has not been assessed.

This thesis addresses the physiological role of Arf6 in Wingless signalling using the Drosophila wing model. I describe a dominant phenotype present in the wing margins of *Arf6* mutants, and show that Arf6 acts cell autonomously in the transduction of the Wg signalling pathway, specifically for the activation of high-level signalling. Arf6 mutant wings are characterised by a loss of wing margin bristles throughout the wing margin, and a corresponding loss of the Senseless-positive proneural clusters that flank the prospective wing margin. Through a series of epistasis experiments, I demonstrate that the Arf6 phenotype is independent of the β-catenin destruction complex, and that Arf6 is required downstream of Armadillo (Arm) stabilisation, at the level of enhanceosome activity. The ability of N-terminally truncated, stabilised Arm to induce *sens* expression and ectopic bristles is dominantly suppressed in Arf6 mutant wings, and it is unable to rescue the loss of bristles in the wing margin. I generated a constitutively activated form of the Pangolin (TCF/LEF orthologue) and find that it is able to rescue the Arf6 phenotype. I find that Arf6 likely regulates downstream processes in Wg signalling, at least in part, through the kinesin-like protein Pavarotti. I also identify a novel, putative role for Arf6 in Notch signalling that is broadly independent of its role in Wg signalling.

My findings establish new framework for the regulation of Wg signal transduction by Arf6 and provide an insight into the processes mediating high level Wg signalling during developmental patterning.

Key words

Drosophila, signalling, Wnt, Wingless, Arf6, Armadillo, Pangolin, Notch, β-catenin

Résumé de la thèse

Les voies de signalisation Wnt et Notch représentent deux des principales voies essentielles à la détermination des destins cellulaires au cours du développement de tous les métazoaires. De nombreux composants et mécanismes conservés de ces voies ont été décrits grâce aux travaux effectués sur l'aile de la *Drosophile*. Le motif précis de la marge de l'aile est établi à travers l'activité synergique et séquentielle de la signalisation Notch (N) et Wingless (Wg), et des défauts dans l'une ou l'autre de ces voies entraînent des phénotypes caractéristiques. Il a déjà été suggéré que la petite protéine G, Arf6, agit en amont dans la signalisation Wnt *in vitro*, mais la pertinence *in vivo* de ces résultats n'a pas encore été évaluée.

Cette thèse aborde le rôle physiologique de Arf6 dans la signalisation Wg en utilisant le modèle d'aile de la *Drosophile*. Je décris un phénotype dominant présent dans les marges de l'aile des mutants *Arf6*, et je montre que Arf6 agit de manière autonome dans la transduction de la voie de signalisation Wg, spécifiquement pour l'activation de la signalisation de haut niveau. Les ailes des mutants *Arf6* sont caractérisées par une perte des soies de la marge alaire dans toute la marge alaire, et une perte correspondante des cellules proneurales exprimant *senseless*, des deux côtés de la marge alaire. À travers une série d'expériences d'épistasie, je démontre que le phénotype Arf6 est indépendant du complexe de destruction de la β-caténine, et que Arf6 est nécessaire en aval de la stabilisation d'Armadillo (Arm), au niveau de l'activité de l'enhanceosome. La capacité de Arm stabilisé et tronqué au niveau de son extrémité N-terminale, à induire l'expression de *sens* et la formation de soies ectopiques est supprimée de manière dominante dans les ailes des mutants *Arf6*, et Arm stabilisé n'est pas capable de sauver la perte de soies dans la marge de l'aile. J'ai généré une forme constitutivement activée de Pangolin (orthologue de TCF/LEF) et j'ai constaté qu'elle est capable de sauver le phénotype *Arf6*. J'ai découvert que Arf6 régule la signalisation Wg, au moins partiellement, via Pavarotti, une protéine de la famille des kinésines. J'ai identifié également un nouveau rôle putatif pour Arf6 dans la signalisation Notch, qui est largement indépendant de son rôle dans la signalisation Wg.

Mes résultats établissent un nouveau cadre pour la régulation de la transduction du signal Wg par Arf6 et fournissent un aperçu des processus de médiation de la signalisation de haut niveau au cours du développement.

Mots Clés

Drosophile, signalisation, Wnt, Wingless, Arf6, Armadillo, Pangolin, Notch, β-catenin

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List of Abbreviations

	A
Ac	Achaete
ACAP1	ArfGAP With Coiled-Coil, Ankyrin Repeat And PH Domains 1
ADAM	A disintegrin and metalloproteinase
Ap	Apterous
AP1/2	Adaptor protein 1/2
APC	Adenomatous polyposis coli
Arf	ADP-ribosylation factor
Arfip	Arfaptin
ARH-GAP10	Rho GTPase-activating protein 10
Arm	Armadillo
ARNO	ARF nucleotide-binding site opener
Arr	Arrow
AS-C	Achaete-scute complex
ASAP	ArfGAP With SH3 Domain, Ankyrin Repeat And PH Domain
awd	Abnormal wing discs
	B
bHLH	Basic helix-loop-helix
BIG	Brefeldin A-inhibited guanine nucleotide exchange protein family
BIO	6-bromoindirubin-30-oxime
BRAG	Brefeldin Resistant Arf GEFs
BSA	Bovine serum albumin
	C
Cindr	CIN85 and CD2AP related
CK1	Casein kinase 1
CK2	Casein kinase 2
CM	Conditioned medium
CSL	*C*BF-1/Suppressor of Hairless/Lag-1
Ct	Cut
	D
D/V	Dorsal/ventral
DKK-1	Dickkopf 1
Dl	Delta
DNA	Deoxyribonucleic acid
Dsh	Dishevelled
Dvl	Dishevelled segment polarity protein
	E
E(spl)	Enhancer of split
E(spl)-C	Enhancer of split complex
EDTA	Ethylenediaminetetraacetic acid
EFA6	Exchange factor for Arf6
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinases
	F

FilGAP	Filamin-A-associated RhoGAP
FIP	Rab11 family-interacting protein
Fng	Fringe
FRET	Fluorescence resonance energy transfer
Fz2	Frizzled2
FZD4	Frizzled class receptor 4
	G
GAP	GTPase activating protein
Garz	Gartenzwerg
GBF1	Brefeldin A-resistance factor 1
GDP	Guanosine diphosphate
GEF	Guanine exchange factor
GEP100	Guanine nucleotide exchange protein 100
GFP	Green fluorescent protein
GGA3	Golgi-localised γ-ear containing, Arf binding protein 3
GGAs	Golgi-localised gamma ear-containing Arf-binding proteins
Git1	G Protein-Coupled Receptor Kinase Interacting ArfGAP 1
Gro	Groucho
GSK3	Glycogen synthase kinase 3
GST	Glutathione *S*-transferases
GTP	Guanosine-5'-triphosphate
	H
HA	Hemagglutinin
HGF	Hepatocyte growth factor
HLH	Helix-loop-helix
Hrs	Hepatocyte growth factor regulated tyrosine kinase substrate
	J
JIP	c-Jun N-terminal kinase (JNK)-interacting protein
	K
KO	Knock-out
	L
L	Larval instar
l'sc	Lethal of Scute
LEF	Lymphoid enhancer factor
Lrp6	Low density lipoprotein receptor-related protein 6
	M
Mam	Mastermind
MEFs	Mouse embryonic fibroblasts
MKLP1	Mammalian kinesin-like protein 1
MT-2	Metallothieonein-2
	N
N	Notch
NaCl	Sodium chloride
NECD	Notch extracellular domain
NEXT	Notch external truncation
NICD	Notch intracellular domain
	P

Pan	Pangolin
PBS	Paxillin binding site
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Phosphatidic acid
PH domain	Pleckstrin homology domain
Phospho	Phosphorylated
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP5K	Phosphatidylinositol 4-phosphate 5-kinase
PLC	Phospholipase C
PLD	Phospholipase D
PNC	Proneural cluster
PtdIns	Phosphatidylinositol
	R
RFP	Red fluorescent protein
Rho	Ras homology family of GTPases
Rho-GAPs	Rho-GTPase activating proteins
RNA	Ribonucleic acid
RNAi	RNA interference
	S
Sc	Scute
Ser	Serrate
Sgg	Shaggy
SHD	Spa-homology domain
shRNA	Short hairpin RNA
Siz	Schizo
Slimb	Supernumerary limbs
SOP	Sensory organ precursor
Spdo	Sanpodo
Step	steppke
Su(H)	Suppressor of hairless
	T
Tb	Tubby
TCF	T cell factor
TLE	Transducin-like Enhancer of split
TNotch	Membrane-tethered form of Notch lacking the NECD
TrCP	Transducin Repeat Containing E3 Ubiquitin Protein Ligase
TRITC	Tetramethylrhodamine
Tum	Tumbleweed
	U
UAS	Upstream activating sequence
	W
Wg	Wingless
WID	Wing imaginal disc
WRE	Wingless responsive element
WT	Wild-type

Introduction

The main objective of my thesis was to study the requirement for the small GTP-binding protein Arf6 and its regulators during development using a *Drosophila* model. My initial observations revealed a dominant defect in the patterning of the wing margins of adult *Arf6* mutant flies. *Drosophila* wing development is a complex, dynamic process orchestrated by the tightly regulated activity of numerous conserved signalling pathways that act synergistically to achieve the precise morphology and patterning of the wing. Two main signalling pathways are responsible for the establishment and patterning of the *Drosophila* wing margin: Notch and Wingless. I have therefore started by broadly introducing these two pathways. Although Notch, Wingless, and their mammalian counterparts, are involved in innumerable processes, my introduction is focussed on their roles in wing patterning.

In order to contextualise the potential roles of Arf6 in Wingless signalling, I have summarised the established links between Arf6 Wnt signalling. There are, to my knowledge, no studies linking Arf6 to the Notch signalling pathway. I have drawn upon the known Arf6 effectors, and mechanisms through which Arf6 has been shown to act to provide speculative insights into the processes that could link Arf6 to N signalling.

Wingless signalling

Wingless (Wg) is a conserved member of the Wnt family of secreted proteins that act as paracrine signalling molecules in a diverse range of developmental processes (Steinhart and Angers, 2018; Swarup and Verheyen, 2012; van Amerongen and Nusse, 2009). Canonical Wg signalling centres around the stability and localisation of the core transducer of the pathway, Armadillo (Arm), a β-catenin homologue. In the absence of Wg, the level of cytoplasmic Arm is suppressed through the activity of the β-catenin destruction complex (Stamos and Weis, 2013) (Figure 1) consisting of the serine/threonine kinases Shaggy (Sgg, a glycogen synthase kinase 3 GSK3 homologue) and Casein kinase 1 (CK1), and the scaffolds Axin and APC (Stamos and Weis, 2013). Cytoplasmic Arm is constitutively recruited to the destruction complex by Axin, where it is phosphorylated by GSK3 and CK1. Phosphorylated Arm is then transferred from the destruction complex to the β-TrCP/Slimb E3 ubiquitin ligase by APC for proteasomal degradation (Su et al., 2008). In the absence of Arm, transcriptional targets of Wg signalling are maintained in a repressed state through the activity of the TCF homologue, Pangolin (Pan) and the TLE homologue Groucho (Gro) (Cavallo et al., 1998; Turki-Judeh and Courey, 2012). Mutating the Sgg phosphorylation sites in Arm generates a form of Arm that

evades phosphorylation and degradation by the destruction complex, and that constitutively activates Wg signalling (Pai et al., 1997).

The binding of Wg to the Frizzled2 (Fz2) receptor and LRP6 homologue Arrow (Arr) co-receptor at the cell membrane triggers the formation of a Fz2/Arr heterodimer, and the polymerisation of Dishevelled (Dsh), increasing its affinity for Axin (Bienz, 2014; Bilic et al., 2007) (Figure 1). The destruction complex is recruited to the Fz2/Arr heterodimer along with Axin, where Sgg phosphorylates the intracellular tail of Arr (Tamai et al., 2004; Zeng et al., 2008b). The phosphorylated cytoplasmic tail of Arr is thought to inhibit Sgg kinase activity, therefore suppressing the destruction complex (Stamos et al., 2014). The complex formed by the recruitment and deactivation of the destruction complex is known as the signalosome (Gammons and Bienz, 2018). The deactivation of the destruction complex alleviates the suppression of Arm, enabling the accumulation of cytoplasmic Arm. Arm then translocates to the nucleus where it acts as a transcriptional co-activator to Pan, initiating the transcription of Wg target genes (Brunner et al., 1997; Peifer et al., 1994). The mechanism through which Arm enters the nucleus is still not fully understood (Fagotto, 2013).

Wg has been described to behave as a morphogen during Drosophila wing development, inducing the expression of disparate target genes in response to the local concentration of the Wg (Struhl and Basler, 1993; Zecca et al., 1996). Although the requirement of a Wg gradient during wing development remains a point of contention (Alexandre et al., 2013; Chaudhary et al., 2019; Martinez Arias, 2003), it is apparent that there is a difference in the sensitivity of Wg target genes to the level of Wg transduction (Baena-Lopez et al., 2009; Song et al., 2009; Zecca et al., 1996). In the developing wing, high level Wg signalling is associated with the induction of *senseless (sens)* expression, while lower-level signalling leads to the expression of low threshold targets such as *distal-less (dll)* and *vestigial (vg)* (Vincent, 2014).

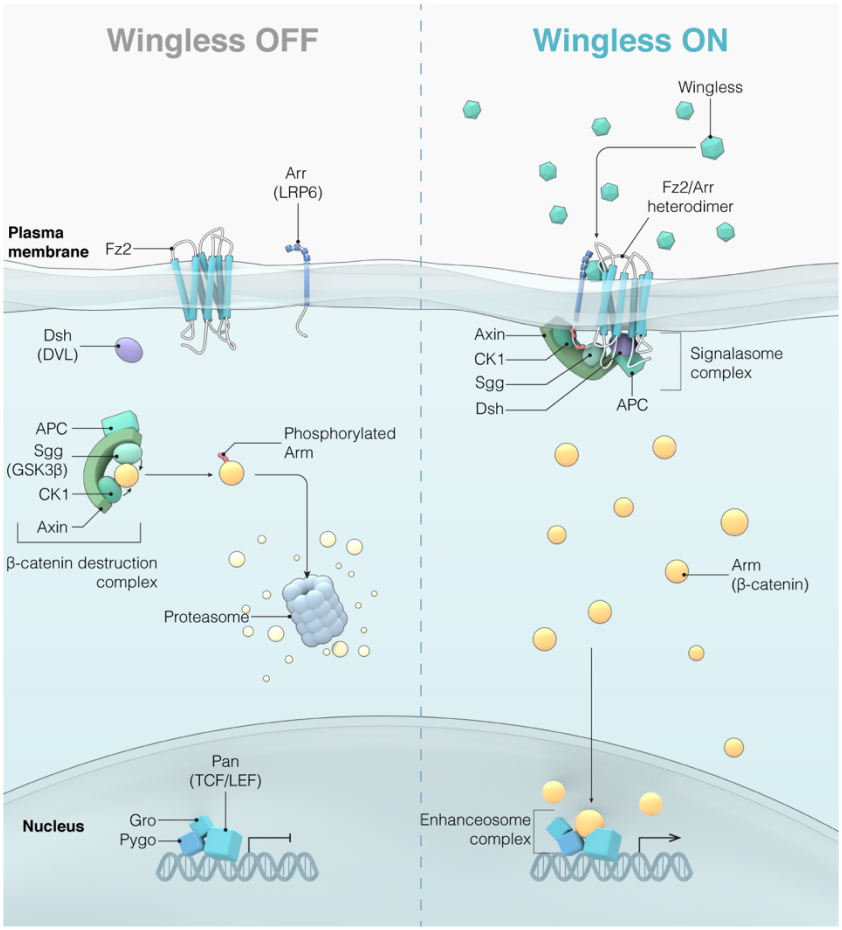

Figure 1 Overview of the Wingless signalling pathway

In the absence of the Wg ligand (Wingless OFF), Arm is constitutively degraded through the activity of β-catenin destruction complex. Wg binds to the Fz2 receptor and Arr co-receptor, leading to the deactivation of the destruction complex and formation of the signalosome complex at the plasma membrane. Stabilised Arm accumulates in the cytoplasm and enters the nucleus, switching Pangolin (Pan) from a transcriptional repressor to a transcriptional activator (as part of the enhanceosome complex), and triggering the expression of Wg target genes.

Notch signalling

Notch is a type 1 transmembrane receptor that is activated in a juxtacrine manner by its membrane-bound ligands, Delta (Dl) and Serrate (Ser) which are present at the membrane of neighbouring cells (Fleming, 1998). The notch receptor comprises a covalently bound heterodimer of the membrane tethered intracellular domain, and the Notch extracellular domain (NECD) (Sanchez-Irizarry et al., 2004) (Figure 2). N signalling is initiated by the binding of the extracellular domains of Dl or Ser to N on the surface of a neighbouring cell. This binding event exposes an ADAM protease cleavage site in the extracellular domain of N, allowing the ADAM metalloproteinase (Kuzbanian in *Drosophila*) mediated cleavage of the extracellular N domain (Lieber, 2002). The N ectodomain is

trans-endocytosed alongside the receptor into the signal-sending cell (Fortini and Bilder, 2009; Parks et al., 2000). The requirement for ligand trans-endocytosis is incompletely understood, however it is likely to induce a mechanical force on the N receptor, revealing the S2 cleavage site (Musse et al., 2012). The remaining transmembrane and intracellular domain (NEXT), forms the substrate for γ-secretase mediated cleavage, releasing the Notch intracellular domain (NICD) from the membrane. NICD translocates to the nucleus, where it binds to the CSL family protein, suppressor of hairless (Su(H)) and Mastermind (Mam), forming the N activation complex target gene transcription (Kovall, 2008). During *Drosophila* wing development, canonical N signalling is responsible for regulating the expression of *wg, cut* and genes of the enhancer of split complex (E(spl)C) (De Celis et al., 1996; Micchelli et al., 1997; Rulifson and Blair, 1995).

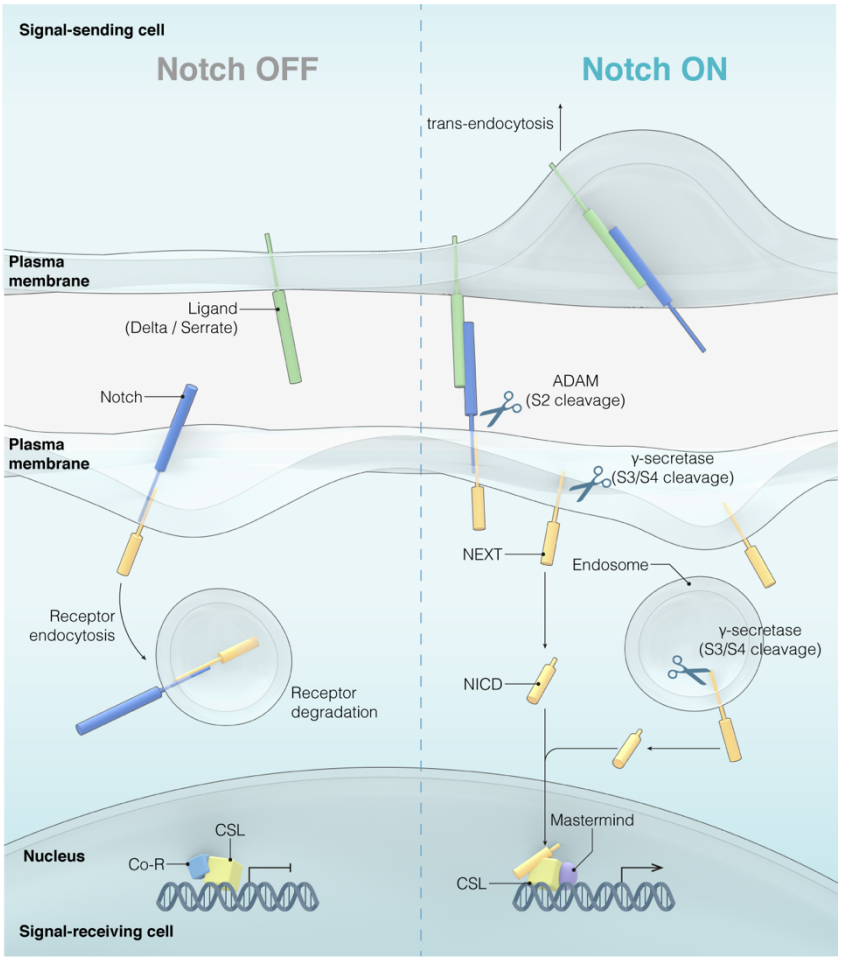

Figure 2 Overview of the core Notch signalling pathway

In the absence of N activation (Notch OFF), the N receptor (Blue and yellow) undergoes rapid turnover at the plasma membrane to tightly control its levels. Target genes are suppressed by a complex of the CSL family proteins (Su(H) in *Drosophila*) and co-repressors (Co-R). Notch activation (Notch ON) occurs when a ligand presented at the membrane of a neighbouring cell (Green, Delta/Serrate in *Drosophila*) binds to the extracellular N domain. The N extracellular domain is cleaved by ADAM proteases (S2 cleavage) and transendocytosed with the ligand. The activated, membrane-bound receptor (NEXT) undergoes a second, ɣ-secretase mediated S3 cleavage, releasing the Notch intracellular domain (NICD). S3 cleavage can take place at the plasma membrane or endosomal membranes. NICD translocates to the nucleus where it interacts with Su(H), and Mastermind, activating target gene transcription.

Development of the *Drosophila* wing margin

Establishment of the wing dorsoventral boundary

The developmental precursors to *Drosophila* adult appendages are known as imaginal discs and originate from small clusters of cells set aside during embryonic development. The imaginal discs

undergo extensive growth and morphological changes during larval development, and it is during this period that the prepattern to the adult structures is established (Modolell and Campuzano, 1998).

The wing imaginal disc (WID) is broadly divided into the anterior, posterior, dorsal and ventral compartments (Garcia-Bellido et al., 1973). The selector gene *apterous (ap)* confers dorsal identity to the cells of the dorsal compartment of the wing (Cohen et al., 1992; Diaz-Benjumea, 1993). The interactions between the dorsal and ventral cells at the D/V boundary are mediated by N signalling: Ap induces the expression of a glycosyltransferase *fringe* (*fng*) and the N ligand *Serrate (Ser)* in the cells of the dorsal compartment of the wing during L2 development (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Irvine and Wieschaus, 1994). Fng glycosylates N, reducing its sensitivity to Ser, whilst increasing its propensity to be to activated by Dl (Blair, 2000; Fleming et al., 1997; Panin et al., 1997). The presence of both Fng and Ser in the dorsal cells prevents Ser from activating N signalling in these cells, therefore limiting N activation by Ser to the ventral boundary cells. Conversely, the increased sensitivity of the N in dorsal cells to trans-activation by Dl enables N signalling activation in the dorsal margin cells by Dl presented by ventral cells (Blair, 2000) (Figure 3).

The activation of N signalling at the D/V leads to the upregulation of *wg* in the presumptive wing margin. During late L3 development, Wg non-autonomously upregulates the expression of both Dl and Ser in the cells flanking the margin, maintaining N activation and therefore *wg* expression at the wing margin in a feedback loop (De Celis and Bray, 1997; Micchelli et al., 1997) (Figure 3). The Dl and Ser present at the membrane of the cells flanking the wing margin bind to N in the same membrane, cell-autonomously suppressing N activation through the process of cis-inhibition (Becam et al., 2010; De Celis and Bray, 1997; Micchelli et al., 1997). This helps restrict *wg* expression to the margin cells and maintain the integrity of the wing margin (De Celis and Bray, 1997; Micchelli et al., 1997; Rulifson et al., 1996).

N signalling at the D/V also induces the expression of the selector gene *cut*, that is expressed in a narrow strip of cells overlapping the D/V boundary (Micchelli et al., 1997). Cut suppresses Wg signalling activity in the margin cells, preventing autocrine Wg activation, and the induction of *Dl* and *Ser* expression in the margin cells (Micchelli et al., 1997). Cut is therefore necessary to maintain *wg* expression and N activity at the D/V boundary (Buceta et al., 2007; De Celis and Bray, 1997; Micchelli et al., 1997).

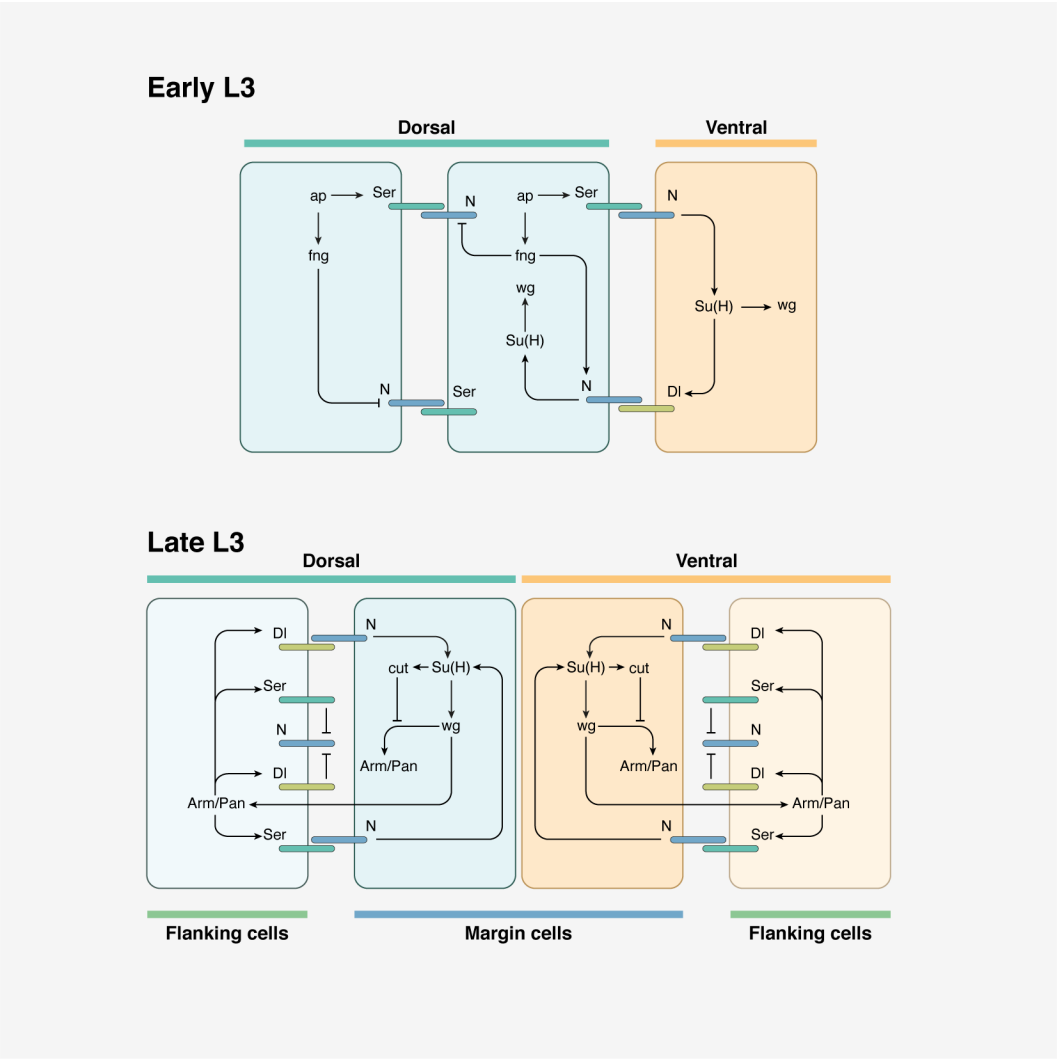

Figure 3 Establishment of the dorsoventral boundary by Notch and Wingless signalling

A simplified scheme outlining the establishment of the wing dorsoventral boundary. During **early L3**, Apterous (Ap) in the dorsal compartment induces *Ser* and *fng* expression. Fng glycosylates N, reducing its ability to be activated by Ser from other dorsal cells. Glycosylated N is more sensitive to activation by Dl from ventral cells. These interactions lead to N activation in the margin cells, triggering the upregulation of *wg* expression. In **late L3,** *wg* and *cut* expression are maintained in the margin cells by N signalling. Cut cell-autonomously blocks Wg signalling, preventing *Dl* and *Ser* expression in margin cells. Wg from the margin drives *Dl* and *Ser* expression in both the dorsal and ventral flanking cells. Dl and Ser cell autonomously inhibit N through cis inhibition, preventing N signalling outside the wing margin cells. Figure based on De Celis and Bray 1997.

Wing margin patterning by Wingless signalling

The margin of the *Drosophila* wing is lined with a regular pattern of innervated bristles in the anterior wing margin and non-innervated bristles in the posterior margin (Hartenstein and Posakony, 1989; Murray et al., 1984) (Figure 4). The innervated bristles in the anterior wing margin represent transducers in the peripheral nervous system, responding to mechanical and chemical stimuli to allow the fly to sense its environment (Hartenstein and Posakony, 1989). The sensory organ precursors (SOPs) arise from clusters of undifferentiated cells, known as proneural clusters (PNCs) that acquire neural potential through the expression of proneural proteins (Cubas et al., 1991; Skeath and Carroll,

1991) (Figure 4A and A'). The proneural proteins are basic helix-loop-helix (bHLH) transcription factors, and can be divided into the Atonal (Atonal and Amos) and the Achaete-scute complex (AS-C) (Achaete, Ac, Scute, Sc, and Lethal of Scute l'sc) families (Lai and Orgogozo, 2004). The proneural proteins form active heterodimers with the class 1 bHLH protein Daughterless, and induce the expression of genes required in neural development (Murre et al., 1989; Reeves and Posakony, 2005).

During the third larval instar (L3), development cells flanking the D/V boundary in the anterior compartment express *ac* and *sc* in response to high level Wg signalling (Couso and Arias, 1994; Phillips and Whittle, 1993). *ac* and *sc* are expressed in the PNCs and SOPs of the slender mechanosensory bristles in the ventral, and recurve chemosensory bristles in the dorsal compartment of the wing margin (Blair, 1992; Couso et al., 1994) (Figure 4B and B'). Flies carrying a deletion of the full AS-C do not form the majority of the external sensory organs throughout the body, including the recurve chemosensory bristles in the wing margin (Jafar-Nejad et al., 2003). Despite the extensive bristle loss, the numbers of stout mechanosensory and non-innervated bristles in the wing margin are only mildly reduced (Garcia-Bellido, 1979; Jack et al., 1991; Jafar-Nejad et al., 2006). This phenomenon led to the discovery that Ac and Sc do not act as the proneural factors for the stout mechanosensory and non-innervated margin bristles; instead Sens plays the role of the proneural factor in this context (Jafar-Nejad et al., 2006). Sens is present in the Ac/Sc positive cells flanking the anterior wing margin, and similar to the *ac* and *sc,* is expressed in response to high level Wg signalling (Jafar-Nejad et al., 2006). In contrast to *ac* and *sc, sens* expression is not restricted to the anterior wing margin, and the two stripes of *sens* expression extend into the posterior compartment of the wing (Jafar-Nejad et al., 2003; Jafar-Nejad et al., 2006) (Figure 4A and A'). Sens therefore plays two independent roles in the patterning of the wing margin, acting both downstream of Ac/Sc to promote bristle development and survival, and independently of Ac/Sc as a bona fide proneural factor in conjunction with Da (Jafar-Nejad et al., 2003; Jafar-Nejad et al., 2006).

Although the margin bristles are morphologically and functionally homologous to the bristles of the mesonotum, they represent exceptions in the established programs of bristle development. The wing margin is one of the few contexts in which *sens* expression is not directly expressed downstream of helix (bHLH) transcription factors (Jafar-Nejad et al., 2006). Similar to the deletion of AS-C, mutant clones for *sens* lack external sensory organs (Nolo et al., 2000). However, the loss of thoracic sensory organs in *sens* mutants is not indicative of a loss of Sens proneural activity, but is instead a result of the requirement of Sens in the SOP developmental program (Jafar-Nejad et al., 2003; Nolo et al., 2000; Reeves and Posakony, 2005). The loss of bristles is a result of a transformation of the pIIa to pIIb , resulting in the formation of supernumerary neuron and sheath cells at the expense

of the external bristle components, the shaft and socket (Jafar-Nejad et al., 2003; Jafar-Nejad et al., 2006).

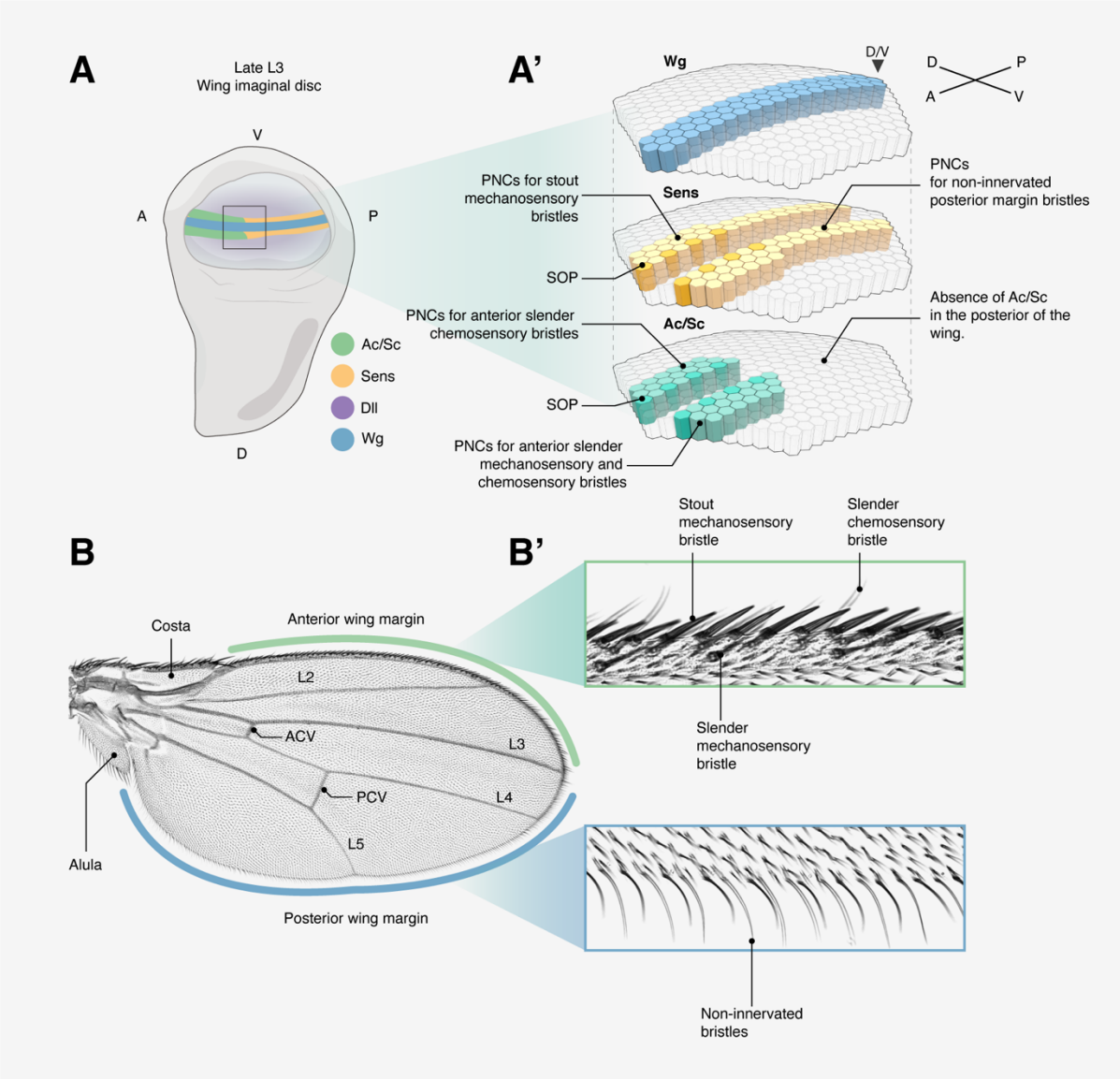

Figure 4 Patterning of the wing margin

Overview of the patterning of the wing margin by Wg signalling. **A** the pattern of Wingless (Wg), Achaete/Scute (Ac/Sc) Senseless (Sens) and Distal-less (Dll) in the L3 wing imaginal disc. **A'** a schematic representation of the boxed region showing details of the proneural clusters (PNCs) and sensory organ precursors (SOPs) for the different classes of bristles found in the adult wing margin (labelled in C'). All SOPs express *sens,* however Sens only plays a proneural role for the mechanosensory and non-innervated bristles (note that ac/sc are not expressed in the posterior wing margin). **C** overview of the morphology of the adult wing. Anterior is towards the top and posterior the bottom, distal is right. The longitudinal wing veins are labelled (L2-L5), as are the anterior and posterior wing margins. Anterior and posterior crossveins are shown (ACV and PCV respectively). **C'** details of the anterior and posterior wing margins are presented. The wing margin bristles are labelled.

There is a complex relationship between Sens and the bHLH transcription factors during sensory organ development. As previously mentioned, *sens* expression is typically directly

downstream of bHLH factors. In turn, high levels of Sens can physically interact with, and promote the activity of the bHLH transcription factors (Acar, 2006). Co-expression of *sens* with *AS-C* genes induces a strong, synergistic increase in the formation of sensory organs (Acar, 2006; Jafar-Nejad et al., 2003; Jafar-Nejad et al., 2006). Conversely, low levels of Sens antagonise the activity of the bHLH proteins (Jafar-Nejad et al., 2003). The bimodal activity of Sens is thought to result from Sens having a greater affinity for DNA binding than binding to AS-C proteins: at low levels of AS-C expression, Sens will directly bind to regulatory elements within the loci of proneural genes, repressing their expression, and potentially acting in tandem with E(spl)-C proteins (Jafar-Nejad et al., 2003). When the proneural bHLH proteins are present at higher concentrations, a greater proportion of Sens will be bound to bHLH proteins, increasing their activity (Acar, 2006; Jafar-Nejad et al., 2003). This phenomenon could help to explain the sensitivity of the wing margin bristles to defects in Wg signalling: the reduced levels of *sens* expression would not only be insufficient for the development of the stout mechanosensory and non-innervated bristles, but could also lead to the suppression of AS-C expression and activity, exacerbating the loss of bristles. This is supported by the finding that inducing moderate levels of Wg signalling do not fully rescue wing patterning in *wg* mutants (Baena-Lopez et al., 2009).

The proneural gene expressing cells of the PNC represent an equivalence group, with each cell holding the potential to become the SOP (Bertrand et al., 2002). SOPs are selected from within the equivalence group through N mediated lateral inhibition (Simpson, 1990). During lateral inhibition, the cells with the highest concentration of proneural protein upregulate *Dl* expression, through which they induce N signalling in their neighbours (Bray, 1998; Sjöqvist and Andersson, 2019)(Figure 5). The increased N signalling suppresses the expression of the proneural genes through the upregulation of genes from *enhancer of split complex (E(spl)-C)*. The reduced proneural gene expression also results in a reduction in *Dl* expression, which in turn increases the cell's propensity to be activated for N signalling, thus amplifying the differences in N responsiveness between the cells and locking in the SOP fate. In addition to being positively regulated by the proneural transcription factors, *sens* expression is also repressed by the E(spl)C proteins (Jafar-Nejad et al., 2003; Nolo et al., 2000). The E(spl)-C proteins can also directly interact with Sens protein, suppressing its proneural activity (Jafar-Nejad et al., 2003). In turn, high levels of Sens repress E(spl)-C expression, allowing SOPs to escape repression by E(spl)-C.

The SOPs for the chemosensory bristles are already selected by late L3 development, whilst the selection for the SOPs for the stout mechanosensory and non-innervated bristles takes place following soon after pupal formation (Jafar-Nejad et al., 2006). At this stage, *cut* begins to be expressed in the SOPs of the wing margin bristles, both in the anterior and posterior wing margins,

and its expression is maintained until the bristles are fully differentiated (Jack et al., 1991). As previously discussed, Cut is required to maintain the integrity of the D/V margin where it is expressed in response to N signalling (Buceta et al., 2007; Micchelli et al., 1997). The loss of function of *cut* leads to a compound phenotype, manifesting in notching of the wing margin and a loss of margin bristles (Jack et al., 1991; Krupp et al., 2005). Interestingly, only the wing notching phenotype is rescued when *wg* is re-expressed in *cut* mutants (consistent with the requirement for Cut to maintain *wg* expression), indicating that Cut has an independent role in wing margin bristle development (Krupp et al., 2005).

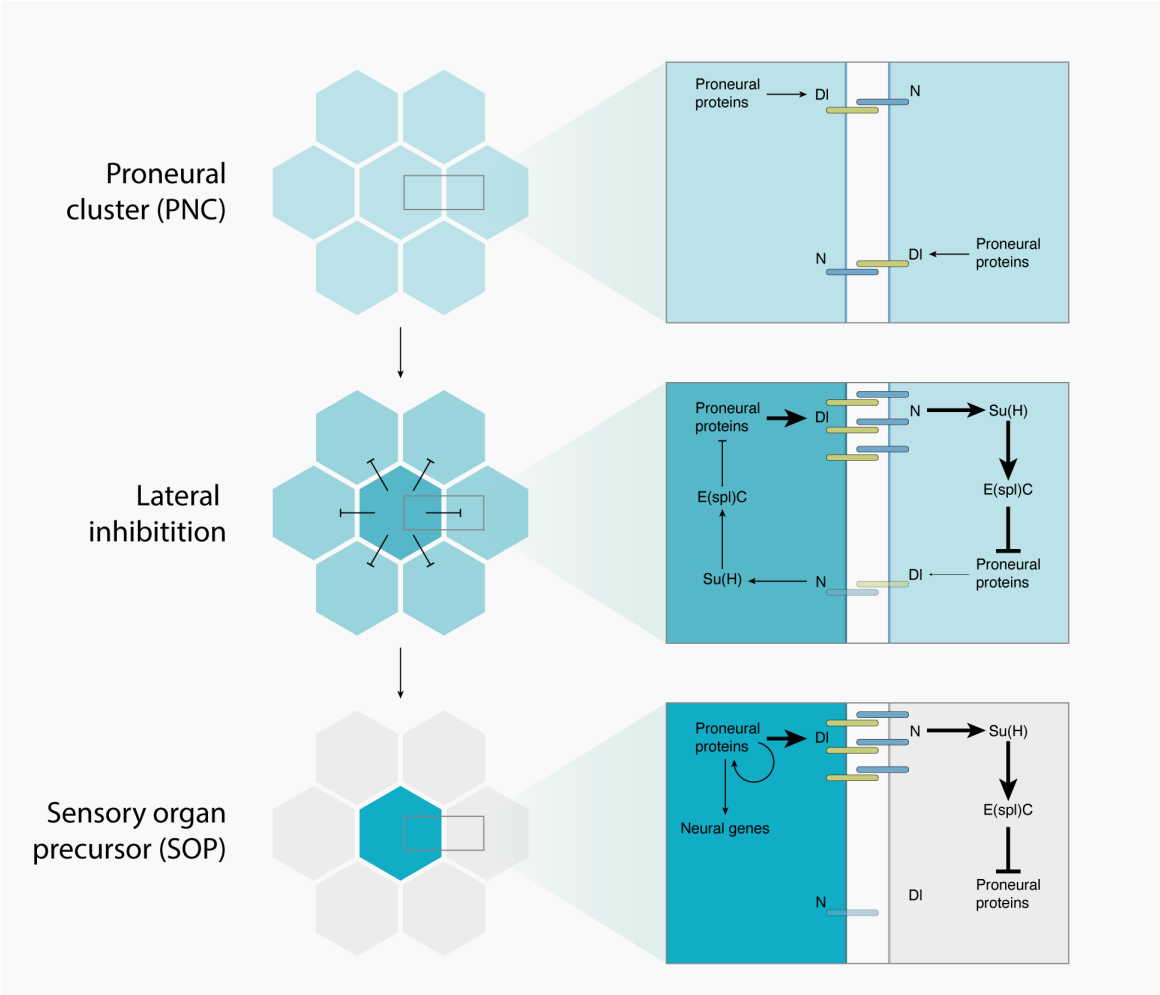

Figure 5 SOP selection through lateral inhibition

Clusters of cells expressing low levels of pro-neural genes represent an equivalence group known as proneural clusters (PNC). Neural potential becomes restricted to a single sensory organ precursor (SOP) through the process of lateral inhibition. During lateral inhibition, a single cell in the cluster begins expressing higher levels of the proneural genes, inducing an increase in *Dl* expression. Dl triggers the N receptor in the neighbouring cells, leading to an upregulation of genes of the enhancer of split gene complex (E(spl)C). E(spl)C proteins repress the expression of proneural factors, suppressing neural fate. The high level of proneural gene expression induces the expression of the genes necessary for neural development (neural genes). The proneural proteins auto-regulate and can maintain

Bristle development

Following the selection of SOPs from within the PNCs, the SOPs undergo a series of stereotyped asymmetric cell divisions, generating the neuron, sheath, shaft and socket that constitute the mature sensory organ (Schweisguth, 2015) (Figure 6). The decision between pIIa and pIIb is mediated through N signalling, with N being active in the pIIa and inactive in the pIIb (Rhyu et al., 1994). The asymmetry in N activity is achieved through the asymmetrical localisation of N regulators along the axis of cell division, resulting in their unequal inheritance by the daughter cells. Numb, a suppressor of N signalling, was the first N regulator identified to be asymmetrically partitioned during SOP division (Rhyu et al., 1994). Although the mechanism through which Numb inhibits N signalling is not fully understood, Numb inhibits N signalling in the pIIb, probably through the regulation of N trafficking and Sanpodo recycling (Spdo, a Notch agonist), reducing the level, and therefore activity, of N at the plasma membrane (Cotton et al., 2013; Couturier et al., 2013). The pIIb cell also inherits the E3 ubiquitin ligase Neuralized (Neur) that ubiquitinates Dl, promoting Dl endocytosis, a step necessary for Dl activity (Musse et al., 2012). Together, Numb and Neur therefore suppress N signalling in the pIIb, while increasing its ability to transactivate N in the neighbouring pIIa.

The pIIa undergoes a second division, giving rise to the accessory cells, consisting of the external components of the sensory organ, the shaft and socket (Schweisguth, 2015). The pIIb also undergoes an additional division, generating a glial cell and the pIIIb. The pIIIb divides a final time, giving rise to the sheath and neuron, while the glial cell undergoes apoptosis, leaving only the shaft, socket, neuron, and sheath (Fichelson and Gho, 2003; Gho et al., 1999).

Although the non-innervated bristles in the posterior compartment are morphologically similar to the slender mechanosensory bristles in the anterior wing margin, they lack a socket, sheath and neuron (Hartenstein and Posakony, 1989). The neural potential of the posterior bristles is not met due to the selective apoptosis of the neurons or their precursors (Blair, 1992). Blocking apoptosis in the wing margin leads to the formation of ectopic neurons in the posterior (Jafar-Nejad et al., 2006). The essential role of N during the fate determinations in the bristle precursors means that both N gain- and loss-of-function phenotypes lead to defects in sensory organ differentiation (Kopan, 1999). Loss of N signalling activity induces the transformation of the pIIa to pIIb, while an increase in N activity transforms the pIIb to pIIa (Heitzler and Simpson, 1991). These conditions lead to characteristic defects in bristle development, however the effect of N gain- and loss of function phenotypes can vary depending on the timing and intensity of the gain or loss of signalling (Kopan, 1999).

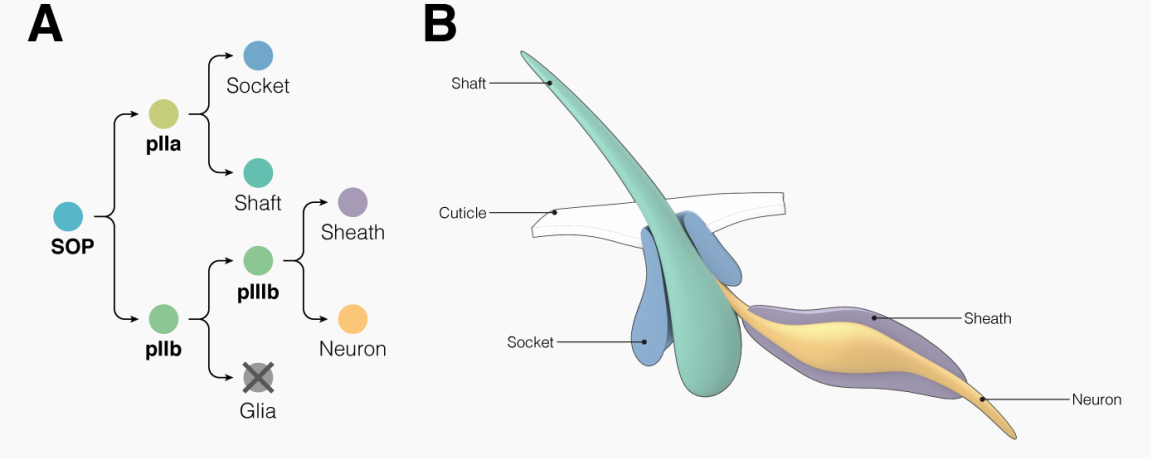

Figure 6 Development of external sensory organs from the sensory organ precursor

A Following its selection, the SOP undergoes two rounds of N-mediated asymmetrical divisions resulting in the formation of the socket, shaft, sheath and neuron that constitute the external sensory organ. Notch is necessary for the selection of the socket and sheath cells. A glial cell is also formed that is subsequently lost through apoptosis. **B** a generalised structure of an adult mechanosensory bristle. The shaft (green) is supported by the socket (blue) and are the two components that are readily visible from the cuticle. The neuron (yellow) is surrounded by sheath cell (purple), and extends its dendrites to the central nervous system.

Convergence of the Wingless and Notch pathways during wing margin development

The hierarchical and repeated requirement for N and Wg signalling during the definition and patterning of the wing margin can lead to phenotypically similar wing defects resulting from perturbations in either pathway, and an increased sensitivity of each pathway to defects in the other (Couso et al., 1994; Gonzalez-Gaitan and Jackle, 1995; Hartenstein and Posakony, 1990; Hing et al., 1994; Phillips and Whittle, 1993). This is particularly apparent later in L3 development when *wg* expression at the D/V is controlled by N signalling, and expression of N ligands in the cells flanking the margin are expressed in response to high level Wg signalling (De Celis et al., 1996; Micchelli et al., 1997; Rulifson and Blair, 1995) (summarised in Figure 3).

There is evidence of a more profound relationship between N and Wg signalling, such that Wg/Wnt and N signalling have been described as forming a single signalling module (Muñoz Descalzo and Martinez Arias, 2012). A non-canonical N signalling pathway has been suggested to act antagonistically to Wg signalling, through which NICD directly binds and represses the activity of Arm (Hayward et al., 2005; Hayward et al., 2006; Hayward et al., 2008). The mechanism through which N is able to suppress Arm activity is not fully understood, but it has been suggested that the complex of NICD and stabilised β-Catenin undergoes lysosomal degradation, independently of the activity of the β-catenin destruction complex (Kwon et al., 2011). This process has been put forward to explain the

similarity between N gain-of-function, and Wg loss-of-function phenotypes in the wing margin (Hayward et al., 2005; Sanders et al., 2009). Although these models have explanatory power, it is extremely difficult to fully disentangle the genetic interactions between N and Wg signalling from potential molecular interaction, as both pathways have complex spatial and temporal activities during wing development.

Arf6 and its regulators

Our findings demonstrate a novel role for the small GTP binding protein Arf6 in the development of the wing margin in response to high level Wg signalling, and a preliminary role for Arf6 in N signalling. To my knowledge, Arf6 has not previously been implicated in wing development, nor in the Wg or N signalling pathways. In the following sections I will provide an overview of Arf6 function and will then discuss what is known regarding the role of Arf6 in the Wnt signalling pathway. Due to the absence of published literature linking N signalling with Arf6 function, I will outline the ways in which Arf6 could be involved in N signalling.

The ADP-ribosylation factors (Arfs) is a family of small GTP binding proteins belonging to the Ras protein superfamily and are central regulators of cell signalling process at multiple levels. The Arfs are involved in a diverse range of cellular processes, including membrane trafficking, modification of membrane lipid composition, and regulation of the actin cytoskeleton (Donaldson and Jackson, 2011). The Arfs are categorised into three subfamilies based on sequence homology: Class I (containing Arf1-3), Class II (containing Arf4-5), and Class III containing the most divergent Arf protein: Arf6. The Arf proteins are involved in regulating intracellular transport through the recruitment of coat proteins for the sorting of cargoes into vesicles, and the modulation of lipid-modifying enzymes (Donaldson and Jackson, 2011). Arf6 is localised at the plasma membrane and endocytic system, where it modulates various steps of endocytosis, exocytosis, recycling, and the organisation of the actin cytoskeleton (D'Souza-Schorey and Chavrier, 2006; Donaldson and Jackson, 2011).

The Arf proteins cycle between an active, GTP-bound, and inactive GDP-bound state (Gillingham and Munro, 2007). The exchange between GDP and GTP is mediated by the guanine exchange factors (GEFs), while the hydrolysis of GTP to GDP is catalysed by the Arf GTPase activating proteins (GAPs) (Figure 7). The Arf-GEFs are essential in not only determining the level of activity of Arfs, but also in modulating the compartmental and temporal specificity of their activity (Donaldson and Jackson, 2000; Jackson and Casanova, 2000). Arf GEFs are characterised by a conserved catalytic Sec7 domain that is sufficient for exchange activity (Chardin et al., 1996; Jackson and Casanova, 2000).

The Sec7 domain acts by physically displacing the GDP from the active site of the Arf, leading to the formation of a nucleotide-free Arf that can once again incorporates GTP (Mouratou et al., 2005). The Arf-GEFs also contain non-catalytic domains, whose architecture and function are less clearly defined than that of Sec7, and that likely contribute to the spatiotemporal activity of the GEFs (Mouratou et al., 2005). The EFA6, Cytohesin and BRAG families contain PH domains that provide them with affinity to specific lipid domains in the plasma membrane. The BRAG and EFA6 PH domains selectively interact with PtdIns(4,5)P_2, while the Cytohesins have high affinity for either PtdIns(3,4,5)P_3, or both PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 depending on the isoform (Jian et al., 2012; Klarlund et al., 2000; Macia et al., 2008). The Cytohesins, BRAG and EFA6 families also have N and C terminal domains. The C-terminal domains mediate the binding with known protein effectors, whilst the function of the N-terminal domains is unknown. Interestingly, the C-terminal domain of EFA6 has been shown to have GEF-independent roles in actin bundling, and in *Drosophila*, an N-terminal tubulin-binding domain has been shown to inhibit microtubule growth (Macia et al., 2019; Qu et al., 2019).

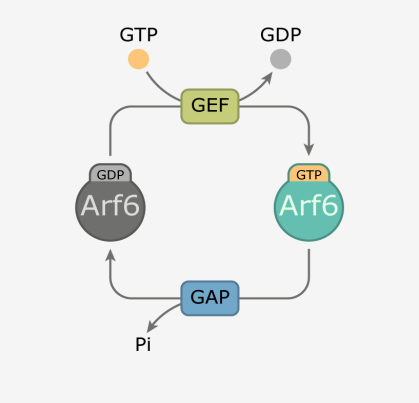

Figure 7 Regulation of Arf6 activity

Arfs undergo cycles of GTP binding, and GTP hydrolysis to GDP, mediated by the GEFs and GAPs respectively. Although the GTP-bound Arf is canonically considered the active form, the cycling between GTP and GDP is an important determinant of Arf activity.

The Arf-GEFs are broadly classified into two classes: the large GEFs (containing GBF1 and BIG families), and the small GEFs (containing the Cytohesin/Arno, Brag/GEP100 and EFA6 families) (Casanova, 2007). The activity of the large Arf-GEFs has been shown to be restricted to class I and II GEFs. Within the small GEFs, the Cytohesins/Arno localise to the Golgi apparatus and the plasma membrane where they predominantly promote exchange for Arf1 (Franco et al., 1998; Macia et al., 2001). The substrate specificity of Brag/GEP100 is not clearly defined; however it has previously been shown that GEP100 (Brag2) can be recruited to the intracellular domain of activated EGFR, and in turn recruits and activates Arf6 in a cellular model of breast cancer invasion (Morishige et al., 2008). The ability of over-expressed GEP100 to increase cellular Arf6-GTP levels was enhanced when treated with

EGF, while Arno/Cytohesin2 and EFA6B increased Arf6-GTP levels independently of the presence of EGF.

EFA6 localises to the plasma membrane and is suggested to act specifically on Arf6 (Franco et al., 1999; Macia et al., 2008). The human genome encodes 15 Arf-GEFs and only 5 Arfs. Although some of the diversity of GEFs can be attributed to tissue specific expression, it also hints at the tight regulation of the Arfs in specific sub-cellular compartments, and in response to different stimuli (Casanova, 2007). The diversity of Arf-GEFs highlights the difficulty in establishing their substrate specificity. This challenge has been approached using *in vitro* assays of GEF activity, and although these studies have provided us with mechanistic insights into GEF activity and regulation, there are limitations to the *in vitro* characterisation of Arf-GEFs that make it difficult to directly translate the findings *in vivo* (Casanova, 2012). The large size of GEF proteins means that assays are often carried out on purified, truncated GEFs that may lack important regulatory domains and post-translational modifications. Furthermore, the activity of GEFs on specific Arfs in *in vivo* is contingent upon their co-localisation in specific subcellular domains; *in vitro* assays on re-constituted membranes may promote interactions that would not otherwise occur, making it difficult to determine their specificity *in vivo*.

Arf6 has been shown to indirectly regulate the activity of the Arf1 through the recruitment and activation of the Arf1 GEF, ARNO (Cohen et al., 2007). The Cytohesin family GEFs have been shown to adopt an auto-inhibited conformation in solution. The inhibition is mediated by the linker between the PH and Sec7 domains, which, in conjunction with the C-terminal helix, block the Arf binding capacity of the Sec7 domain (DiNitto et al., 2007). Arf6-GTP was shown to bind to the ARNO PH domain, acting synergistically with membrane lipids to alleviate its autoinhibitory state (Stalder et al., 2011). In turn, activated ARNO could mediate Arf1 nucleotide exchange. Arf1-GTP was then shown to be able to promote the activation of ARNO, suggesting the existence of an Arf activation cascade (Stalder et al., 2011).

Although the GTP-bound form of the Arfs is often considered the active form, Arf6 activity has been shown to be mediated by the rate of cycling between the GTP and GDP bound states, and the GTP-locked form of Arf6 does not fully recapitulate the activity of WT Arf6, emphasising the importance of the GDP-GTP cycle (Klein et al., 2006). Due to the negligible intrinsic GTPase activity of the Arf proteins, the hydrolysis of Arf-bound GTP to GDP requires the activity of the GTPase activating proteins (Arf-GAPs). The Arf-GAPs are a large protein family characterised by a conserve zinc-finger catalytic GAP domain and can be classified into three groups based on their domain structure: Arf-GAP1, AZAP and Git (Randazzo and Hirsch, 2004). Arf-GAP1 members have a GAP domain in the N terminus, while AZAP members have an N terminal PH domain and C-terminal flanking the GAP

domain. The GIT class have 3 C-terminal ankyrin repeats followed by a Spa-homology domain (SHD) and paxillin binding site (PBS). Each of these groups is further subdivided based on the presence of additional protein domains (Jackson et al., 2000; Randazzo and Hirsch, 2004). As with the Arf-GEFs, the localisation of the Arf-GAPs helps in determining the localised activity of the Arf proteins, and the specificity of GAPs to Arfs is still being assessed (Sztul et al., 2019). Several Arf-Gaps have been suggested to act on Arf6, including ACAP1/2, Git1/2 based on *in vitro* assays (Randazzo and Hirsch, 2004).

Arf6 carries out its functions through interaction with a wide range of effectors that can be classified into 4 main categories: lipid modifying enzymes (such as PLD, PIP5K and potentially PI4K), Rho GTPase regulators, the Arf-GAPs, and a large group of miscellaneous protein interactors (D'Souza-Schorey and Chavrier, 2006; Donaldson and Jackson, 2011). Arf6-GTP has been shown to recruit and activate the lipid modifying phosphatidylinositol 4-phosphate 5-kinase (PIP5K) and phospholipase D (PLD), which catalyse the formation of phosphatidylinositol-4,5-bisphosphate (PIP_2) and phosphatidic acid (PD) respectively. Phosphatidic acid helps in promoting PIP5K activity, allowing Arf6 to dramatically increase the levels of PIP_2 at the cell membrane (D'Souza-Schorey and Chavrier, 2006). PIP_2 is important for the sorting of membrane proteins, the recruitment of clathrin coat components for the generation of clathrin pits, and the recruitment and activation of Rho-GTPases to regulate actin dynamic (D'Souza-Schorey and Chavrier, 2006; Donaldson and Jackson, 2011; Singh et al., 2019). Arf6 also regulates actin dynamics through the regulation of Rho regulators. Arf6 was shown to directly recruit the Rac1-GEF Kalirin 5, and indirectly recruit the Rac1-GEF Dock180 to the membrane, leading to an increase in Rac1 activity (Koo et al., 2007; Santy et al., 2005). Arf6 has also been shown to interact with the Rho-GAPs ARH-GAP10 and FilGAP in a GTP dependent manner, providing a potential process through which Arf6 can suppress Rho-GTPase activity (Dubois et al., 2005; Kawaguchi et al., 2014).

In addition to the Arf-GAPs acting to catalyse GTP hydrolysis on the Arfs, they also represent the largest family of Arf effectors. The role of Arf-GAPs as effectors is highlighted by their large number of protein interaction domains which would suggest that they can act as scaffolds for the formation of protein complexes (Kahn et al., 2008). For example, GIT1 has been shown to cooperate with Arf6 in regulating the endocytosis of GPCRs through both clathrin dependent and independent endocytosis (Moore et al., 2007). Similarly, the Arf-GAPs ASAP1 and ASAP2 bind to Arf6-GTP, recruiting them to sites of high Arf6 activity (Hashimoto et al., 2004; Onodera et al., 2005). ASAP1 binds to cortactin, paxillin, and protein kinase D2, promoting cortical actin remodelling and integrin recycling, leading to epithelial-mesenchymal transition (EMT) (Hashimoto et al., 2016; Hirano et al., 2008; Onodera et al., 2012).

The final group of Arf6 effectors does not fit neatly into a single category, highlighting the huge variety of Arf6 interactors, and the range of mechanisms through which Arf6 can function. Arf6-GTP was shown to specifically interact with, and recruit, adaptor protein 2 (AP2) but not AP1, a component of the clathrin coat necessary for clathrin-dependent endocytosis and the selection of cargo molecules (Paleotti et al., 2005). The recruitment of AP2 was potentiated by the presence of PIP_2 in the membrane, highlighting the synergistic mechanisms through which Arf6 promotes AP2 both directly, and through the induction of PIP_2 formation (Krauss et al., 2003). Another adapter protein, GGA3, a member of the Golgi-localised gamma ear-containing Arf-binding proteins (GGAs) was shown to act as an Arf6 effector. GGA3 mediates the Rab4-depenent recycling of Met receptor tyrosine kinase in response to HGF treatment (Parachoniak et al., 2011). Arf6 was also activated in response to Met activation and was suggested to recruit GGA3, forming a GGA3-Met complex, promoting Met recycling at the expense of degradation (Parachoniak et al., 2011). Arf6 can also interact with a series of scaffold proteins, JIP3/4, and FIP3/4 necessary for linking cargo proteins to kinesin and dynein motor proteins for cargo transport along microtubules (Fielding et al., 2005; Montagnac et al., 2009). The binding of JIP3 or JIP4 to Arf6-GTP triggers them to release kinesin and to bind instead to dynein, switching the direction of travel along the microtubule (Montagnac et al., 2009).

Arf6 and cytokinesis

Cytokinesis is initiated at the start of anaphase, during which an actomyosin ring forms at the equator of the two dividing cells, constricting the plasma membrane at the interface of the two daughter cells through the ingression of the cleavage furrow. As the cleavage furrow progresses, anti-parallel overlapping microtubules at the cell midzone also constrict, forming the central spindle (Fededa and Gerlich, 2012). Central-spindle assembly depends on the centralspindlin complex, comprising the mammalian kinesin-like protein 1 (MKLP1) (the homologue of Pavarotti in *Drosophila*) and the Rho-family GAP, RacGAP1 (the homologue of Tumbleweed in *Drosophila*) (Mierzwa and Gerlich, 2014). The cleavage furrow continues to ingress until the cytoplasm of the two daughter cells is divided into two compartments, and only a narrow intercellular bridge remains, connecting the two daughter cells (Fededa and Gerlich, 2012). A dense complex known as the midbody forms at the midpoint of the intercellular bridge, and provides a site for the assembly of machinery necessary for the final stage of cytokinesis and the individualisation of the two daughter cells: cell abscission (Fededa and Gerlich, 2012).

The completion of abscission requires the trafficking, and fusion of endosomal vesicle at the intercellular bridge, where they contribute to the formation of a secondary membrane furrow that will eventually form the scission site (Frémont and Echard, 2018; Goss and Toomre, 2008). Arf6 has been shown to localise to the midbody during cytokinesis, where it contributes to the completion of cytokinesis in both mammalian somatic cells and *Drosophila* spermatids (Dyer et al., 2007; Schweitzer and D'Souza-Schorey, 2005). Arf6 has been suggested to regulate the trafficking of Rab11 and FIP4 positive vesicles to and from the intercellular bridge through the JNK-interacting proteins JIP3 and JIP4 (Montagnac et al., 2009). Arf6-GTP was shown to bind to JIP3 and JIP4, decreasing their affinity for Kinesin-1, whilst increasing their affinity for the dynactin complex (Montagnac et al., 2009). This interaction suggests that the Arf6 GDP-GTP cycle can switch the direction of Rab11, FIP3 vesicle transport along microtubules. Arf6 may also promote the docking of FIP4 vesicles at the midbody through a direct interaction with FIP4 and the exocyst complex (Fielding et al., 2005).

The localisation of Arf6-GTP at the midbody is contingent upon MKLP1, and the two proteins have been shown to form a heteromeric complex (Makyio et al., 2012). The Arf6-MKLP1 heterodimer was suggested to link the plasma membrane to the midbody microtubules, with MKLP1 binding to the microtubules, and Arf6 binding to the membrane (Makyio et al., 2012). The crystal structure revealed that in the complex, the Arf6 N-terminal helix and myristates are accessible for membrane binding, and although the authors suggested that this mediated binding to the plasma membrane, it could potentially mediate binding to endosomal membranes. Importantly, the MKLP1-Arf6 binding interface requires Arf6 to be in a GTP-bound conformation (Makyio et al., 2012). Although it is not clear how Arf6 is regulated at the midbody, EFA6 has also been shown to transiently localise to the cleavage furrow, so could provide a source of Arf6-GTP that would then be recruited to the midbody by MKLP1 (Ueda et al., 2013). Although the interaction between Arf6 and MKLP1 is likely to be conserved in *Drosophila,* Arf6 only appears to be necessary during spermatid cytokinesis (Dyer et al., 2007).

Intriguingly, the centralspindlin components Pavarotti (Pav) and Tumbleweed (Tum) have been shown to act as nuclear repressors of Wg signalling during embryonic patterning, acting at the level of Pan activity (Jones and Bejsovec, 2005; Jones et al., 2010). Our findings suggest that the conserved interaction between Arf6 and Pav could provide a novel regulatory axis for Wg signalling.

Drosophila Arf6

The Arf family is highly conserved throughout the Metazoa, and a single representative of each subfamily is encoded by the *Drosophila* genome, Arf79F (Arf1), Arf102F (Arf4) and ARf51F (Arf6) (we will refer to Arf51F as Arf6) (Figure 8A) (Gillingham and Munro, 2007). The cloning of *Drosophila Arf6* revealed a gene that was strikingly similar to that of the mammalian *Arf6,* and that encoded a protein with 96% sequence identity to its mammalian counterpart (Figure 8B) (Lee et al., 1994).

The number of Arf6-GEFs are similarly reduced in *Drosophila,* with only a single representative of each GEF family (Figure 8C). Importantly, the putative *Drosophila* Arf6-GEFs contain two highly conserved motifs, the first of which contains an invariant Glutamic acid, that are essential for the catalytic activity of the Sec7 domain (black arrowhead Figure 8D) (Jackson and Casanova, 2000). Although this does not guarantee that the *Drosophila* Arf6-GEFs are functional, it supports the presence of active Sec7 domains. *Drosophila* Arf6 has since been implicated in a range of disparate developmental and cellular processes in *Drosophila*. In the following section I will provide an overview of what is known regarding the role of Arf6 during *Drosophila* development and the mechanisms through which Arf6 has been shown to act.

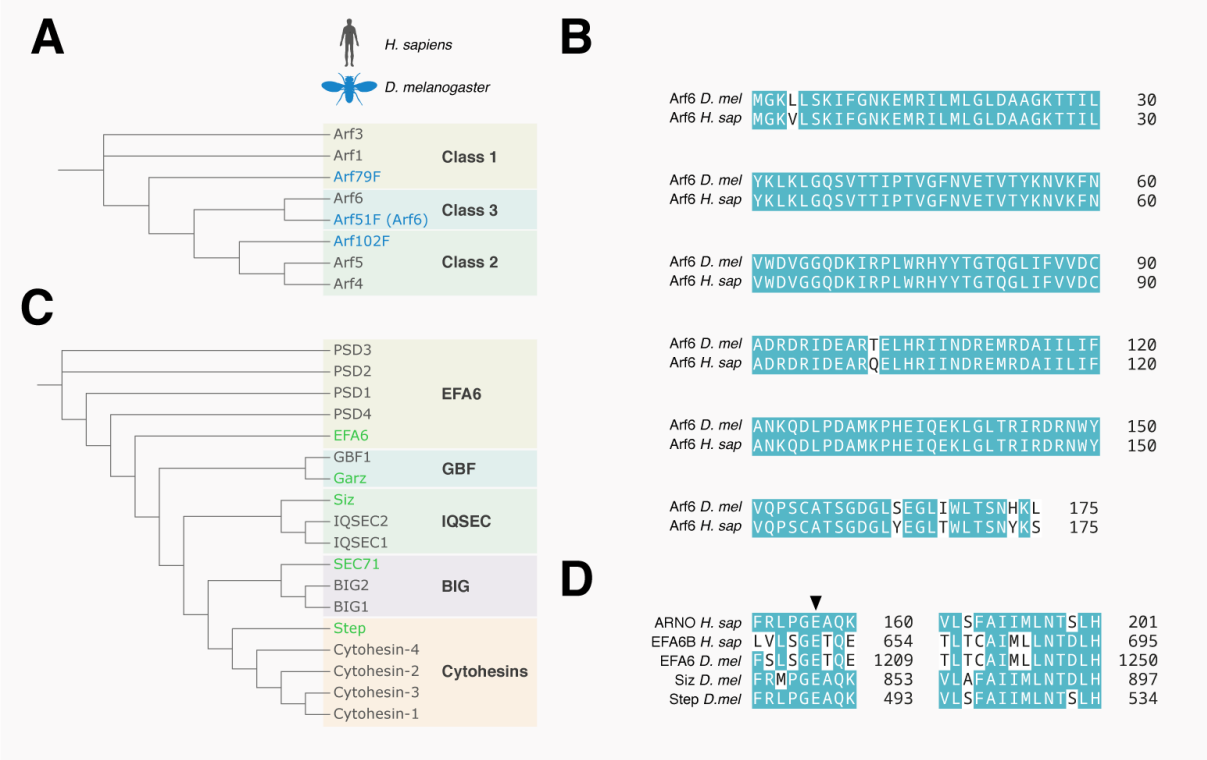

Figure 8 Conservation of Arf6 and Arf6-GEFs in *Drosophila*

A The Arf family of GTP binding proteins are highly conserved between Human and *Drosophila*. A single representative of each Arf family is present in *Drosophila*, whose primary sequences are more similar to their Human counterparts than to the other *Drosophila* Arfs. **B** *Drosophila* and human Arf6 has 97% sequence identity conservation. **C** The putative Arf6 GEFs are also conserved in *Drosophila*. Similar to the Arfs, a single representative of each Arf-GEF family is present in *Drosophila*. **D** two conserved motifs present in the Sec7 domain of the mammalian and *Drosophila* GEFs. The black arrowhead indicates a Glu residue essential for the catalytic activity of Arf-GEFs. The two motifs are suggested to form a hydrophobic groove necessary for exchange activity. The conservation of these domains indicates that these proteins could act as Arf-GEFs.

A screen for genes required during muscle development identified the Arf-GEF Schizo (Siz), an orthologue of GEP100/BRAG2, as being required for myoblast fusion during embryogenesis (Chen et al., 2003; Donaldson, 2003). The presence of sec7 domain in Siz, combined with its sequence similarity to GEP100 indicated that Siz was a potential Arf-GEF. This was supported by the ability of the over-expression of full-length Siz, but not forms lacking a functional Sec7 domain to rescue the *siz* mutant phenotype. The Siz sec7 domain was found to be sufficient to promote GDP release on *Drosophila* Arf6, but not Arf1, however, the assay was carried out in the absence of a reconstituted membrane, potentially limiting its activity. Over-expressing a dominant negative form of Arf6 ($Arf6^{T27N}$) in embryonic myoblasts phenocopied the *siz* mutant phenotype. The membrane localisation of Rac was found to be perturbed in both the *siz* mutant and in response to the $Arf6^{T27N}$. Due to the requirement for actin cytoskeleton re-arrangement during myoblast fusion, Arf6 was suggested to be required to regulate Rac recruitment during this process (Chen et al., 2003).

Siz was also shown to be required in axon guidance in embryonic development, during the midline crossing of commissural neurons, with fewer axons crossing the midline in *siz* mutants (Onel et al., 2004). Importantly, blocking endocytosis through the expression of a dominant negative form of Shibire (a dynamin homologue) partially phenocopied the *siz* phenotype, suggesting the phenotype could be a result of trafficking defects. Expressing dominant negative Arf6 once again phenocopied the *siz* phenotype, indicating that in this context, Siz primarily acts through Arf6 activation (Onel et al., 2004).

The requirement for Arf6, and the ability of Siz to act as an Arf6-GEF were later called into question (Dottermusch-Heidel et al., 2012; Dyer et al., 2007): although myoblast fusion defects were confirmed in *siz* mutants, the effects of expressing dominant negative Shibire did not phenocopy the *siz* mutants, instead causing cell fate defects (Dottermusch-Heidel et al., 2012). Furthermore, expressing the GTP-locked $Arf6^{Q67L}$ in null *siz* embryos did not rescue the phenotype, suggesting Arf6 does not act downstream of Siz activity, or that the GTP-GDP cycle is necessary for Arf6 activity in this context. These findings were supported by a yeast two-hybrid screen in which Siz specifically interacts with GDP locked Arf1 ($Arf1^{T31N}$), but not GTP locked Arf1, Arf2 or Arf6. This would support a model in which Siz does regulate Arf6 activity. The dispensability of Arf6 and Arf1 was supported by the lack of myoblast fusion phenotype in response to the expression of $Arf6^{T27N}$, or in double mutant *Arf6*, *Arf1* embryos. It is therefore likely that the phenotype induced by dominant negative Arf6 is due to the non-specific and neomorphic effects induced by the expression of either GTP-locked or dominant-negative forms of Arf6 (Macia, 2004). The lack of phenotype in homozygous Arf6 mutant embryos could have been explained by the maternal contribution of Arf6, however the lack of myoblast fusion defects was also confirmed in zygotic and maternal null *Arf6* mutants (Dyer et al., 2007). These studies highlight the difficulties that can arise during the translation of biochemical GEF activity data using truncated proteins, into an *in vivo* context.

Studies using two independently generated null *Arf6* alleles, $Arf6^{1}$ and $Arf6^{KO}$ (Dyer et al., 2007; Huang et al., 2008; Huang et al., 2009) revealed that although null *Arf6* mutants were viable, males presented a recessive sterility phenotype. This was shown to be a result of a requirement for Arf6 during spermatocyte cytokinesis (Dyer et al., 2007). Arf6 was shown to be necessary for recycling membrane at the cleavage furrow to allow the progression of cytokinesis. Removing *Arf6* led to cytokinetic failure, and the formation of polynucleated spermatids and male sterility (Dyer et al., 2007).

Arf6 has also been implicated in the behavioural response of adult *Drosophila* to ethanol, with *Arf6* mutant flies showing greater levels of sensitivity to ethanol (Gonzalez et al., 2018; Peru y Colon

de Portugal et al., 2012). Arf6^{Q67L} was found to interact with the *Drosophila* Arfaptin homologue, Arfip, that *arfip* mutants also showed heightened sensitivity to ethanol. *Arf6, arfip* double mutants did not show increased ethanol sensitivity relative to *Arf6* mutants alone, indicating that *arfip* acts genetically downstream of Arf6. The authors found that Arf6 and Rac1 could be immunoprecipitated with Arfip both *in vitro* and *in vivo*. EFA6D/PSD3 was suggested to activate Arf6 in the context of ethanol tolerance (Gonzalez et al., 2018). Homozygous *Arf6* and *EFA6* mutants showed the same level of ethanol sensitivity to double Arf6, EFA6 mutants, suggesting the *EFA6* phenotype was a result of the loss of *Arf6.* Indeed, the relative level of GTP-loaded Arf6 was strongly reduced in fly heads of homozygous *EFA6* mutants, indicating that the exchange activity of EFA6 on Arf6 is conserved in *Drosophila*, and that EFA6 is the major Arf6 GEF in this context (Gonzalez et al., 2018). The potential roles of other putative *Drosophila* Arf6-GEFs in regulating Arf6 activity were not tested in this study.

EFA6 and Arf6 have also been shown to act together during eye development, during which reduced Arf6 function (as a result of RNAi in a WT or *Arf6^{KO}* background) resulted in defects in ommatidial patterning (Johnson et al., 2011). Knocking-down the *Arf-GAPs*, *arf-GAP3* and *ASAP*, or the Arf-GEFs, *EFA6* and *siz* equally led to patterning defects, phenocopying the *Arf6* mutant phenotype. Although Arf1 was also found to be involved in eye patterning, knocking-down a*rf1* did not recapitulate the phenotypes observed in the *Arf6* knockdown, suggesting that they are necessary in distinct developmental steps during eye patterning. The similarity between the knockdown of both Arf-GEFs and Arf-GAPs suggests that the cycling of Arf6 and/or Arf1 is required for their activity in eye patterning, rather than just their GTP or GDP bound forms. The requirement for Arf1 in different developmental steps to Arf6 during eye development hints at the possibility that Arf6 could regulate Arf1 activity in this context; however the effect of removing both Arf1 and Arf6 function during eye development was not tested.

During eye development, the CD2AP homologue Cindr regulates cytoskeletal changes to coordinate cell movements to help establish the precise patterning of the eye (Johnson et al., 2008). Cindr was found to co-immunoprecipitate with Arf-GAP3 and ASAP, and the *Arf6* eye patterning defects were mildly enhanced in a background carrying a deficiency containing *cindr* (Johnson et al., 2011). The eye patterning defects induced in the *Arf6* RNAi were dominantly enhanced in *Rac* and *Rho1* mutant background, suggesting that Arf6 may be mediating eye patterning through the activity of the Rho-GTPases on cytoskeletal dynamics (Johnson et al., 2011).

Together, these studies demonstrate that although Arf6 participates in a wide range of seemingly disparate developmental processes, the cellular mechanisms through which it is likely to act, and the regulators modulating its activity, appear to be conserved between mammals and

Drosophila. The viability of null *Arf6* mutants is both beneficial, as it allows the study of mutant animals throughout development, and surprising due to the apparent pleiotropic effects of *Arf6* mutants, and the unviability of mammalian *Arf6* mutants (Suzuki et al., 2006). The reduced number of Arfs and Arf regulators encoded by the *Drosophila* genome is likely to reduce the potential for the redundancy that often poses a challenge in the characterisation of Arf function and regulation in mammals (Sztul et al., 2019). Furthermore, the striking conservation of Arf6 and its regulators between mammals and *Drosophila* make the findings in *Drosophila* more likely to be relevant to the mammalian system, and gives the *Drosophila* model huge potential to gain a greater *in vivo* understanding of Arfs and their regulators.

Arf6 and Wnt signalling

In the following sections I will provide an overview of what is known regarding the role of Arf6 and its regulators during Wnt signalling. Although there are very few reports implicating Arf6 in Wnt signalling, there is evidence that Arf6 activation can be promoted by Wnt activation, and that Arf6-GTP regulates the upstream steps in the Wnt signalling cascade. I have presented these data independently in the following sections and have summarised the proposed mechanisms through which Arf6 regulates Wnt signalling in Figure 9.

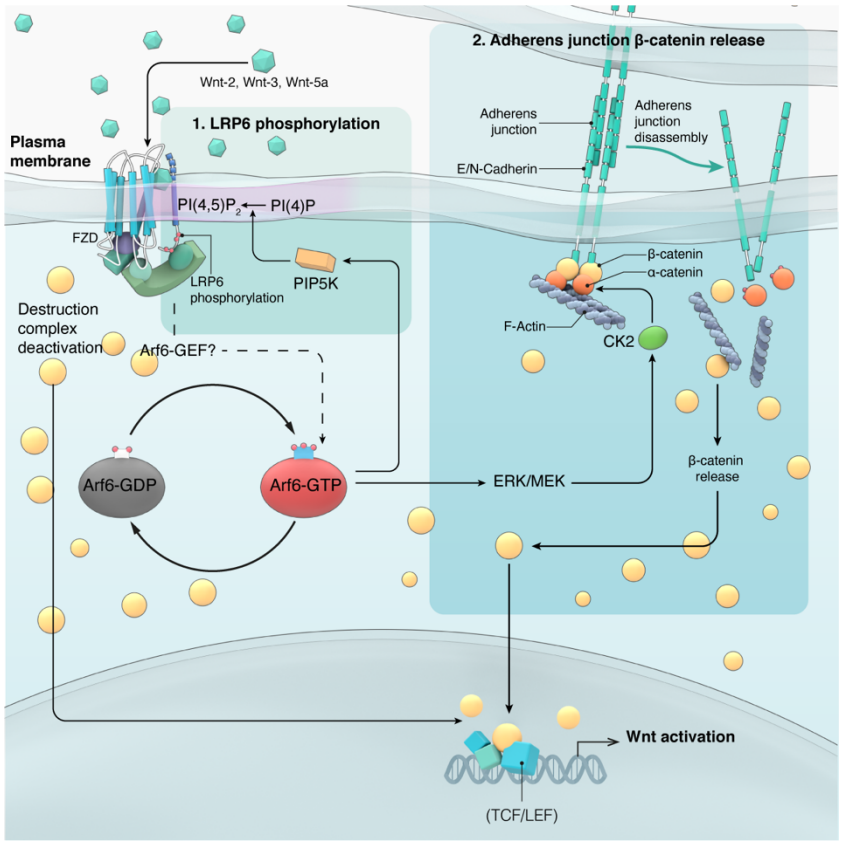

Figure 9 Models of Arf6 in Wnt signalling

Two potentially non-mutually exclusive models have been proposed to link Arf6 function to the canonical Wnt signalling pathway. Both suggest that Arf6 is activated downstream of Wnt binding to the FZD/LRP6 (dashed line). **Model 1** (LRP6 phosphorylation) suggests that Arf6-GTP leads to increased membrane PI(4,5)P_2 (purple shading) levels through PIP5K activity. The increase in PI(4,5)P_2 promotes LRP6 phosphorylation, and subsequently destruction complex suppression and β-catenin accumulation. **Model 2** (adherens junction β-catenin release) suggests that Arf6-GTP promotes the CK2-mediated phosphorylation of α-catenin through the activation of ERK/MEK signalling. α-catenin phosphorylation leads to the destabilisation of the adherens junctions, releasing β-catenin into the cytoplasm, enhancing canonical Wnt signalling.

The regulation of Arf6 by Wnt signalling

The ability of Wnt activation to promote GTP-loading on Arf6 was a finding common to several studies (Grossmann et al., 2013; Kim et al., 2013; Pellon-Cardenas et al., 2013). The relative levels of GTP-bound Arf6 were measured using a GST-tagged GGA3 (Golgi-localised γ-ear containing, Arf binding protein 3) pulldown, an Arf effector protein with increased affinity for GTP-bound Arf6 and Arf1 (Santy and Casanova, 2001), or a metallothieonein-2 (MT-2) pulldown, a protein found to have high affinity for Arf6-GTP (Schweitzer and D'Souza-Schorey, 2002). An *in vitro* FRET (fluorescence resonance energy transfer) assay was also developed based on the affinity of GGA3 for GTP-bound Arf6 and Arf1, which could provide an insight into the subcellular localisation of Arf6 activation (Pellon-Cardenas et al., 2013).

Treating cells with Wnt3a, Wnt2, or Wnt5a conditioned medium (Wnt CM) resulted in a relative increase in the level of Arf6-GTP (Grossmann et al., 2013; Kim et al., 2013; Pellon-Cardenas et al., 2013), and in the case of Wnt3a, an increase in Arf1-GTP (Pellon-Cardenas et al., 2013). The increase in Arf6- and Arf1-GTP levels was found to grow rapidly following the treatment with Wnt3a CM, peaking at between 10 and 30 minutes (Pellon-Cardenas et al., 2013). The total levels of Arf6 were not found to change in response to Wnt activation, suggesting that the effect was not a result of transcriptional regulation of *Arf6* or changes in Arf6 stability induced by Wnt signalling (Grossmann et al., 2013; Kim et al., 2013; Pellon-Cardenas et al., 2013). Monitoring Arf6-GTP levels using an *in vivo* FRET assay following Wnt treatment provided comparable results, with a rapid increase in fluorescence from Arf1 and Arf6 GGA3 FRET following the addition of Wnt3a CM (Kim et al., 2013). Together, these data suggest that the GTP-loading of Arf6 is a common response to multiple Wnts.

The increase in Arf6-GTP was found to depend on components of the signalosome: Lrp6, FZD4 and Dvl, as knocking them down, or inhibiting LRP6 activity using DKK-1 reduced Arf6-GTP levels in the presence of Wnt CM (Grossmann et al., 2013; Pellon-Cardenas et al., 2013). Arf1-GTP levels were also found to depend on LRP6, Dvl1, Dvl2 and Dvl3 (Pellon-Cardenas et al., 2013). These data are indicative that a functional signalosome is necessary to induce GTP loading onto both Arf1 and Arf6 following Wnt treatment. To test whether a downstream step in Wnt signalling was required for the GTP-loading of Arf6, LOX cells were treated with an AXIN stabiliser, *endo*-IWR1, the tankyrase inhibitor XAV-939, or the GSK3β inhibitor, BIO (Grossmann et al., 2013). Stabilising AXIN is expected to suppress Wnt signalling, whereas inhibiting tankyrase or GSK3b activity is expected to block β-catenin destruction complex activity, de-repress the cytoplasmic pool of β-catenin, triggering high level Wnt activation. No increase in the levels of either the total- or GTP-bound Arf6 were observed in response to these treatments (Grossmann et al., 2013).

These data indicate that Arf6 activity is independent of destruction complex activity or a downstream transcriptional regulation by the Wnt pathway. However, the treatments were carried out in LOX melanoma cells, that are endogenously expressing Wnt5a and Wnt2 (Grossmann et al., 2013). The presence of a canonical Wnt activation, and presumably partial assembly of a functional signalosome in these cells, presents a confounding effect that makes it difficult to interpret the result, as the signalosome could be sufficient to saturate Arf6-GTP levels.

The requirement for Wnt activity, and signalosome components for the increase in Arf6- and Arf1-GTP levels suggest that Wnt pathway components regulate the activity of Arf-GEFs. To identify the GEF linking Wnt activation to Arf6, cells were treated with molecules commonly used to inhibit Arf-GEF activity: Brefeldin-A and Secin-H3 (Grossmann et al., 2013; Kim et al., 2013). SecinH3 inhibits the Sec7 domain, blocking exchange activity, and has been suggested to be particularly active against the Cytohesin family of Arf-GEFs (Benabdi et al., 2017). Treating LOX melanoma cells with SecinH3 induced a reduction in GTP-bound Arf6 and phenocopied the *Arf6* knock-down, reducing both the cytoplasmic pools of β-catenin and the activity of the Wnt activity reporter (Grossmann et al., 2013). The results regarding the identity of the Arf6-GEF were inconclusive due to conflicting results arising from the use of siRNA and small molecule inhibitors.

Taken together, these data independently demonstrate that GTP loading of Arf6 is induced downstream of the binding of Wnts to the FZD receptor and LRP6 co-receptor, convincingly linking the Wnt activation to Arf6 activity (Grossmann et al., 2013; Kim et al., 2013; Pellon-Cardenas et al., 2013). Arf6 GTP loading was contingent upon signalosome components. Although GEP100 was suggested to be responsible for activating Arf6 in this context, the identity of the Arf6-GEF to promote exchange on Arf6 downstream of Wnt activation, and the mechanism linking Wnt activation to the recruitment and activation of Arf6-GEFs are not clear (Grossmann et al., 2013; Pellon-Cardenas et al., 2013).

The regulation of Wnt signalling by Arf6

Arf6 has been shown to regulate the upstream steps of canonical Wnt signalling at the level of the signalosome activity, and of β-catenin availability. The first putative link between Arf6 and Wnt signalling was made during a screen for small molecule modulators of Wnt signalling (Zhang et al., 2007). The authors identified QS11 based on its ability to enhance a Wnt gain-of-function in both cell culture assays induced by Wnt-3a CM, measured with TOPFlash (a synthetic reporter of Wnt transcriptional activity, consisting of TCF responsive elements that drive luciferase expression in

response to Wnt activation (Korinek, 1997)) and in *Xenopus* embryos transfected with XWnt-8 RNA. QS11 was suggested to specifically inhibit ArfGAP1, which in turn regulates Arf1 and Arf6 activity. QS11 treatments resulted in increases in both Arf1 and Arf6 in the GTP bound state. In a later study, inconsistencies between the ability of the molecular analogues to inhibit ArfGAP1 and to promote the activity of the TOPFlash assay led the authors to question the specificity of QS11 to ArfGAP1, raising the possibility of ArfGAP1 independent effects being responsible for the enhancement of Wnt signalling (Singh et al., 2015).

A study aiming to gain a mechanistic understanding of the link between Arf1, Arf6 and Wnt signalling found that expressing Arf1 or Arf6 in HEK293T cells potentiated TCF reporter activity following treatment with Wnt3a CM (Kim et al., 2013). Accordingly, knocking down either *Arf1* or *Arf6* with shRNA reduced Wnt reporter activity, and simultaneously knocking down both *Arf1* and *Arf6* led to a further reduction in Wnt signalling. Importantly, the knockdown of either transcript resulted in a reduction in β-catenin accumulation following treatment with Wnt3a CM. Without knowing the efficiency of suppression achieved with the shRNA, it is difficult to distinguish between a potential functional redundancy between Arf6 and Arf1, two distinct roles for Arf6 and Arf1, or activation of Arf1 in response to Arf6 activation through an Arf cascade.

The authors suggested that Arf1 and Arf6 are necessary to recruit PIP5K in order to increase PIP_2 levels at the plasma membrane in response to Wnt activation, promoting LRP6 phosphorylation and signalosome activity (Kim et al., 2013). The levels of PIP_2 were measured using a $PI(4,5)P_2$ FRET sensor fused to the PH domain of phospholipase C (PLCδ) which has a high affinity for PIP_2 (Nishioka et al., 2008). Treating cells with Wnt3a CM induced a rapid increase in the level of PIP_2 reporter activity. The increase in PIP_2 probe fluorescence was suppressed in cells in which Arf1 and Arf6 were simultaneously knocked-down (Kim et al., 2013). These results suggest that Arf6 and / or Arf1 are necessary for induction of PIP_2 synthesis in response to Wnt signalling. However, the shArf6 and shArf1 were simultaneously expressed under a constitutive promoter (H1-RNA) in stably expressing cell lines (Brummelkamp et al., 2002; Kim et al., 2013). Cells in which the expression of *Arf6* and *Arf1* are simultaneously reduced are likely to have a range of physiological defects unrelated to Wnt signalling, making it difficult to interpret these results (D'Souza-Schorey and Chavrier, 2006; Donaldson and Jackson, 2011). These results also do not allow us to decouple the potential roles of Arf1 and Arf6 in regulating the Wnt-induced increase in PIP_2. PIP_2 increases in response to Wnt activation have been suggested to be required to induce the phosphorylation of the Wnt co-receptor LRP6, necessary for the formation and activity of the signalosome complex (Pan et al., 2008). The knockdown of *Arf6/Arf1*

led to a reduced level of LRP6 phosphorylation, suggesting that Arf6 or Arf1 could regulate Wnt signalling through the activity of LRP6 (Kim et al., 2013).

Under physiological conditions, the levels of cytoplasmic β-catenin are suppressed by the activity of the β-catenin destruction complex, whilst a pool of stabilised β-catenin is found bound to the cytoplasmic tail of E-cadherin in the adherens junctions. The dual roles of β-catenin naturally led to the possibility of a dynamic exchange between structural and signalling β-catenin (Heuberger and Birchmeier, 2010; Valenta et al., 2012).

Arf6 has been suggested to promote Wnt signalling by mediating the dissociation of β-catenin in the adherens junctions, supplementing the cytoplasmic pool of β-catenin available for canonical signal transduction (Grossmann et al., 2013; Pellon-Cardenas et al., 2013). Madin-Darby Canine Kidney (MCDK) cells treated with Wnt3a CM showed increased levels of E-cadherin endocytosis, an effect that was blocked by transfection with $Arf6^{T27N}$ (Pellon-Cardenas et al., 2013). The levels of both the cytoplasmic and nuclear fractions of β-catenin were also observed to increase in response to the Wnt3a treatment, consistent with Wnt pathway activity. Importantly, expressing $Arf6^{T27N}$ suppressed the increase in β-catenin in response to Wnt3a, without affecting the basal levels of β-catenin. Expressing the GTP-locked $Arf6^{Q67L}$ cell autonomously induced a strong increase in both the cytoplasmic and nuclear fractions of β-catenin, independently of Wnt3a treatment. These experiments were supported by the subcellular accumulation and relocalisation of hypophosphorylated β-catenin, and TOPFlash assays.

Without using live imaging, or selective labelling, we cannot be certain of the origin of the intracellular pool of E- cadherin. Moreover, it is difficult to attribute an increase in cytoplasmic β-catenin and Wnt signalling directly to β-catenin released from the disassembly of the adherens junctions. The sufficiency of $Arf6^{Q67L}$ to lead to increases in both cytoplasmic and nuclear β-catenin levels, and induce TOPFlash activity, is surprising: in the absence of Wnt3a stimulation, the β-catenin released from the adherens junctions should be expected to be rapidly degraded by the β-catenin destruction complex (Valenta et al., 2012). This is supported by previous studies showing that simply removing E-cadherin could only potentiate the expression of Wnt responsive genes in the presence of Wnt activation and alone is not sufficient to activate Wnt signalling (Heuberger and Birchmeier, 2010; Kuphal and Behrens, 2006; Van De Wetering et al., 2001). Alternatively, the sufficiency of $Arf6^{Q67L}$ to trigger Wnt signalling may be a result of Wnt-independent LRP6 phosphorylation leading to destruction complex deactivation (Cselenyi et al., 2008; Kim et al., 2013).

ERK-CK2 signalling activity was suggested to provide the functional link between Arf6 and the increase in cytoplasmic β-catenin (Pellon-Cardenas et al., 2013). Phospho-ERK levels were increased in response to Wnt3a or Arf6^{Q67L}, while expressing Arf6^{T27N} blocked the increased Phospho-ERK phosphorylation in response to Wnt3a. The increase in ERK activation was then suggested to lead to the CK2-mediated phosphorylation of α-catenin, destabilising the adherens junctions and increasing the cytoplasmic pool of β-catenin (Pellon-Cardenas et al., 2013). Arf6 would potentially act by promoting the internalisation of c-Met, triggering ERK phosphorylation on endosomes (Pellon-Cardenas et al., 2013).

Although these findings are suggestive of a role of Arf6 in supplementing the cytoplasmic pool of β-catenin, potentiating the Wnt signalling response, the ability of Arf6^{T27N} to fully suppress the cytoplasmic increase in β-catenin in response to Wnt CM is surprising. This is either indicative of Arf6^{T27N} inducing a complete loss of responsiveness to the Wnt3a ligand, or alternatively, that the increase in β-catenin levels in response to Wnt signalling are exclusively a result of β-catenin release from the adherens junctions. Together with the ability of Arf6^{Q67L} to cell autonomously activate Wnt signalling, these results hint that Arf6 could be involved in regulating several steps of the Wnt signalling pathway.

A similar role of Arf6 was described to occur in LOX melanoma cells (Grossmann et al., 2013). LOX melanoma cells endogenously express high levels of Wnt5a and Wnt2, and can therefore induce Wnt signalling in an autocrine manner, driving an invasive phenotype (Grossmann et al., 2013; You et al., 2004). Knocking-down Arf6 was shown to induce an increase in the membrane-associated β-catenin fraction, with a corresponding decrease in both the nuclear and cytoplasmic fractions (Grossmann et al., 2013). Reducing *Arf6* expression was also associated with a reduction in Wnt signalling activation, revealed using a Wnt activity reporter (7TFP-luciferase, a modified form of TOPFlash (Fuerer and Nusse, 2010)) and the reduced expression of an endogenous Wnt signalling target, *AXIN2.* Conversely, expressing Arf6^{Q67L} was shown to rescue the reduction in cytoplasmic and nuclear β-catenin, and *AXIN2* expression resulting from knocking-down *Wnt5a* (Grossmann et al., 2013). The ability of Arf6^{Q67L} to induce Wnt signalling is once again surprising in the absence of the deactivation of the β-catenin destruction complex. In the context of LOX melanoma cells, the endogenous Wnt2 expression may be sufficient to partially suppress the destruction complex, permitting the β-catenin released from the adherens junctions to activate Wnt signalling. The deactivation of the destruction complex could also potentially be explained by a cell autonomous role of Arf6 on the phosphorylation of LRP6, that could act in concert with Wnt2 to induce Wnt activation (Kim et al., 2013). Although knocking down Arf6 did not affect the levels of LRP6 phosphorylation, the

effect of Arf6^{Q67L} expression on LRP6 phosphorylation was not tested. The effect of knocking-down *Arf6* on the levels of nuclear β-catenin appears dramatic, with almost a complete loss of signal, whereas the reduction in cytosolic β-catenin is much milder. The response of the TOPFlash assay to knocking down *Arf6* is also comparable to that of knocking down *Wnt5a.* Following the model presented in the paper, these findings would again suggest that the β-catenin from the adherens junctions provides the majority of the β-catenin necessary for Wnt signalling, with negligible contribution of the suppression of the β-catenin destruction complex, contrary to established models of canonical Wnt signalling (Stamos and Weis, 2013).

A similar suppression of Wnt signalling and cytoplasmic β-catenin accumulation was observed in uveal melanoma cells in response to knocking-down *Arf6* (Yoo et al., 2016). Arf6^{Q67L} expression increased the cytoplasmic and nuclear fractions of β-catenin, and an increase in TOPFlash activity, however this was not tested in the absence of endogenous Wnts. Knocking down *CK2α* phenocopied the *Arf6* knockdown, suggesting that the ERK-CK2 axis may also be responsible for the destabilisation of the adherens junction in this context (Pellon-Cardenas et al., 2013; Yoo et al., 2016). Although Arf1 was suggested to redundantly contribute to promoting LRP6 phosphorylation, the requirement for Arf1 in the release of β-catenin from the adherens junctions was not assessed, making it difficult to exclude the possibility that Arf6 acts through Arf1 in this context (Kim et al., 2013; Pellon-Cardenas et al., 2013).

These studies present an intriguing possibility that Arf6 acts as a mediator of the exchange between the roles of β-catenin in adhesion and signalling. Although the adherens junctions appear to serve as a sink for cytoplasmic Arm/β-catenin, the ability of β-catenin from the adherens junction to activate Wnt signalling remains a contentious issue (Heuberger and Birchmeier, 2010; Van Der Wal and Van Amerongen, 2020). Importantly, knocking-down E-cadherin is not sufficient to induce Wnt signalling in the absence of Wnt activation, or inactivating mutations in the destruction complex, indicating that while the β-catenin destruction complex is active, β-catenin released from the adherens junctions is not sufficient to activate Wnt signalling (Heuberger and Birchmeier, 2010). The ability of β-catenin from the adherens junctions to activate Wnt signalling is predominantly based on a correlation between the reduced levels of β-catenin in the adherens junctions, and an increase in the cytoplasmic and nuclear pools. However, it is difficult to directly attribute the increase in Wnt signalling to the activity of junctional β-catenin. Further studies in which E/N-Cadherin binding deficient β-catenin is expressed in the absence of Arf6 would need to be tested. Alternatively, cell-lines deficient in *cadherin* expression such as L-cells could provide a viable model in which to test for

cadherin-independent roles of Arf6 in Wnt signalling (Nagafuchi et al., 1987). The relative contribution of Arf1 during this process is also not clear.

In contrast to the requirement for Arf6 in signalosome assembly and β-catenin availability, *Drosophila* Arf1 has been suggested to be required in Wg signalling, for the endocytosis of the Wg ligand, a step proposed to be necessary for Wg activation (Hemalatha et al., 2016). Knocking-down the *Drosophila Arf1,* or the Arf1-GEF, *garz*, led to an accumulation of extracellular Wg at the plasma membrane in the wing imaginal disc, suggesting that Wg internalisation was impaired. The *garz* loss of function also induced defects in fluid-phase uptake. Specifically knocking-down *garz* in the posterior compartment of the wing imaginal disc induced a reduction in the high-level Wg signalling target, *sens.* Although these results are indicative of a role of Arf1 and Garz in Wg signalling, similarly to the role of junctional β-catenin to the activation of Wnt signalling, the requirement for ligand internalisation for initiation of Wg signalling remains controversial. This is fuelled by the pleiotropic effects resulting from the prolonged disruption of endocytic pathways, and the non-specific effects of small molecule inhibitors (Gagliardi et al., 2008; Gagliardi et al., 2014; Seto and Bellen, 2006).

Together these studies indicate that Arf6 is likely to play a role in the upstream steps of the transduction of canonical Wnt signalling (Grossmann et al., 2013; Grossmann et al., 2016; Kim et al., 2013; Pellon-Cardenas et al., 2013), potentially through two complementary roles in signalosome assembly and activation, and through the provision of β-catenin for Wnt signalling. The relative contributions of both mechanisms have not been assessed, for example, by inhibiting or knocking-down *LRP6,* while expressing $Arf6^{Q67L}$. The studies do not exclude the possibility that Arf6 is also required in a downstream step in the transduction of Wnt signalling, as a reduction in signalling-competent β-catenin will mask a potential downstream role for Arf6. The regulation of Wnt/Wg signalling by Arf6, and the reciprocal regulation of Arf6 by Wnt signalling have not been studied in an *in vivo,* physiological context. The potential developmental relevance of these processes has also not been assessed and are the subject of my thesis.

Arf6 and Notch signalling

Our findings are indicative of a putative role of Arf6 in Notch signalling. Due to the lack of literature discussing the roles of Arf6 in N signalling, I have provided some of the steps in the regulation of N signalling in which Arf6 could potentially be involved. The information has been divided into the possible roles of Arf6 in signal sending, and signal receiving cells for clarity.

Arf6 in the N signal-sending cell

The N ligand Dl has been found in endocytic compartments in cells of *Drosophila* embryos and wing imaginal discs (Kooh et al., 1993). These observations led to the finding that Dl signalling activity depends on its internalisation, and that disrupting endocytosis in signal-sending cells using endocytic mutants led to a loss of Dl activity, and an accompanying disruption of N signalling (Seugnet et al., 1997). Although Dl endocytosis is known to be required in N signalling, the reason for which Dl activity requires its internalisation is still not fully established (Le Borgne et al., 2005). Two non-mutually exclusive models have been proposed: Dl internalisation could be required prior to its presentation at the cell surface, during which the ligand may be modified or trafficked to a specific membrane domain. Alternatively, the internalisation of Dl bound to the surface of a neighbouring cell could exert a physical force on the N receptor, promoting S2 cleavage (Musse et al., 2012). I will not provide a comprehensive discussion of the two models (A discussion of the evidence is provided in (Kandachar and Roegiers, 2012; Musse et al., 2012)).

Dl is ubiquitinated at the plasma membrane by the partially redundant E3-ubiquitin ligases Neuralized and Mind bomb (Le Borgne, 2006). Ubiquitinated Dl is recognised by the endocytic adaptor protein Epsin that promotes the Dl internalisation (Wang and Struhl, 2004). Both Epsin and Neuralized contain $PI(4,5)P_2$ binding domains (Skwarek et al., 2007; Wendland, 2002). The interaction between Epsin and $PI(4,5)P_2$ has been suggested to act in tandem with ubiquitinated epitopes to recruit Epsin to the plasma membrane (Claudio Aguilar et al., 2003; Itoh, 2001). The role of $PI(4,5)P_2$ binding of Neuralized is more enigmatic, as it is not necessary for Dl ubiquitination, but is required for the Dl internalisation (Skwarek et al., 2007). Arf6 could promote Epsin and Neuralized recruitment through the activation of PIP5K and PLD, increasing the levels of membrane $PI(4,5)P_2$ (Donaldson and Honda, 2005). The mechanisms of N ligand endocytosis are incompletely understood, but appear to require clathrin and dynamin activity (Nichols et al., 2007). The role of Arf6 in regulating clathrin mediated endocytosis suggests that Arf could also regulate Dl internalisation (Donaldson and Jackson, 2011).

During the asymmetrical division of SOPs, Neuralized becomes asymmetrically segregated between the daughter cells, with a greater amount of Neuralized being inherited by the pIIb than by the pIIa (Le Borgne and Schweisguth, 2003). This asymmetry induces a greater level of Dl ubiquitination in the pIIa, increasing driving N activation in the neighbouring pIIa cell. Intriguingly, there is also an asymmetrical division of Rab11 recycling endosomes between the pIIa and pIIb (Emery et al., 2005). This asymmetry is dependent upon the recruitment of the *Drosophila* FIP3/4 homologue Nuclear fallout (Emery et al., 2005). Intriguingly, Arf6 interacts directly with Rab11 and FIP3 during the late stages of cytokinesis where both promote cell abscission (Takahashi et al., 2011). Arf6 also

regulates the direction FIP3 endosomes travel along microtubules (Montagnac et al., 2009). This would suggest that in some contexts, Arf6 may contribute to the Rab11/FIP3 mediated activity of Dl. It is not clear whether this mechanism could more generally couple N signalling to cytokinesis, or whether the mechanism would be restricted to asymmetrical cell division.

Arf6 in the signal receiving cell

Endocytosis of the N receptor can promote or repress its activity, and plays an essential role in regulating the dynamics of N signalling (Conner, 2016) (Figure 2). The role of trafficking in the regulation of N signalling has been reviewed elsewhere (Le Borgne, 2006; Yamamoto et al., 2010). In this section I will specifically address the potential implication of Arf6 in the regulation of N signalling.

The endocytic adaptor protein Numb antagonises N signalling during SOP asymmetrical division (Schweisguth, 2015). During the asymmetrical division, Numb is inherited by the pIIb, leading to the cell autonomous suppression of N signalling (Rhyu et al., 1994). Although it is still not fully understood how Numb suppresses N signalling, the genetic and molecular evidence suggests that Numb regulates the endocytosis and trafficking of N and Sanpodo (Spdo) (a positive N regulator), potentially leading to N degradation, or the slowing of the recycling of N/Spdo complexes (Cotton et al., 2013; Couturier et al., 2012; Couturier et al., 2013). EFA6 directly binds to, and can be regulated by the Numb (Zobel et al., 2018). This interaction is necessary to regulate the activity of Arf6 during the recycling of cargos implicated in the control of polarised cell protrusions. Furthermore, the authors also demonstrated that Numb could also be co-immunoprecipitated with the Arf6-GAP, ACAP1, suggesting that Numb could act as a core scaffold for the regulation of Arf6 activity (Zobel et al., 2018). The potential role of a Numb-EFA6-Arf6 axis in N regulation has not yet been tested.

The nucleoside diphosphate kinase Nm23-H1 has been shown to be involved in Rac1 activation and dynamin dependent endocytosis (Palacios et al., 2002). Arf6-GTP was found to interact with, and recruit Nm23-H1 to sites of cell-cell contacts, where it promoted the endocytosis of E-cadherin (Palacios et al., 2002). The *Drosophila Nm23-H1* homologue *awd* is necessary for N signal transduction during wing and oocyte development (Ignesti et al., 2014). Loss of function *awd* clones showed an accumulation of NICD in early endosomes, suggesting that Awd is required for the transition of NICD from the early to late endosome (Ignesti et al., 2014). Expressing *NEXT* in *awd* clones did not rescue signalling, supporting a model in which Awd is necessary downstream of S2 cleavage. Although this contrasts with the role of Nm23-H1 in promoting dynamin dependent endocytosis, it does not preclude the possibility that Arf6 regulates the recruitment of Awd to sites of N activity.

Arf1 has been suggested to regulate N signalling during *Drosophila* haematopoiesis (Khadilkar et al., 2014). Depleting Arf1 or its exchange factor, *gartenzwerg (garz,* a *GBF1* orthologue*),* in the larval lymph glands induced a trapping of NICD in hrs positive endosomes and an increase in the number of differentiated crystal cells, indicative of an increase in N signalling. Although the authors suggest that Arf1 could regulate the trafficking of NICD through the endocytic regulator Asrij, the mechanistic link is not clear (Khadilkar et al., 2014).

Neither *Arf6*, nor its regulators have previously been identified in genome-wide RNAi screens for regulators of the N signalling pathway (Guruharsha et al., 2012). Although this alone does not stand as evidence for an absence of a role for Arf6 in N signalling, it does suggest that any involvement of Arf6 in the N signalling pathway is likely to either be context specific, or non-essential.

Thesis objectives

The striking level of conservation of Arf6 in *Drosophila,* combined with the availability and viability of null *Arf6* mutants prompted me to test for a role for Arf6 during *Drosophila* development. The requirement for Arf6 in Wnt/Wg signalling has not previously been tested in an *in vivo* model, and the potential developmental implications of the previously proposed models of Arf6 activity in the Wnt signalling pathway had not been assessed. These questions form the basis of my thesis project. The main focus was on whether Arf6 is required in the wingless signalling pathway during *Drosophila* development. I next aimed to characterise the mechanism through which Arf6 acts in Wg signalling. I further aimed to complement these data with a biochemical and developmental characterisation of the putative Arf6-GEFs in *Drosophila*.

Main results (manuscript)

The core results generated during my thesis are presented in the form of a manuscript entitled "*Arf6 is necessary for high level Wingless signalling during Drosophila wing development*". In the following sections, I will present complementary data to those included in the manuscript. These data were not included in the main manuscript due to their inconclusive nature. In the manuscript we described an Arf6 phenotype indicative of a defect in high level Wg signalling and demonstrated that Arf6 acts genetically downstream of stabilised Arm for the activation of the Sens throughout the wing blade. The *Arf6* phenotype was ameliorated in a *pav* mutant background. Based on the previously described interactions between Arf6 and Pav/MKLP1, we proposed that Pav could be in part responsible for the *Arf6* phenotype. There are two main components missing in the story: the mechanisms regulating Arf6, and in turn the mechanism through which Arf6 regulates Wg signalling. The data I will present here represent the initial steps I took in order to begin establishing a model with which to explain how Arf6 integrates into the Wg signalling pathway. I will also present the results of complimentary but independent experiments, putatively linking Arf6 to Notch signalling. These data are once again inconclusive and represent ongoing work.

Arf6 is necessary for high level Wingless signalling during *Drosophila* wing development

Julien Marcetteau[1], Tamàs Matusek[1], Frédéric Luton[2*], Pascal P. Thérond[1*#]

[1] Université Côte d'Azur ; UMR7277 CNRS; Inserm 1091 ; Institut de Biologie de Valrose (iBV); Parc Valrose ; 06108 Nice cedex2, 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

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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 co-receptor, 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 stripe 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 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. In contrast to the previously suggested upstream roles of Arf6 in Wnt signalling (Grossmann et al., 2013; Kim et al., 2013; Pellon-Cardenas et al., 2013), our data indicate that Arf6 is necessary downstream of Arm stabilisation for the activation of high level Wg signalling. Moreover, we show that Arf6 acts upstream, or at the level of Pan activity. These findings represent a novel function for Arf6 in Wg signalling during wing margin development, and is the first demonstration of an *in vivo* role for Arf6 in Wg/Wnt signalling.

Materials and Methods

Fly genetics

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

Clone induction

Clones were generated by crossing males of either FRT42B, $Arf6^{KO}$/ CyO, Tb::RFP or FRT42B, $Arf6^{1}$/ CyO, Tb::RFP with virgins of y, w, hsFLP; FRT42B, ubi-nlsGFP. Heat shock induction was carried out for 30 minutes in a water bath at 37ºC, 48h after egg lay. Larvae carrying $Arf6^{1}$ or $Arf6^{KO}$ 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: w^{1118} (Bloomington #3605) served as a wild-type control and the source of wild-type chromosomes. $Arf51F^{GX16w-}$ ($Arf6^{KO}$) (Bloomington #60585 (Huang et al., 2009)), $Arf6^{1}$ (Dyer et al., 2007) (A kind gift from Marcos Gonzalez Gaitan) are both independently generated null alleles of *Arf6* lacking the full coding region. $Arf6^{KO}$ was initially recessive lethal, so we introgressed both Arf6 null alleles into a *w-* background and reconfirmed the presence of the deletions by PCR. $Arf6^{KO}$ and *Arf1* 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 $Arf6^{KO}$ background (Huang et al., 2009). High level Wg activation was induced using *UAS-dsh::myc* (Bloomington #9453), $UAS\text{-}sgg^{A81T}$ (Bloomington #5360)(Bourouis, 2002) , $UAS\text{-}Arm^{S10}$ (encoding Arm lacking amino acids 37-84 in the N-terminus, Bloomington #4782) (Pai et al., 1997), $vgMQ\text{-}arm^{NDel}$ (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^{CX4}* (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 stripe overlapping the D/V boundary) (Bloomington #43343), *tub-gal80^{TS}; hh-gal4*/TM6b in the posterior compartment. Mitotic clones were induced *using y,w,hsFLP; FRT42B, ubi-GFP^{NLS}* (derived from Bloomington #5826), and *Arf6^{KO}; 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^{963}* (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' XbaI 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, 2013).

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 37ºC for 30 minutes then heat inactivated at 95ºC for 2 minutes using a thermocycler. 1µl of the resulting extraction was used as the PCR template.

The defficiency described for *Arf6*^{1} was validated using PCR (figure S1B') and the primer combinations shown in (figure S1B). *Arf6*^{KO} has previously been characterised in (Huang et al., 2009) Primer sequences used are provided in the materials and methods. 2x GoTaq Green Master Mix (M7121, Promega) was used for the PCR reactions. The following primers were used to validate the *Arf6*^{1} allele:

Primer name	**Sequence**
Arf6_A	GATCTGCGGGTCCACTGAAA
Arf6_D	TGTCTCGCAAATTGAGGCAGA

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, 2013). Stout bristle counts in the anterior margin were modelled using Generalised Linear Models (GLM) with Poisson errors. Counts of ectopic bristles were modelled using GLM with Poisson errors, and Zero-Inflated negative binomial GLMs were used to compensate for the large number of wings that contain no ectopic bristles **(in figures 3B and D)**(Zeileis et al., 2008). Pairwise contrasts between genotypes for both margin and ectopic bristles were calculated using the emmeans package (Lenth, 2021). The p values resulting from multiple comparisons were corrected for Type 1 error using Bonferonni correction. 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 and Discussion

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 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 (figure 1A, A').

Wg-dependent Senseless expression is suppressed in an Arf6 mutant

The zinc finger transcription factor Sens acts as the proneural factor for 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 expression 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 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 loss of Sens expression is due to a defect in *wg* expression, we analysed the pattern of Wg in *Arf6* 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 $Arf6^{KO}$ 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 $Arf6^{KO}$ clones, an intermediate level in heterozygous tissue and the WT levels in the WT 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 expression (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* GSK3β 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 an *Arf6* mutant background (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} at 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 WT *dsh* also induced ectopic bristles which were suppressed by the $Arf6^{KO}$ (closed orange arrow, figure S5B, B'). *dsh* over-expression also enhanced of the $Arf6^{KO}$ phenotype (compare figure S5C, S5B, S5C'). This is unlikely to be due to a dominant negative effect of Arm^{S10} or Dsh as expressing these constructs in a WT 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 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 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 $vgArm^{NDel}$ in either a WT (figure 3F) or heterozygous $Arf6^{KO}$ background (figure 3F'). Clusters of ectopic Sens positive nuclei were apparent far from the wing margin in WT wings expressing $vgArm^{NDel}$ (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^{NDel} is still able to activate low level signalling in the *Arf6* mutant background, its ability to activate Sens expression is strongly attenuated. Importantly, the *Arf6* margin phenotype and suppression of high level Arm activity are independent of Arf1 (figure S7A, A', B, B') indicating that Arf1 and Arf6 play distinct roles in Wg signalling (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 proneuronal clusters of the wing margin due to a suppression of high level Wg signalling.

Arf6 regulates Wg signalling 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) (figure S9A, S9A'). Expressing *pan-VP16* in a WT background only induced low levels of ectopic Sens (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

D/V boundary (figure S9C, C'). Despite its greater ability to induce Sens expression, expressing *Arm*^{S10} with *C96-gal4* in a heterozygous *Arf6*^{KO} did not rescue Sens expression (figure S9D, D'), whilst expressing *pan-VP16* in the same conditions resulted in a substantial rescue of Sens throughout the wing margin (figure 4C, C').

We have described a novel role for Arf6 in regulating high level Wg signalling, downstream of Arm stabilisation but upstream or at the level of Pan activity. The *Arf6* wing phenotype is particularly striking due to its dominance, as even mutations in core Wg pathway components do not dominantly induce wing margin defects (Couso et al., 1993; Couso et al., 1994). We therefore posit that the *Arf6* phenotype represents the derepression of negative regulators of the Wg pathway. Pav could provide a functional link between Arf6 and nuclear Wg signalling; Pav and Arf6 have previously been shown to cooperate during the spermatid cytokinesis (Dyer et al., 2007), however, Arf6 is dispensable for somatic cytokinesis (Dyer et al., 2007)(this study), and the suppression of Wg phenotype by Pav is independent of its role in cytokinesis (Jones et al., 2010). Arf6 could therefore conditionally sequester Pav in endosomal structures outside the nucleus, preventing its repressive activity in Wg signalling. Although previous studies have shown direct interactions between Arf6GTP to Pav/MKLP1 (Dyer et al., 2007; Makyio et al., 2012), further work is required to test whether this interaction is required for the suppression of high level Wg signalling we observe in the Arf6 mutant, and the mechanisms regulating Arf6 activity in this context**.**

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Author contributions

J. M. conducted all experiments except for Fig. S9 C-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.

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Figure 1

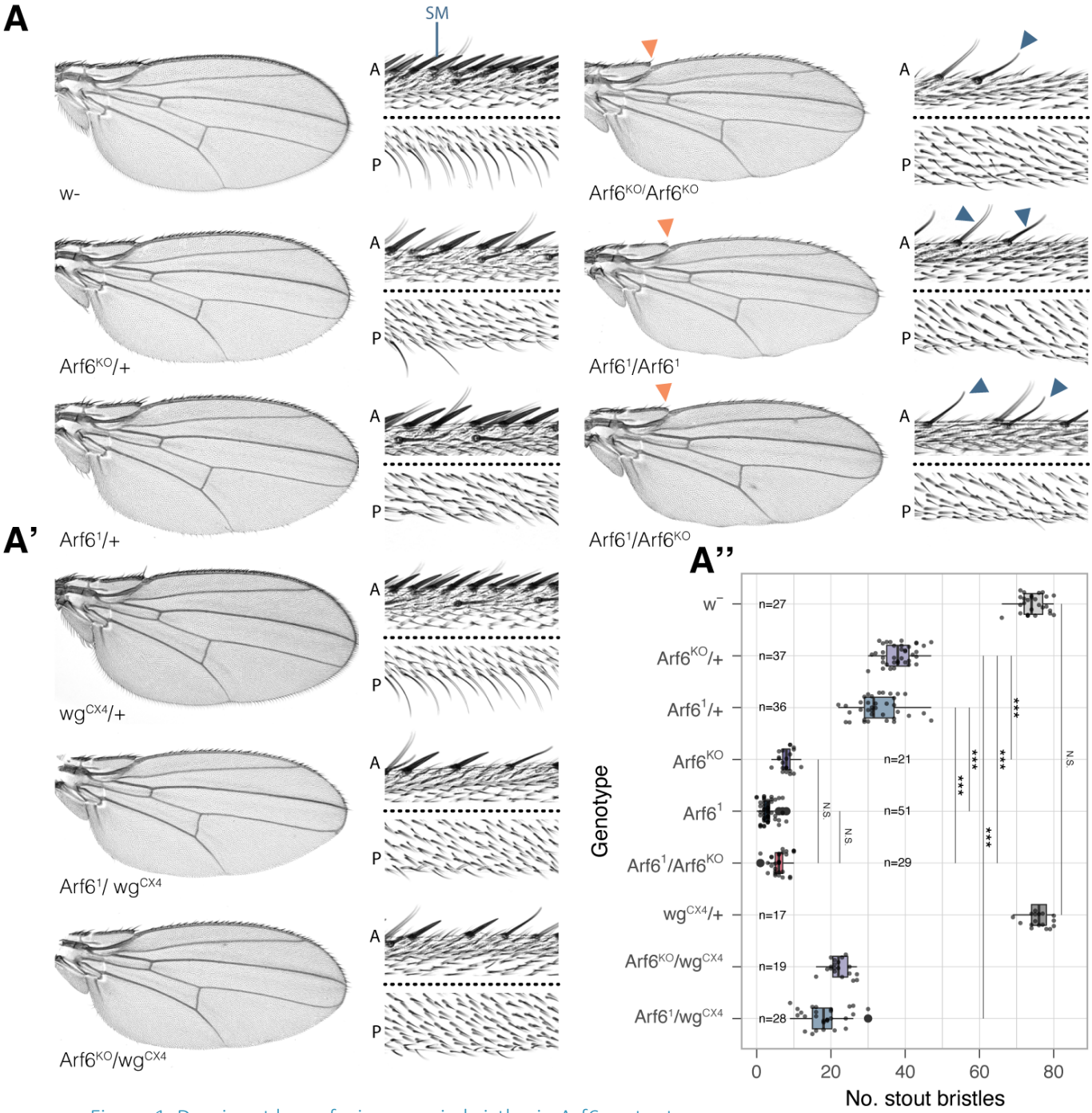


Figure 1. Dominant loss of wing margin bristles in Arf6 mutants.

(**A-A'**) Representative wing blades and wing margins of control (*w*$^{-}$), *Arf6*^{KO}, *Arf6*^{1} and *wg*^{CX4} 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 modelled using a GLM with Poisson errors. Significance values resulting from post-hoc pairwise contrasts are reported by the following abbreviations: N.S. = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.001$, *** = $p \leq 0.001$.

Figure 2

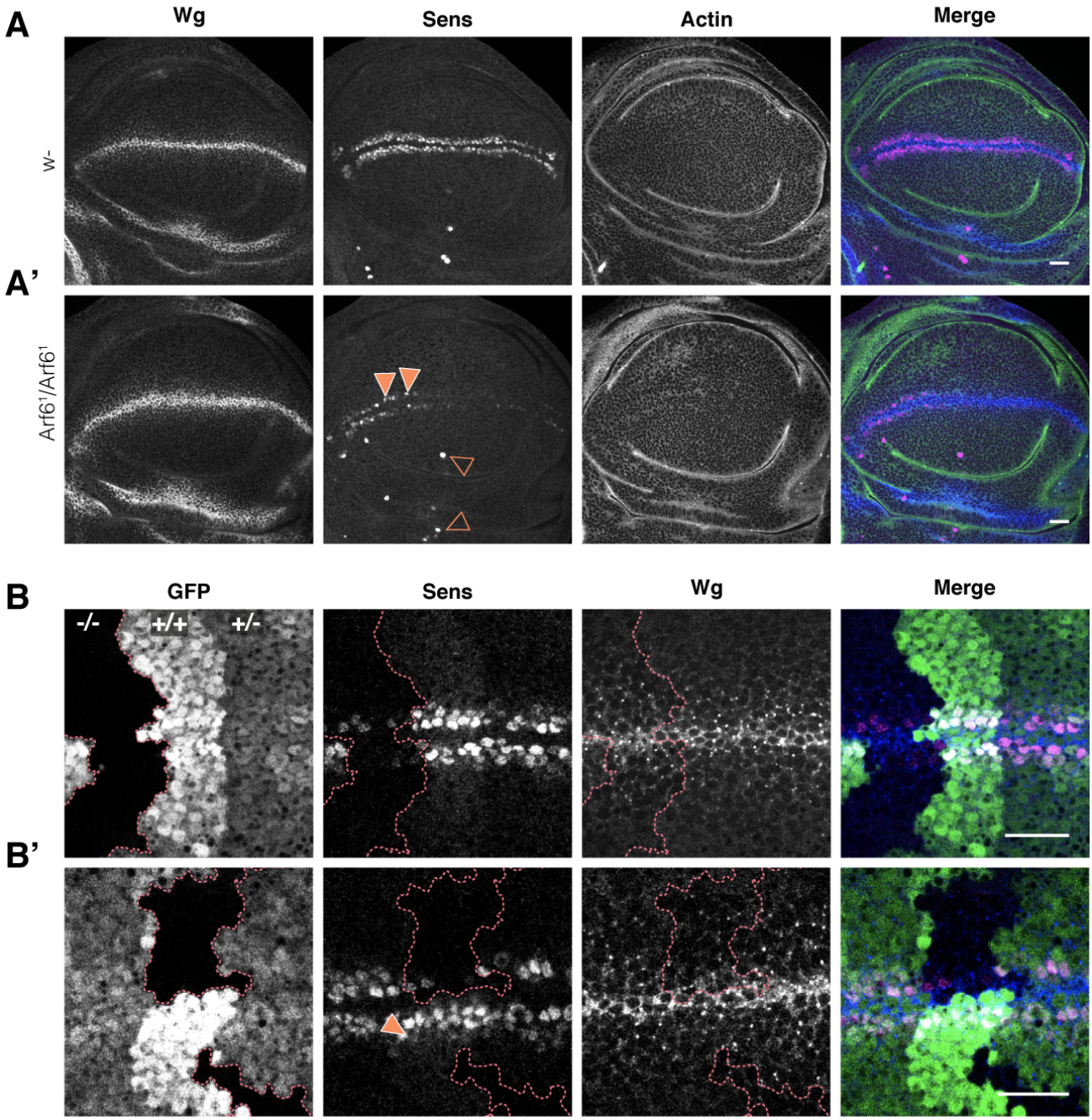


Figure 2. The level of Sens expression is strongly reduced in the absence of Arf6.

(**A**) Wg and Sens staining in control (w^-) and $Arf6^1$ 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 $Arf6^1$ mutant. Sens is also observed in the prospective ventral radius and campaniform sensilla (open orange arrows). WT n=10, $Arf6^1$ n=10. (**B**) Sens and Wg staining in *$Arf6^1$* mutant clones is marked by the absence of GFP (-/-). Heterozygous and homozygous WT tissue 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

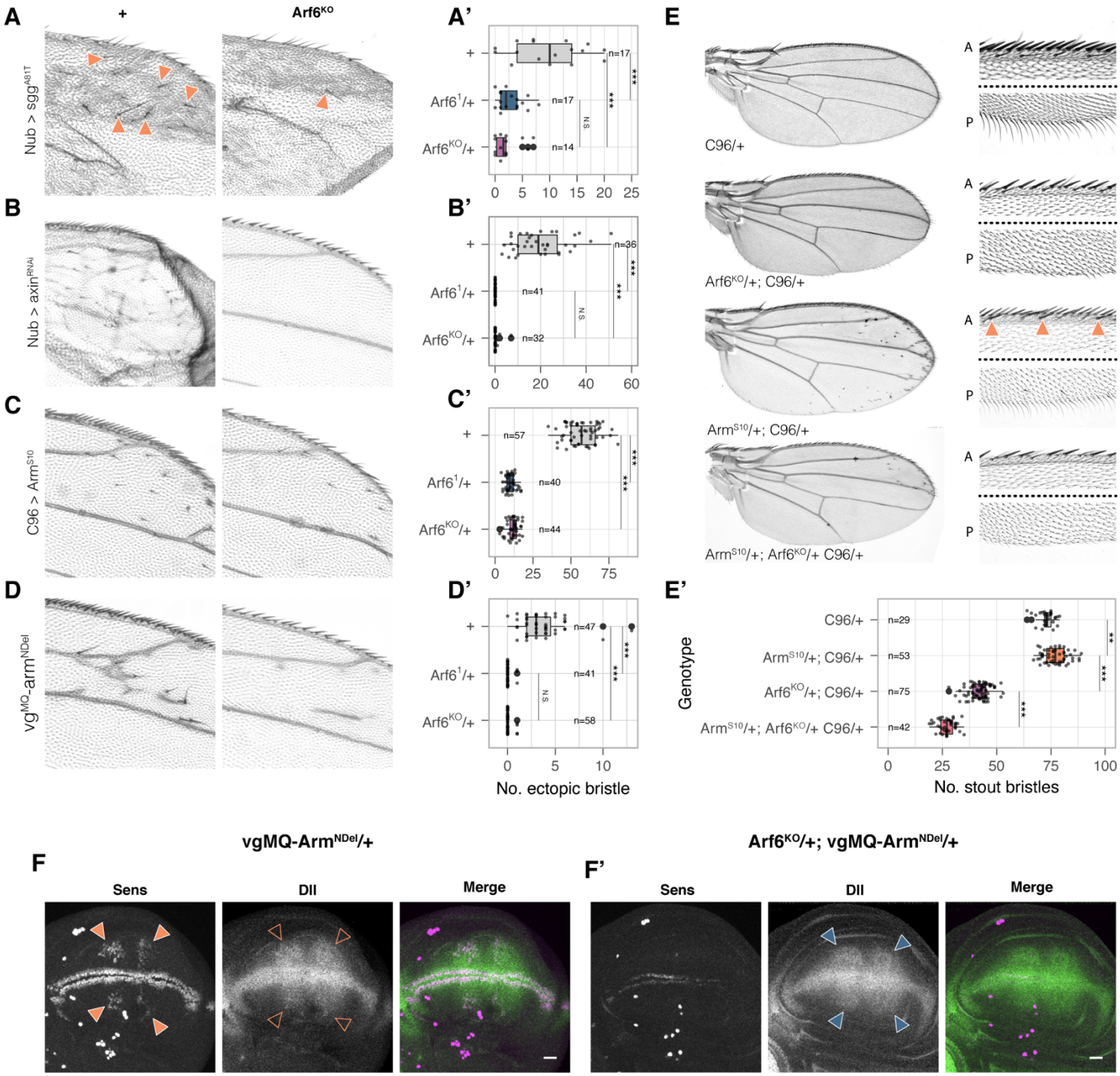


Figure 3. Epistatic analysis shows that Arf6 acts downstream of Arm stabilisation.

(**A)** Dominant negative Sgg (sgg^{A81T}) expressed 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-gal) 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 WT background (solid orange arrowheads). (**E')** Quantification of stout mechanosensory bristles. Significance values resulting from post-hoc pairwise contrasts are reported by the following abbreviations: N.S. = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.001$, *** = $p \leq 0.001$. Ectopic bristles numbers were modelled using Zero Inflated negative binomial GLM. (**F)** vgMQ-Arm^{NDEL} induces ectopic Sens (closed orange arrowheads) and Dll (open orange arrowheads) in a WT background. (**F')** Ectopic Sens, but not Dll (closed blue arrows) is suppressed in the Arf6^{KO} background. In the merges, Sens is in magenta, Dll in green.

Figure 4

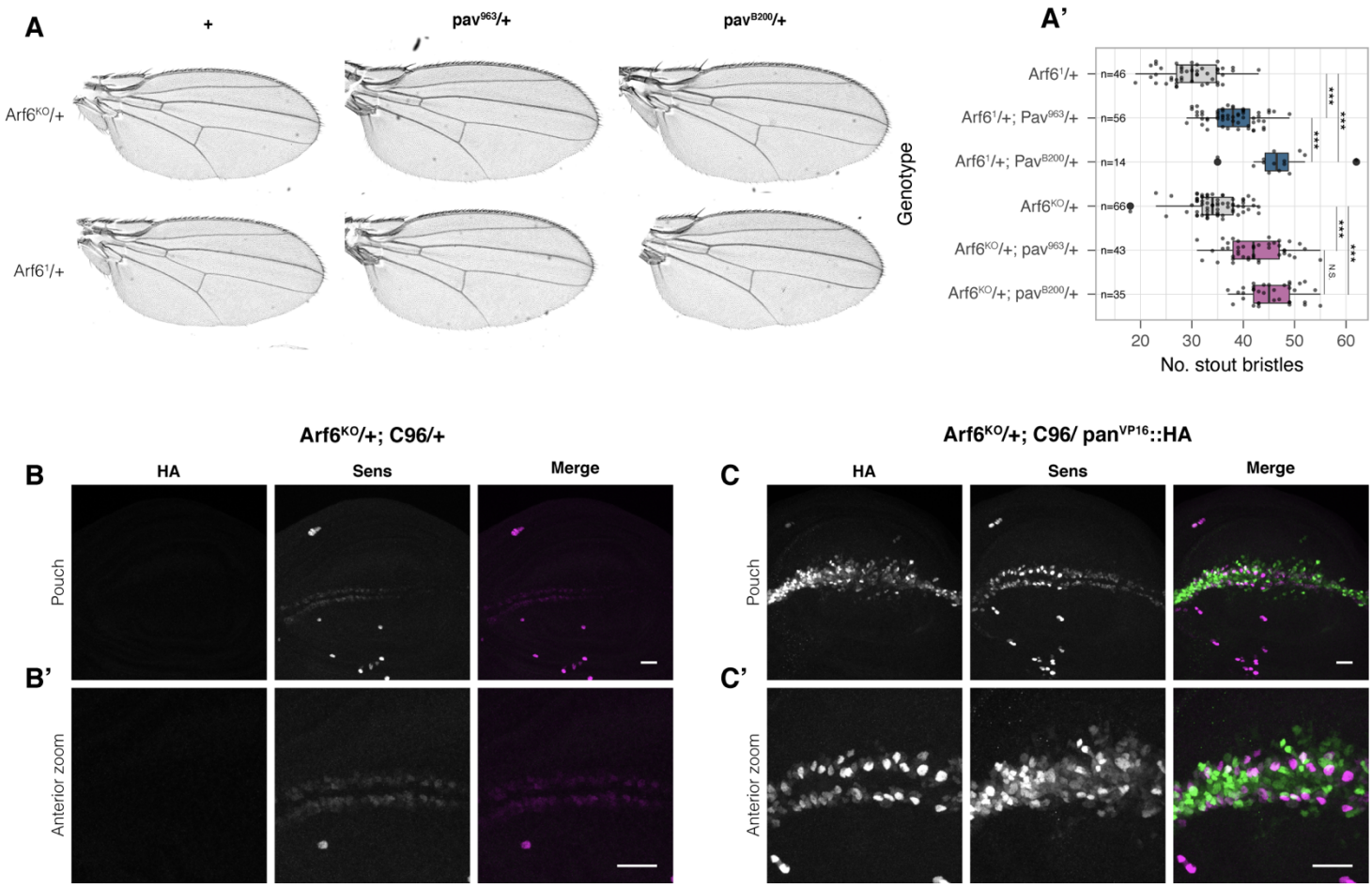


Figure 4. Pan-VP16 rescues Sens expression at the wing margin of *Arf6* mutant.

(**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, Pan-VP16::HA is magenta and Sens is green).

Figure S1

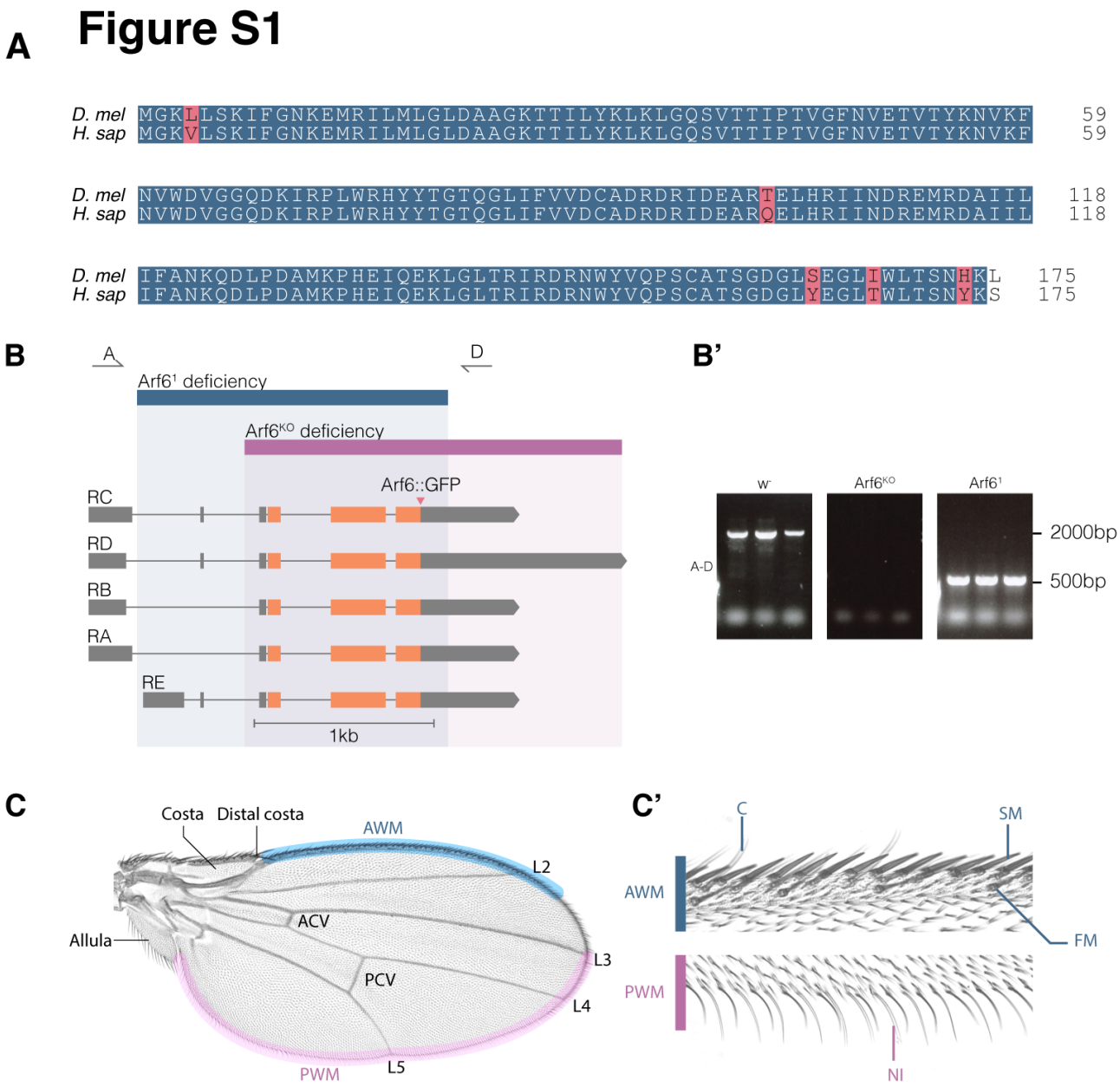


Figure S1. *Arf6*^{1} and *Arf6*^{KO} are null alleles delete.

(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 (*Arf1* 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 in 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 the *Arf6*^{1} deletion. Three biological replicates are shown for each genotype. The deficiency in *Arf6*^{KO} was analysed in detail 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.

Figure S2

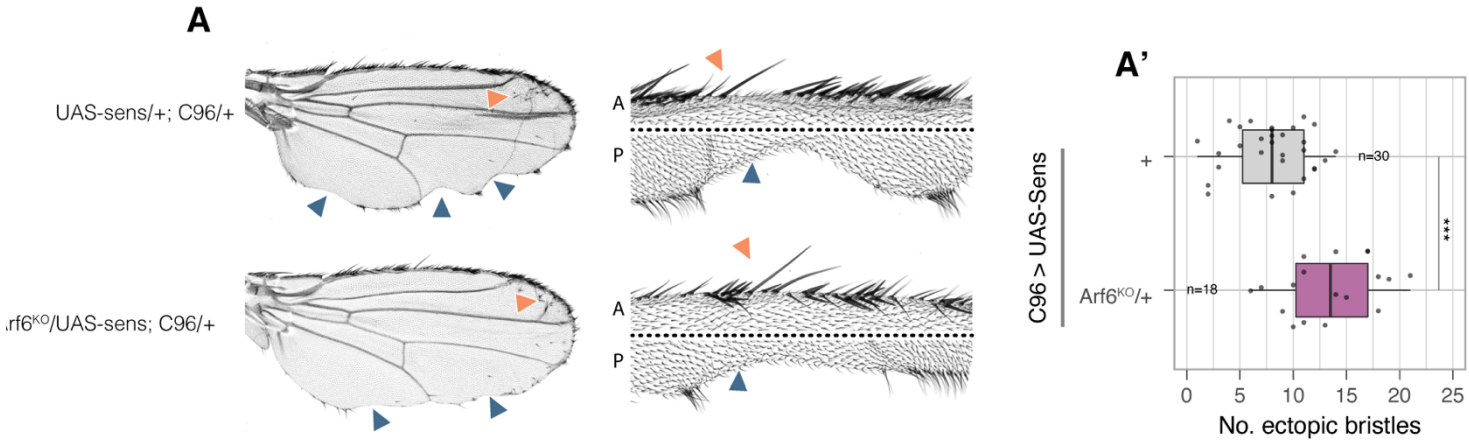


Figure S2. Sens proneural activity is not impaired in *Arf6* mutants

(A) Over-expressing wild-type Sens induces ectopic bristles (closed orange arrowheads) and wing margin notching (closed blue arrowheads) in both a *WT* and *Arf6^{KO}* background, resembling the previously described hypermorphic *sens^{Lyra}* 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 the absence of *Arf6*. Ectopic bristle numbers were modelled using a GLM with Poisson errors. Significance values resulting from post-hoc pairwise contrasts are reported by the following abbreviations: N.S. = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.001$, *** = $p \leq 0.001$.

Figure S3

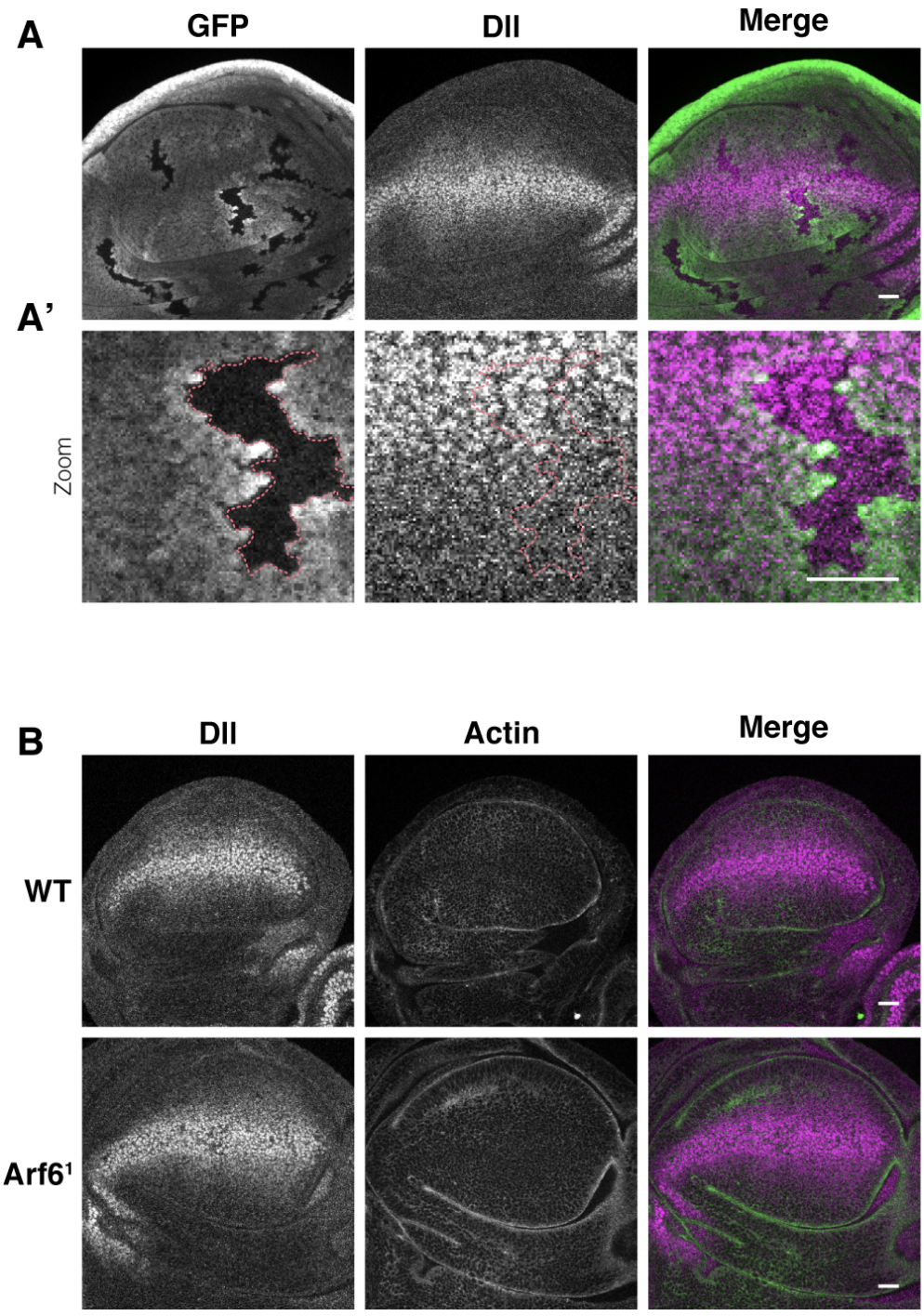


Figure S3. Distal-less expression is not affected in the absence of Arf6.

(A) Dll staining in *Arf6*[1] clones (labelled by the absence of GFP) with zoom shown in **(A')** n=23**. (B)** Dll staining in both *WT* and full *Arf6*[1] mutant discs. In the merges, Dll is in magenta, GFP in green (A-A') and actin in green (B). All scale bars represent 20μm. WT n=5, *Arf6*[1] n = 8.

Figure S4

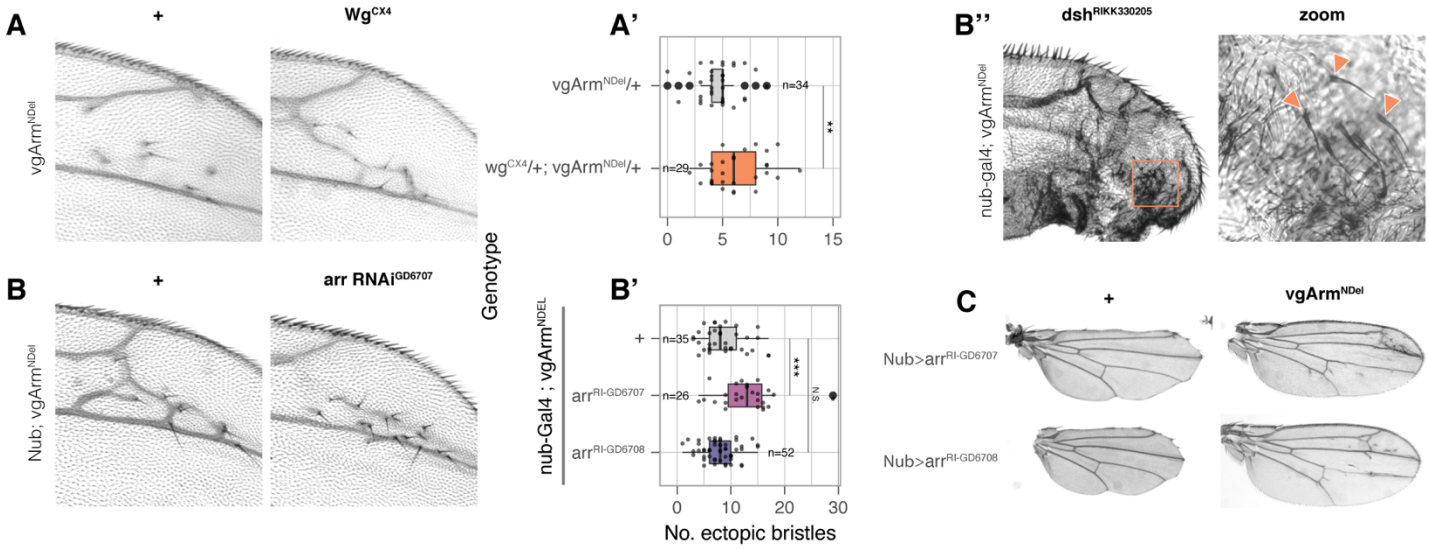


Figure S4. vg_{MQ}-Arm^{NDel} acts independently of endogenous Wg signalling

(A) The ectopic bristles induced by *vg_{MQ}-Arm^{NDel}* (*$vgArm^{NDel}$*) are not dominantly reduced in a heterozygous *wg* background (*wg^{CX4}/+*) indicating that *$vgArm^{NDel}$* 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 *vgMQ-Arm^{NDEL}*, again suggesting that the signalling activity of *$vgArm^{NDel}$* 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 vgMQ-Arm^{NDEL}. No quantification is shown due to the difficulty of reliably discerning ectopic bristles in this context. **(C)** *$vgArm^{NDEL}$* is able to rescue the loss of the wing margin induced by knocking down *arr* with *nub-gal4*. This shows that $vgArm^{NDel}$ can recapitulate WT canonical Wg signalling activity and is sufficient to rescue a moderate Wg wing margin phenotype induced by the *arr* knock-down.

Figure S5

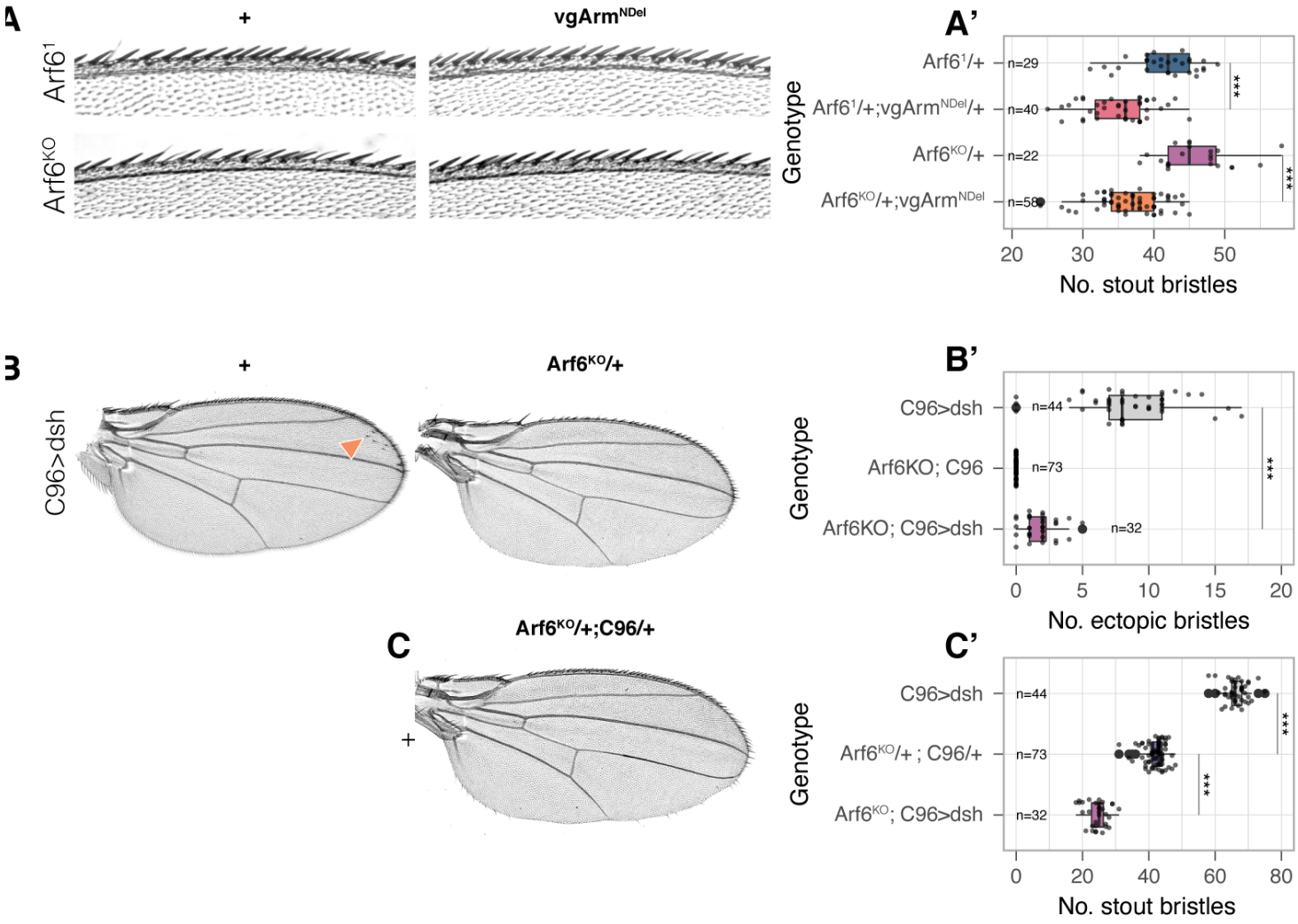


Figure S5. Wg activation by vgArm^{NDel} and dsh over-expression enhance the Arf6 phenotype

(A) The loss of stout wing margin bristles in an *Arf6* mutant background is enhanced by the expression of *vgArm^{NDEL}* (quantified in **A'**). **(B)** *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 *Arm^{NDEL}*, the number of stout mechanosensory bristles is reduced by the over-expression of *dsh* in an *Arf6^{KO}* background **(**quantified in **C').**

Figure S6

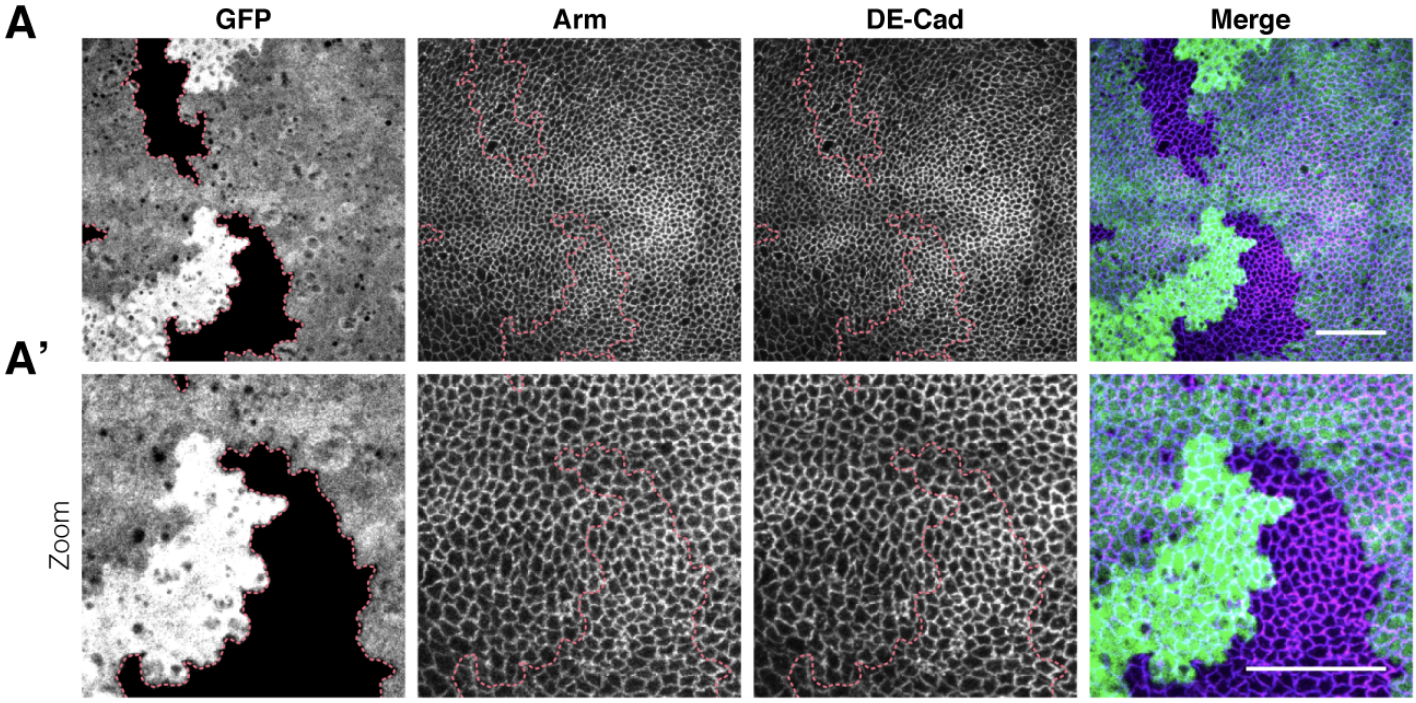


Figure S6. Arm and DE-Cadherin localisation is not affected in Arf6 mutants

(A) Staining for the adherens junction components Arm and DE-Cadherin in *Arf6*^{KO} clones. (**A'**) A zoom of the *Arf6*^{KO} mutant clone is shown. In the merges, GFP is in green, Arm is in blue and DE-Cad is in magenta. n = 28.

Figure S7

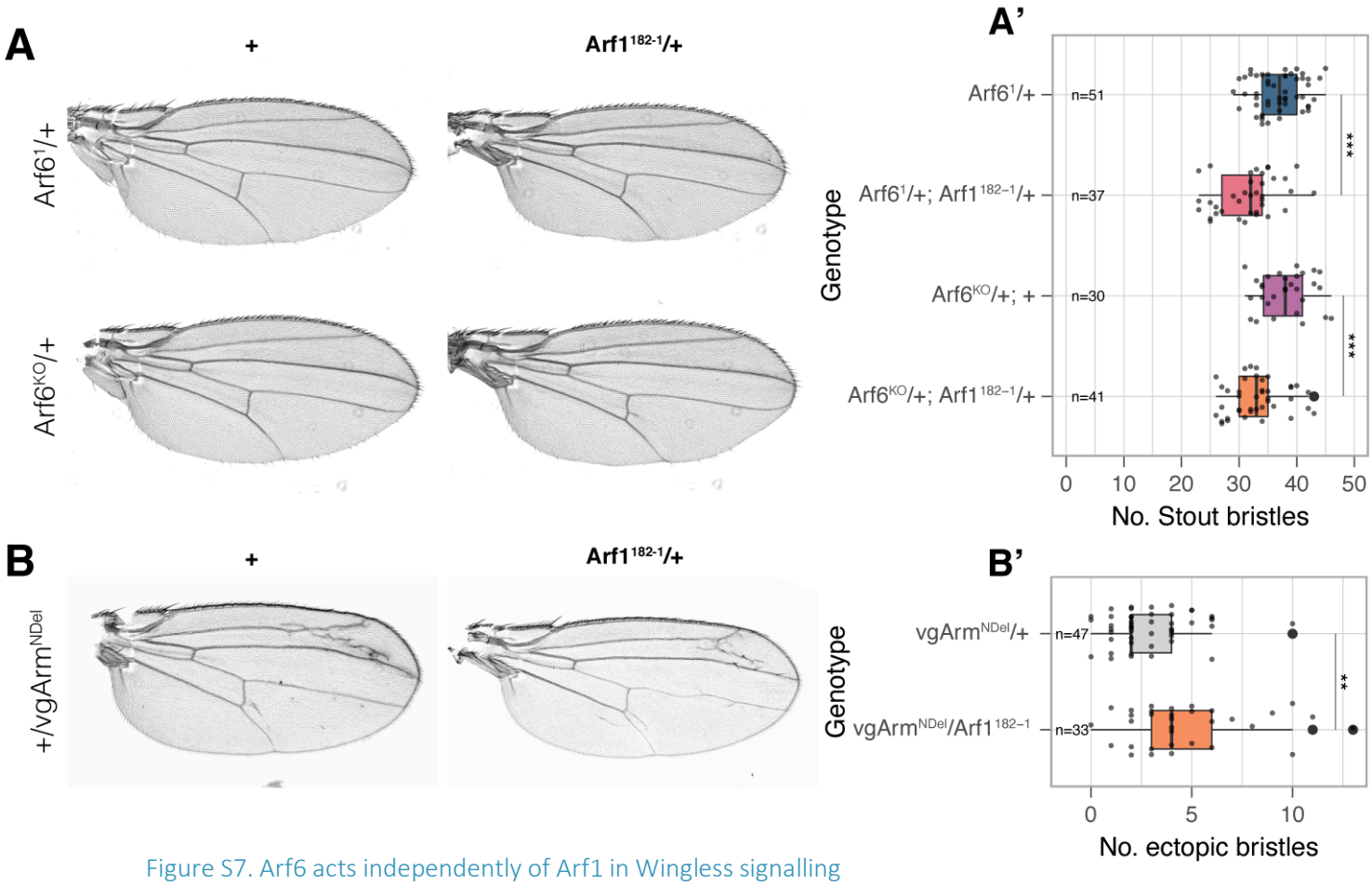


Figure S7. Arf6 acts independently of Arf1 in Wingless signalling

(**A)** Consistent with an upstream role of Arf1 in Wg signal transduction (Hemalatha et al., 2016), removing a copy of *Arf1* (*Arf1*$^{182\text{-}1}$ (West et al., 2017)) in an *Arf6* mutant background mildly enhances the loss of anterior stout mechanosensory bristles **(**quantified in **A'). (B)** Unlike the *Arf6* mutations, the *Arf1* mutation does not reduce the number of ectopic bristles induced by *vgMQ-Arm*^{NDEL} **(**quantified in **B').** This indicates that Arf1 and Arf6 play distinct roles in Wg signalling, and that the margin bristle phenotype we observed is specific to *Arf6*.

Figure S8

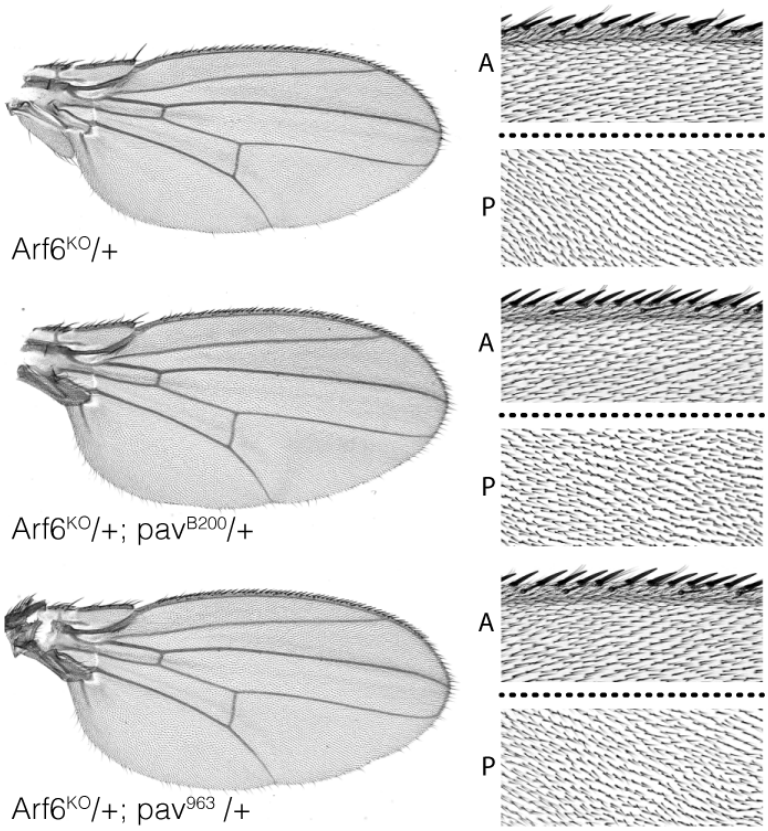


Figure S8. The genetic interaction between Arf6 and pav does not induce cytokinesis defects

(**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.

Figure S9

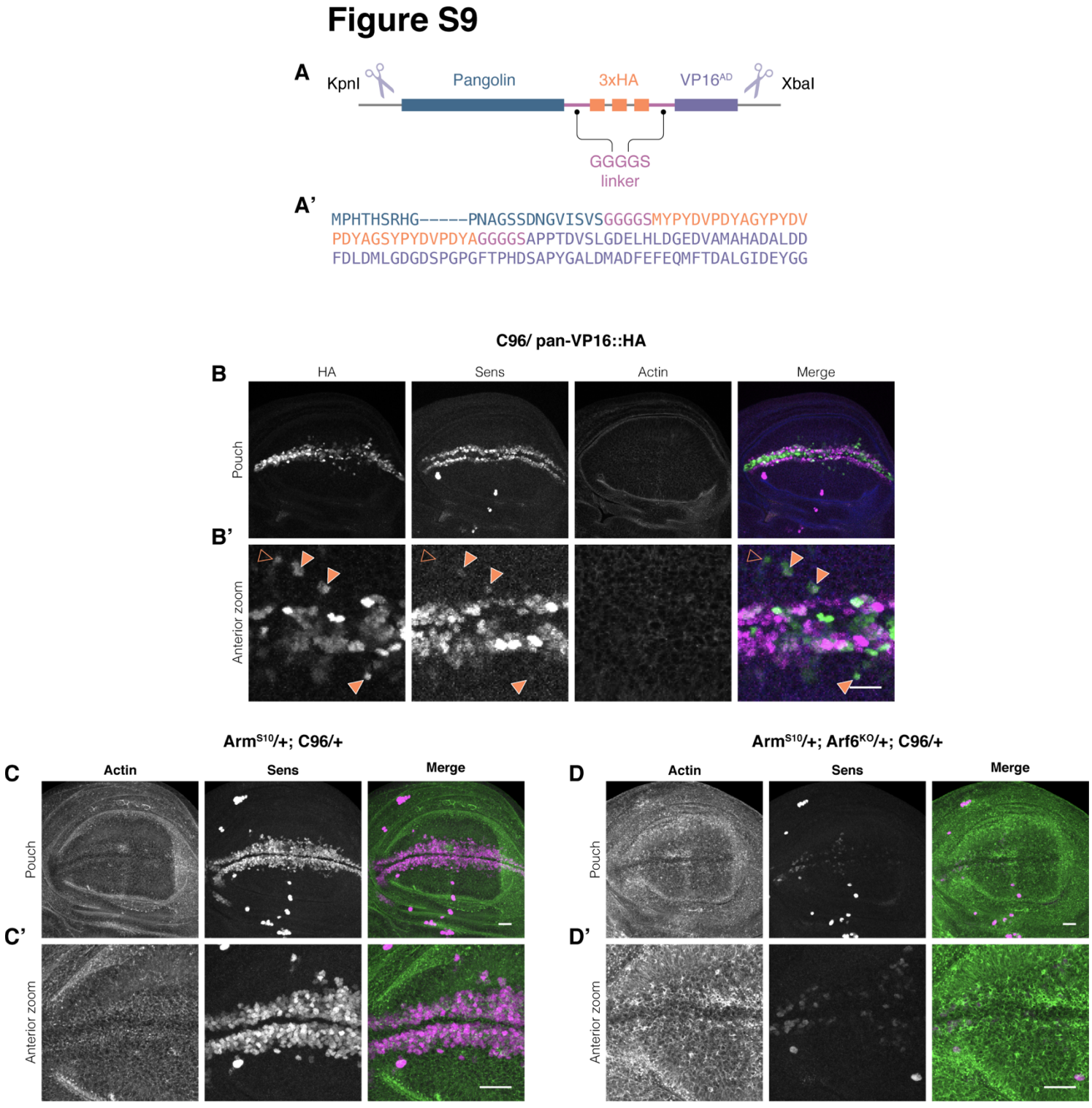


Figure S9. Structure and activity of Pan-VP16

(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 correspond to the colours in the schematic shown in **(A). (B)** expressing *pan-VP16* at room temperature using *C96-Gal4* induces a low level of ectopic Sens (closed orange arrowhead) close to the wing margin, but is not sufficient to activate Sens further from the margin (open orange arrowheads) (*Pan-VP16::HA* is shown in green and Sens in magenta in the merge). **(C)** Expressing Arm^{S10} under the same conditions as *pan-VP16* leads to extensive ectopic Sens surrounding the D/V margin. **(C')** zoom of the anterior margin is shown. **(D)** expressing Arm^{S10} in a heterozygous $Arf6^{KO}$ wing imaginal disc does not rescue endogenous Sens. Ectopic Sens is almost completely suppressed. **(D')** zoom of the anterior compartment is shown.

Supplementary references

Hemalatha, A., Prabhakara, C. and Mayor, S. (2016). Clathrin-Independent Endocytosis of Wingless via Clic/Geec Pathway is Necessary for Effective Signalling in Drosophila Wing Discs. *Proc. Natl. Acad. Sci.* **110**, 595a.

Huang, J., Zhou, W., Dong, W., Watson, A. M. and Hong, Y. (2009). Directed, efficient, and versatile modifications of the Drosophila genome by genomic engineering. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 8284–8289.

Nolo, R., Abbott, L. A. and Bellen, H. J. (2001). Drosophila Lyra mutations are gain-of-function mutations of senseless. *Genetics* **157**, 307–315.

West, J. J., Zulueta-Coarasa, T., Maier, J. A., Lee, D. M., Bruce, A. E. E., Fernandez-Gonzalez, R. and Harris, T. J. C. (2017). An Actomyosin-Arf-GEF Negative Feedback Loop for Tissue Elongation under Stress. *Curr. Biol.* 27, 2260-2270.e5.

Complementary Results

The role of Arf6 in Wingless signalling

How did we identify Pavarotti as a potential candidate?

Arf6 has not previously been implicated in the Wg signalling pathway, and the previously described mechanisms for the activity of Arf6 in Wnt signalling were not compatible with our data (Summarised in Figure 9). To identify Arf6 effectors that could potentially provide the functional link with Wg signalling, I began with a broad bioinformatic approach. I carried out a literature review to identify proteins whose loss- or gain of function had been shown to modulate the activity of a constitutively active form of Armadillo, Arm^{S10} (Pai et al., 1997) (listed in Table 1)**.** I then generated a high-confidence protein-protein interaction map including these factors, known Wg pathway components (based on Flybase pathway annotations) and Arf6 (Figure 10) based on experimentally tested interactions. This process revealed that Arf6 integrates into the network through a direct interaction with Pavarotti (Pav), the *Drosophila* homologue of Mammalian Kinesin Like Protein I (MKLPI) (Makyio et al., 2012). Pav has previously been shown to act as a negative, nuclear regulator of Wg signalling during embryonic development (Jones et al., 2010). Importantly, the activated Arf6-GTP and Pav were shown to physically interact (Dyer et al., 2007), and the crystal structure of a heterotetrametric complex of MKLP1 and mammalian Arf6-GTP has been resolved (Makyio et al., 2012). This approach is strongly biased towards the identification of known Wg pathway regulators and relies on previously described protein-protein interactions.

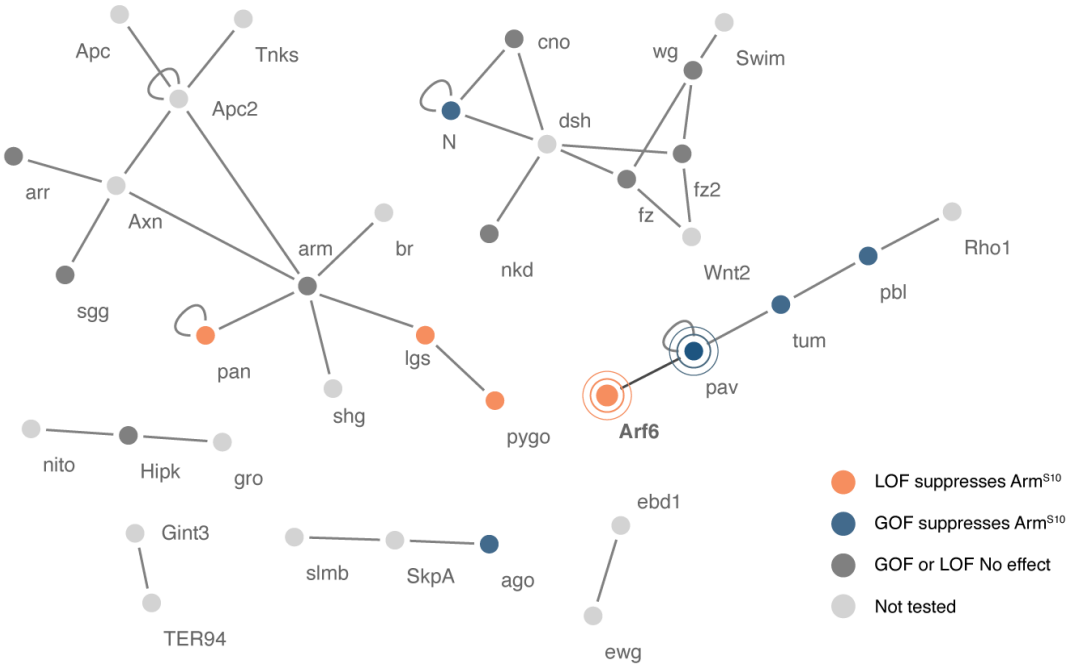


Figure 10 Identification of candidates linking Arf6 to Wg signalling

A protein-protein interaction network containing regulators of canonical Wg signalling, and proteins whose gain or loss of function have previously been shown to repress the activity of ArmS10 based on a literature review (Table 1). The protein-protein interaction network was generated using the Molecular Interaction Search Tool (MIST), filtering out low ranking results and detached nodes (Hu et al., 2018). Protein interactions are based on data from the FlyBi project (https://flybi.hms.harvard.edu/results.php**).** Protein-protein interactions are determined through sequential yeast two-hybrid assays, producing high confidence interactors. The figure was generated using CytoScape, in which node colour was mapped to the ability of the protein to suppress ArmS10 activity either through its over-expression (blue circles) or loss of function (orange circles). Protein not previously tested in an ArmS10 background are represented in light grey, while dark grey circles indicate proteins that were tested, but that did not affect the ArmS10 phenotype. Arf6 integrates into the network through a direct physical interaction with the MKLP1 homologue, Pavarotti (Pav).

Protein	Condition	Effect on Arm^{S10}	References
Ago	GOF	S	(Nam and Cho, 2020)
Als	LOF	NS	(Reim et al., 2014)
Arf51F	LOF	S	This study
Arm	LOF	NS	(Pai et al., 1997; Thompson, 2004; Tolwinski and Wieschaus, 2001)
Arr	LOF	NS	(Widmann and Dahmann, 2009)
Cno	LOF	NS	(Carmena et al., 2006)
Cut	GOF	NS	(Buceta et al., 2007)Buceta et al 2007
Cut	LOF	NS	(Buceta et al., 2007)
Ebd1	LOF	S	(Benchabane et al., 2011)
Flw	LOF	S	(Hall et al., 2019; Luo et al., 2007)
Ft	GOF	E	(Jaiswal et al., 2006)
Fz	LOF	NS	(Tolwinski et al., 2003)
Fz2	LOF	NS	(Tolwinski et al., 2003)
Hipk	LOF	NS	(Lee et al., 2009)
Hyd	LOF	S	(Flack et al., 2017)
Ift-a	LOF	NS	(Balmer et al., 2015; Vuong et al., 2018)
Klp64d	LOF	NS	(Vuong et al., 2018)
Lgs	LOF	S	(Hoffmans and Basler, 2004; Hoffmans and Basler, 2007; Hoffmans et al., 2005; Kramps et al., 2002)
N	GOF	S	(Hayward et al., 2005; Langdon et al., 2006; Muñoz-Descalzo et al., 2011; Sanders et al., 2009)
Nkd	GOF	NS	(Rousset, 2001)
Nmo	GOF	S	(Mirkovic et al., 2011)
Pan	LOF	S	(Balmer et al., 2015; Hoffmans and Basler, 2004; van de Wetering et al., 1997)
Pav	GOF	S	(Jones et al., 2010)
Pbl	GOF	S	(Greer et al., 2013)
Pp1	LOF	NS	(Luo et al., 2007)
Pygo	LOF	S	(Thompson et al., 2002)
Sgg	LOF	NS	(Pai et al., 1997)
Sgg	GOF	NS	(Steitz et al., 1998)
Sgl	LOF	NS	(Häcker et al., 1997)
Sno	GOF	NS	(Quijano et al., 2010)
Tsh	LOF	S	(Waltzer et al., 2001)
Tum	GOF	S	(Jones et al., 2010)
Tws	LOF	NS	(Bajpai, 2004)
Wg	LOF	NS	(Cox and Baylies, 2005; Larsen et al., 2008; Pai et al., 1997; Tang et al., 2012)
Wnk	LOF	NS	(Serysheva et al., 2014)
Yan	GOF	S	(Olson et al., 2011)

Table 1 Proteins known to affect ArmS10 activity

Proteins shown to affect the signalling activity of the constitutively active Arm^{S10}. Proteins are classified based on whether their gain-of-function (GOF) or loss-of-function (LOF) was studied. The effect on Arm^{S10} is shown as either suppression (S), enhancement (E), or no suppression (NS). Proteins were identified through a literature review. The relevant publications are listed.

The two *pav* mutant alleles also induced an increase in the number of wing margin bristles in a homozygous Arf6 mutant background, (Figure 11A and A'). However, the rescue was less

pronounced than in the double heterozygous combination. This result is consistent with Pav acting genetically downstream of Arf6 in wing margin development.

Tumbleweed (Tum) is a Rho GAP that, together with Pav, forms the centralspindlin complex necessary for the formation of the central spindle during cytokinesis (Mishima et al., 2002). Tum was identified alongside Pav as a downstream repressor of wingless signalling during embryonic development (Jones and Bejsovec, 2005; Jones et al., 2010) (Figure 10). Based on this I tested whether Pav and Tum act redundantly in the context of wing margin development, and whether reducing *tum* expression could also rescue the *Arf6* phenotype in the same manner as I observed in the *Pav* mutant. In contrast to *pav,* the genetic interaction between *Arf6* and two independent *tum* alleles caused a strong enhancement of the Arf6 mutant phenotype (Figure 11B and B'). This indicates that in contrast to their roles in embryonic development, there is a lack of functional redundancy between Pav and Tum in the development of the wing margin (Jones et al., 2010). It is not clear from the results presented by Jones et al. (Jones et al., 2010) whether Pav and Tum act redundantly, or whether their interaction is required for their non-canonical activity as Wg regulators (Jones and Bejsovec, 2005; Jones et al., 2010). Double mutant *pav* / *tum* embryos could not be phenotypically distinguished from single mutants, suggesting a more complex interaction than a simple functional redundancy (Jones et al., 2010). Mutations affecting *tum* have been shown to lead to a relocalisation of Pav *in vivo* suggesting that reducing the expression of *tum* could interfere with the ability of Pav to interact with Arf6, 'relieving' Pav of its suppression (Mishima et al., 2002; Somers and Saint, 2003). Although we can conclude that Pav acts genetically downstream of Arf6, the nature of this interaction is not known.

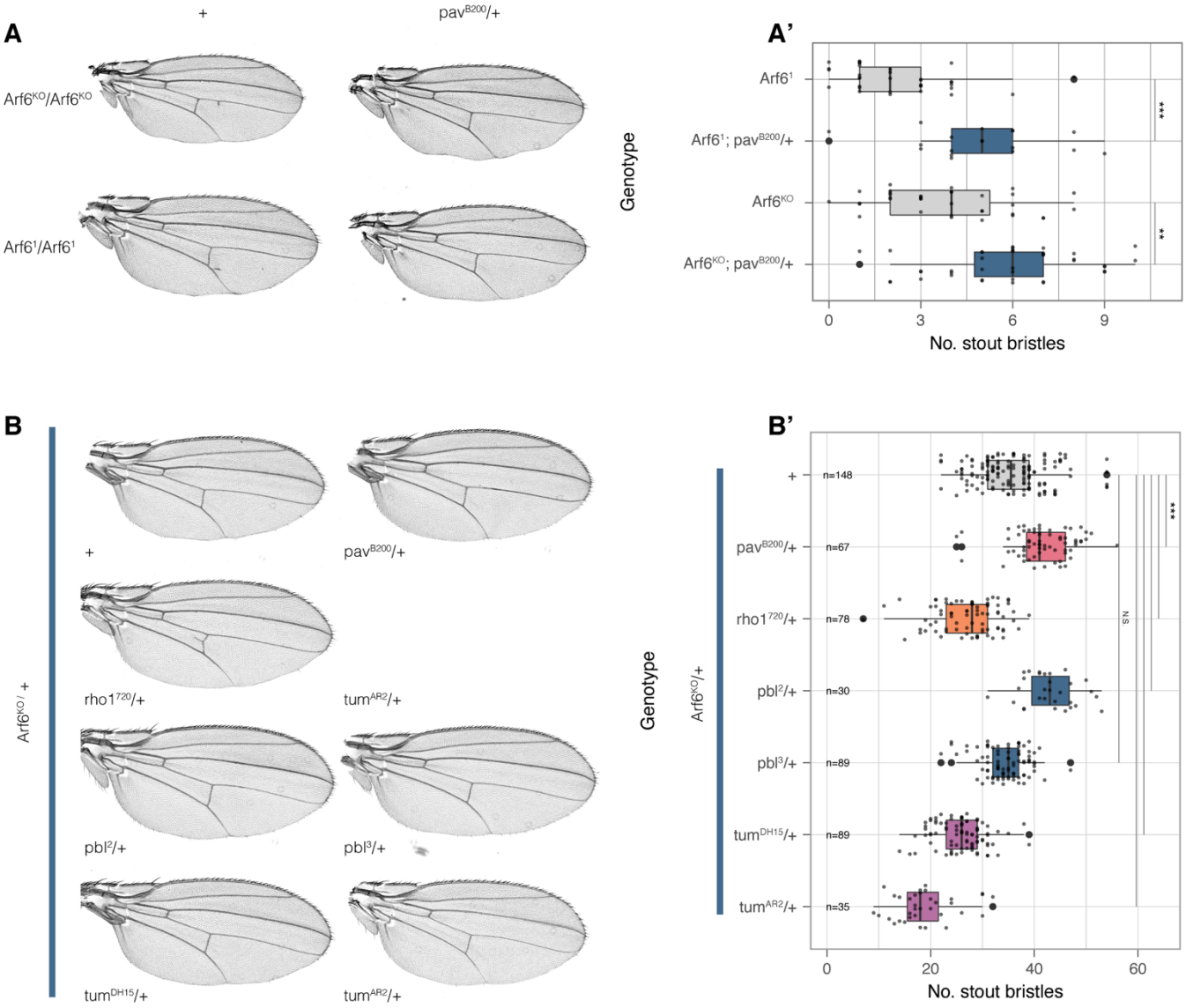


Figure 11 Genetic interaction between *pav*, *tum* and *Arf6*

A Heterozygous *pav*^{B200} or *pav*^{963} in a homozygous *Arf6*^{KO} background provides a mild, but statistically significant increase in the number of stout margin bristles in the anterior wing margin. Quantified in **A'.** **B** wings resulting from the dominant genetic interactions between *Arf6*^{KO} and *pav*, *tum, Rho1*, and *pbl*, alleles. Stout mechanosensory bristles are quantified in **B'.**

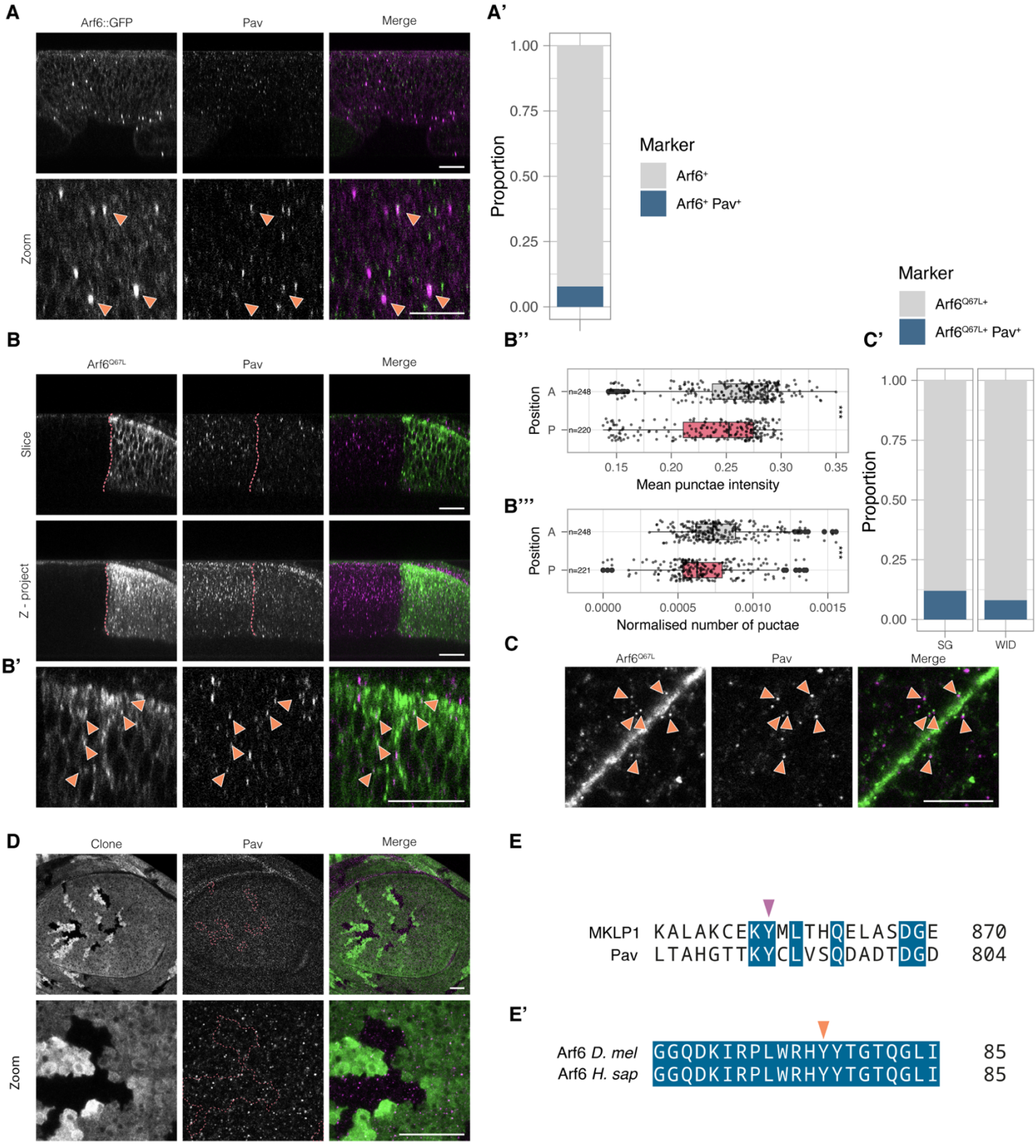
We next assessed whether the previously described interaction between Arf6 and Pav/MKLP1 was involved in the Arf6 phenotype. Our model suggests that Arf6 suppresses the non-canonical activity of Pav as a Wg regulator, and that this Pav function would be de-repressed in the Arf6 mutant, inducing a Wg loss of function phenotype (Jones et al., 2010). Based on the direct interaction that has previously been described between Arf6 and Pav/MKLP1 (Dyer et al., 2007; Makyio et al., 2012), I began by staining the wing imaginal discs of flies carrying endogenously GFP-tagged Arf6 (Arf6::GFP (Huang et al., 2009)) with antibodies raised against endogenous Pav (Lie-Jensen et al., 2019). Despite

both proteins having a predominantly punctate distribution in the wing imaginal disc, I observed a low level of co-localisation between them (Figure 12 A, A'). Intriguingly, when Arf6 and Pav punctae did overlap, the overlap was often incomplete (orange arrowheads figure Figure 12). The formation of the Arf6-MKLP1 heterodimer depends on Arf6 being in the GTP-bound conformation (Makyio et al., 2012). Under physiological conditions, only a small proportion of Arf6 is found in a GTP-bound form, which could help explain the low level of co-localisation between Pav and Arf6.

I therefore tested the effect of expressing the GTP-locked form of Arf6, Arf6^{Q67L} on the localisation of endogenous Pav (Figure 12, B'). I drove the expression of Arf6^{Q67L}::GFP over-night in the posterior compartment of the wing imaginal disc using hh-gal4. Arf6^{Q67L} localised to the cell membrane, cortex and in large punctae (Figure 12, B', C). Pav punctae were again observed adjacent to, and overlapping with, Arf6^{Q67L} punctae, however the level of co-localisation was similar to that of WT Arf6::GFP (orange arrowheads Figure 12 B', quantified in C'). Arf6^{Q67L} expression induced a reduction in both the number and the mean intensity of the Pav punctae compared to the anterior domain in which Arf6^{Q67L} was not expressed (Figure 12 B, B'', B'''). To gain greater subcellular resolution, I also stained for Pav in the large epithelial cells of salivary glands expressing Arf6^{Q67L} (figure Figure 12 C, quantified in C'). Cytoplasmic punctae with co-occurring Pav and Arf6^{Q67L} were once again readily visible. Although these data show that a large pool of Pav is not associated with Arf6, Arf6^{Q67L} it is able to regulate the subcellular levels of Pav in the wing imaginal disc. Interestingly, Pav punctae are still present in the posterior compartment of the wings expressing Arf6^{Q67L}. This can be interpreted as Arf6^{Q67L} specifically affecting only a portion of Pav, or Arf6^{Q67L} affecting the steady-state dynamics of Pav, leading to an apparent reduction in Pav levels. Live imaging of the effects of Arf6, or Arf6^{Q67L} on Pav dynamics could provide an insight into this process. Furthermore, the identity of the subcellular compartments in which Pav and Arf6 colocalise is not known. Staining wing discs for Arf6, Pav and markers of early (Rab5), recycling (Rab4 and Rab11), and late (Rab7) endosomes would provide valuable information regarding the potential mechanisms linking Arf6 and Pav. Arf6 has previously been shown to colocalise with Rab11, Rab4 and Pav at the central spindle, however only Pav was present at endogenous levels, whilst *Rab4*, *Rab11* and *Arf6* were expressed under ubiquitous promoters (Dyer et al., 2007). Although I can compare between the anterior and posterior compartments of the wing, I cannot directly compare the effect of endogenous Arf6::GFP with the effect of expression Arf6^{Q67L} expression due to the differences in expression levels. A control in which WT Arf6::GFP is expressed under the same driver as Arf6^{Q67L} would need to be carried out.

To test whether the *Arf6* loss of function affects the distribution of Pav, I stained for Pav in *Arf6* mutant clones. The loss of Arf6 did not induce any apparent change in the localisation of Pav

(Figure 12**D)**. These results indicate that under physiological conditions, Arf6 is not a major regulator of Pav localisation. This is compatible with the dispensability of Arf6 for the completion somatic cytokinesis in *Drosophila,* and potentially mammals (Jackson and Bouvet, 2014). I have so far only considered the possibility that Arf6 regulates a non-canonical role of Pav as a regulator of Wg signalling. It has been proposed that Pav is responsible for recruiting Arf6-GTP to the Flemming body during cytokinesis (Makyio et al., 2012). It is possible that this mechanism has been co-opted in the context of Wg signalling, and that Pav is responsible for Arf6 localisation, or that Arf6-GTP sequesters a small pool of Pav involved in Wg signalling. The residues required for the binding of Arf6 and MKLP1 are conserved in *Drosophila* Arf6 and Pav (closed pink and orange arrow heads respectively Figure 12E) (Makyio et al., 2012)**.** This suggests that it would be possible to generate Arf6-binding deficient Pav (Pav^{Y792A}) and Pav-binding deficient Arf6 ($Arf6^{Y77A}$) based on constructs described by Makyio et al. (2012). Generating the Y77A mutation in an $Arf6^{Q67L}$ background would provide a means of testing whether the change in Pav localisation depends on the direct binding of Arf6 and Pav, or whether Arf6 indirectly affects Pav localisation through its trafficking. It may be possible to produce these mutations in the endogenous loci, as the binding of Pav to Arf6 is not necessary for somatic cytokinesis, and the *Arf6* mutants are viable (this assumes that there are no pleiotropic effects of either mutation). Whether introducing an endogenous, Arf6-binding deficient Pav into an *Arf6* mutant background can rescue the *Arf6* mutant phenotype would provide strong evidence for the potential requirement for the direct binding of Arf6 and Pav.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Arf6::GFP
Pav
Merge
Zoom
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Proportion
1.00
0.75
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Marker
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B
Arf6Q67L
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D
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Pav LTAHGTTKYCLVSQDADTDGD 804
E'
Arf6 D. mel GGQDKIRPLWRHYYTGTQGLI 85
Arf6 H. sap GGQDKIRPLWRHYYTGTQGLI 85

Figure 12 Characterising Pav localisation in the wing imaginal disc

A Arf6::GFP L3 wing imaginal discs stained for endogenous Pav. Z-sections are shown. There is a low level of co-localisation between the two proteins (7.7% of Arf6 punctae are positive for Pav) (closed orange arrowheads **A**). The proportion of Arf6 punctae labelled with Pav is quantified in **A'**. **B** expressing a GTP-locked form of Arf6 (Arf6Q67L) in the posterior compartment of the wing imaginal disc induces a reduction in both the average intensity and number of Pav punctae (quantified in **B''** and **B'''** respectively. Colocalising punctae are shown in **B'**, and quantified in **C'**). **C** Partial co-localisation of $Arf6^{Q67L}$ and endogenous Pav is also observed in the epithelial cells of the salivary glands. (colocalization quantified in **C'**). **D** Pav localisation is not noticeably affected in Arf6KO clones (marked by the absence of GFP). **E** An alignment of a region of Pav with MKLP1 involved in binding with Arf6. A conserved tyrosine residue essential for binding with Arf6 is highlighted (pink arrowhead). **E'** a region of Arf6 necessary for binding with MKLP1. A conserved tyrosine residue essential for binding is indicated with an orange arrowhead. The residues necessary for binding of Arf6 with MKLP1 are based on those described in by Makyio et al. (2012). Scale bars represent 20 µm.

Which Arf6-GEFs regulate Arf6 during wing margin patterning?

The manuscript was predominantly focussed on the role of Arf6 in Wg signalling and did not address the mechanisms through which Arf6 is activated in the context of wingless signalling. I began by testing whether knocking down the predicted *Drosophila* Arf6 GEFs, *EFA6*, *steppke (Step,* encoding a Cytohesin family Arf-GEF*)* and *schizo (Siz,* encoding a GEP100/BRAG2 homologue*)* was sufficient to phenocopy the Arf6 mutant phenotype. EFA6 is considered to act as a specific Arf6-GEF, and shows limited exchange activity on Arf1 *in vitro* (Franco et al., 1999). Conversely, the Cytohesins typically show a greater level of activity on Arf1 than Arf6 (Macia et al., 2001). The Arf-GEFs all contain a catalytic Sec7 domain necessary to mediate exchange activity on the Arfs, and a PH domain necessary for their recruitment to lipid membranes (Jackson and Casanova, 2000). GTP-bound Arf6 was previously shown to recruit and activate the Arf1 GEF cythoesin-2 to the plasma membrane, leading to an increase of Arf1 activity (Stalder and Antonny, 2013). This regulatory cascade indicates that Arf1 activation can be coupled to Arf6 activity.

Knocking down *EFA6* and *siz* did not phenocopy the Arf6 phenotype (Figure 13A). Knocking down *step* throughout the wing induced a reduction in the number of bristles in the anterior wing margin (Figure 13A, A'), and small portions of the posterior wing margin lacking bristles (blue closed arrowheads, Figure 13A). The *step* knock-down also introduced occasional duplications of stout bristle shafts in the anterior margin (orange closed arrowhead, Figure 13A), a phenotype not present in *Arf6* mutant wings. The loss of wing margin bristles suggested that Step could be responsible for regulating the activity of Arf6 in the context of wing margin development. Knocking down *step* in a heterozygous *Arf6* mutant background enhanced the *Arf6* margin phenotype, but also introduced an apparent

planar cell polarity defect in the wing blade, potentially due to a suppression of Arf1 activity (Carvajal-Gonzalez et al., 2015) (Figure 13B, B').

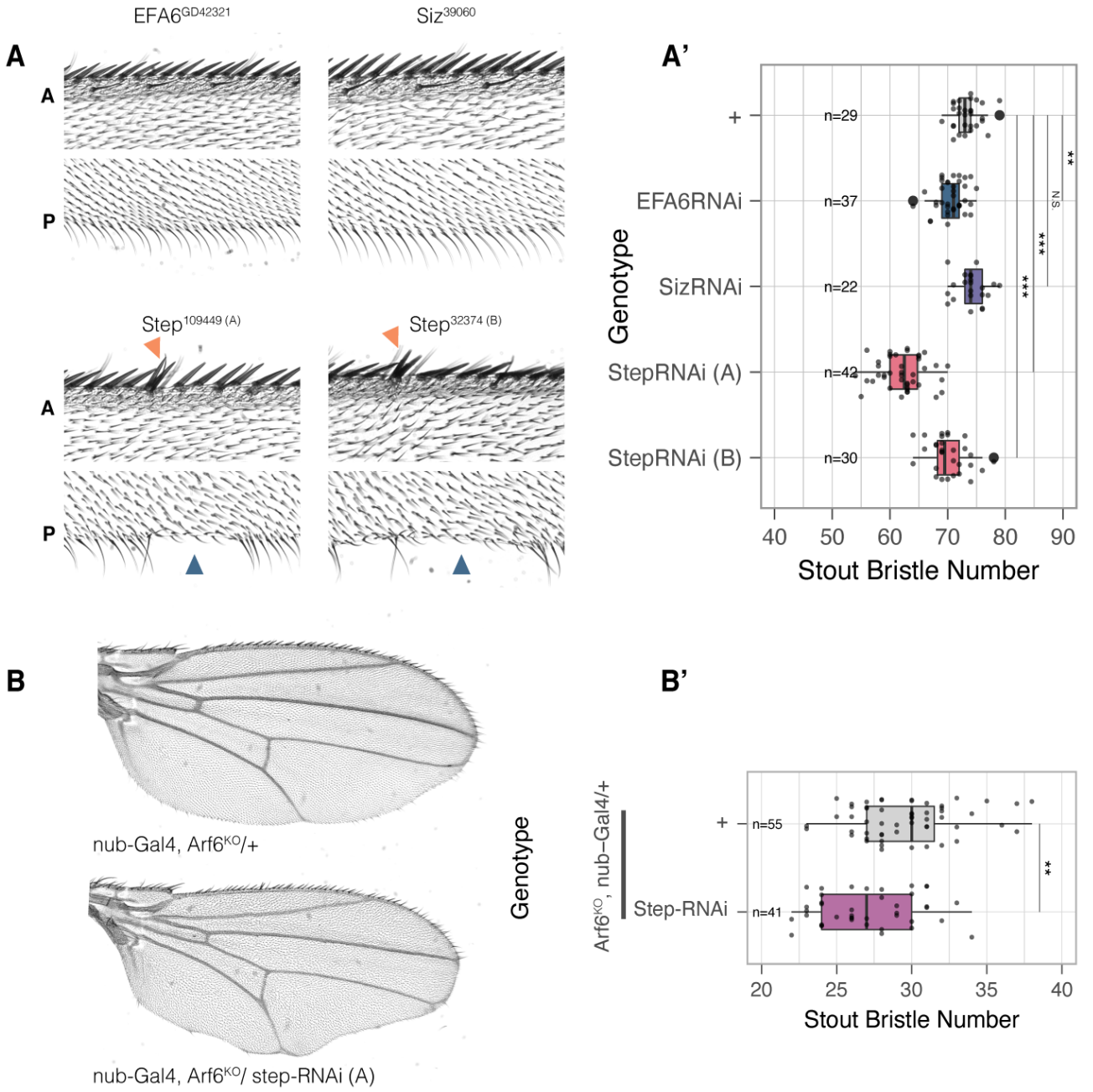
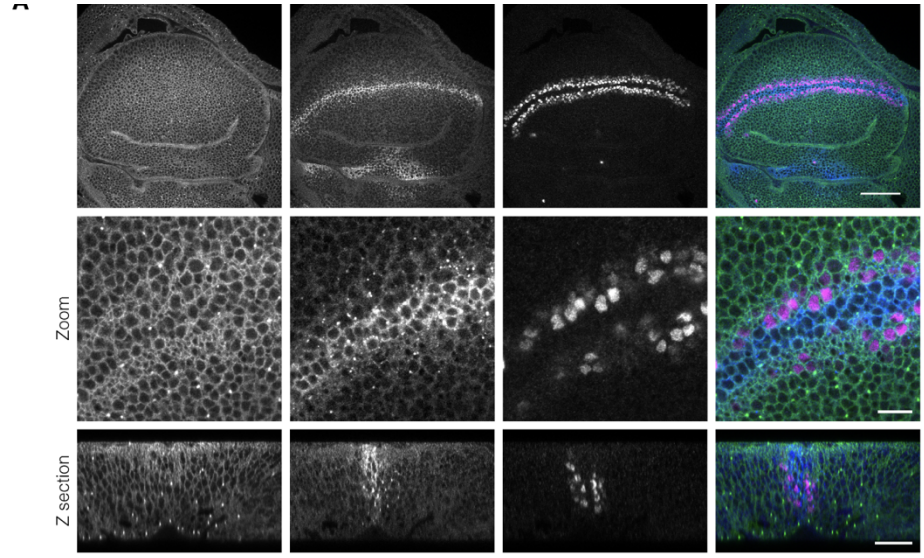
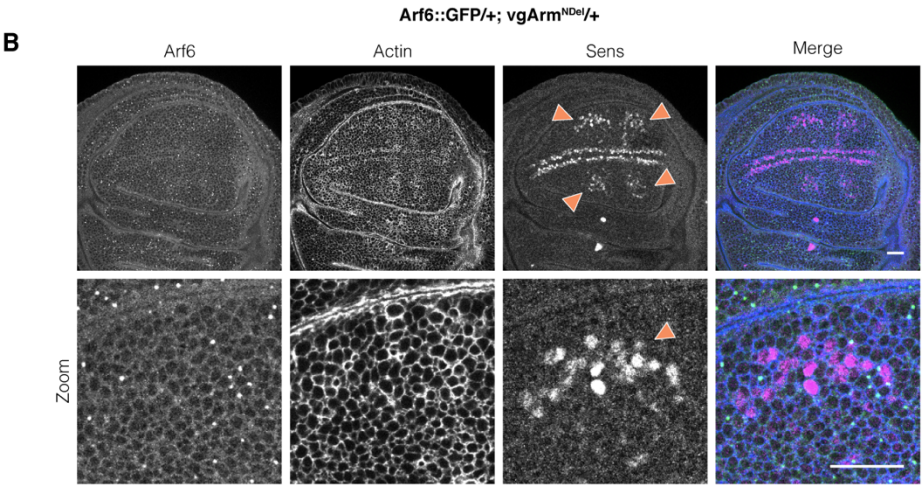


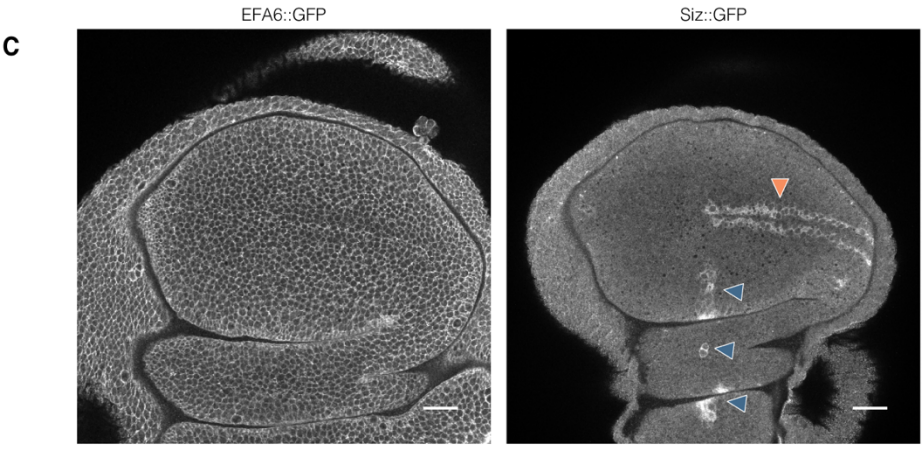
Figure 13 The effect of knocking down Arf6 GEFs on wing margin development

A The wing margins of wings in which the Arf6-GEFs (*EFA6, schizo (siz), steppke (step)* have been knocked down. The anterior (A) and posterior (P) wing margins are juxtaposed. Knocking down *EFA6* or *siz* does not induce wing margin bristle defects (the number of stout mechanosensory bristles is quantified in **A'**). Knocking down *step* leads to a mild reduction in the number of mechanosensory bristles, duplications of the mechanosensory bristle shafts (closed orange arrows) and missing bristles in the posterior wing margin (indicated by closed blue arrowheads). **B** Knocking down *step* in a heterozygous *Arf6^{KO}* background enhanced the *Arf6* phenotype, but also causes additional phenotypes not associated with the *Arf6* mutant alone, including wing notching and a mild PCP defect. The number of stout anterior bristles is quantified in **B'.**

Arf6 is distributed homogenously throughout the wing imaginal disc, localising predominantly at the cell cortex cortical, large punctae and at the membrane (Figure 14A)**.** Although this distribution is not suggestive of a target of Wg signalling, we tested whether the expression of vg-Arm^{NDel} affected the distribution of Arf6 in the wing pouch (Figure 14B). We did not observe any change in the level of Arf6 response to active Arm. This does not test a potential role for upstream components of the Wg signalling pathway (such as signalosome components) from recruiting Arf6. The localised activation of Arf6 is predominantly achieved through localisation of Arf6 GEFs (Casanova, 2007). An endogenous EFA6 knock-in (Huang et al., 2009) displayed a ubiquitous membrane localisation in the wing. We did not have a way of visualising Step distribution, however previous reports have described Step as being present throughout the wing, localising to cortical punctae (Hahn et al., 2013). Interestingly, Schizo (visualised with an endogenous Siz::GFP protein trap (Nagarkar-Jaiswal et al., 2015)) was concentrated in two stripes flanking the anterior wing margin (orange arrow, Figure 14C) and in the PNCs of wingless independent sensory organs (blue arrows, Figure 14C). This is consistent with previous reports that *schizo* is expressed in response to proneural proteins (Reeves and Posakony, 2005). The distribution of EFA6 and Siz were not modified by the expression of Arm^{NDel} (Figure 14D).


Zoom
Z section


Arf6::GFP/+; vgArm^NDel/+
Arf6
Actin
Sens
Merge
Zoom


EFA6::GFP
Siz::GFP

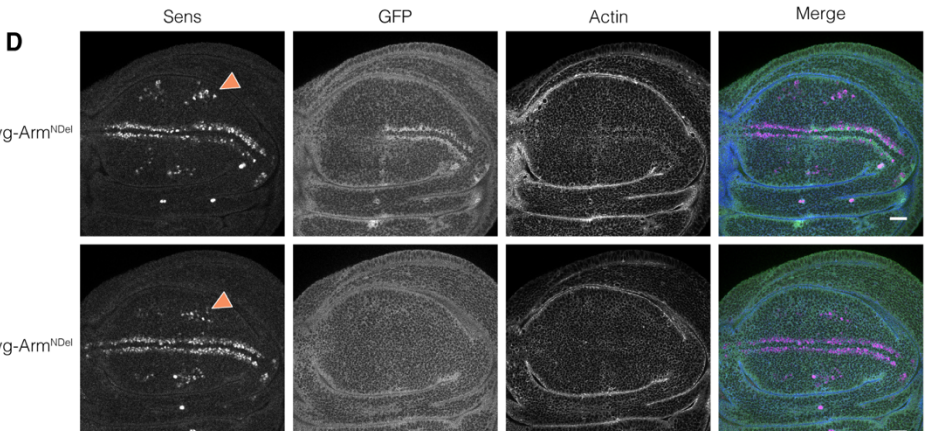

Sens
GFP
Actin
Merge
Siz::GFP / vg-Arm^NDel
EFA6::GFP / vg-Arm^NDel

Figure 14 Localisation of Arf6 and Arf6-GEFs in the wing imaginal discs

A Arf6 is ubiquitously expressed throughout the wing imaginal disc and does not show the typical pattern of a Wg target (heightened expression at the D/V boundary). Arf6 is predominantly found at the cortex and plasma membrane, and is also present in large punctae that are mainly located basolaterally (visible on the Z-section). **B** Arf6 localisation is not affected by the hyperactivation of the Wg signalling pathway induced by the expression of $vgArm^{NDel}$ and visualised by the ectopic expression of Sens far into the dorsal and ventral compartments (closed orange arrowheads). **C** EFA6 is located at the plasma membrane throughout the wing imaginal disc. Similarly to Arf6, EFA6 does not have a distribution that would suggest that it is regulated by Wg signalling. Siz expression is enhanced in two stripes flanking the anterior wing margin (closed orange arrowhead) and in the PNCs of other sensory organs (closed blue arrowheads). **D** high level activation of Wg signalling through the expression of $vgArm^{NDel}$ does not lead to ectopic expression of either Siz or EFA6, again suggesting that they are not directly regulated by Wg signalling. We were unable to visualise Step localisation due to a lack of working markers or antibodies. Scale bars represent 20 µm.

Together, these findings provide a preliminary impression of the regulation of Arf6 during wing margin development. Although Step appears to be the most likely candidate as the Arf6-GEF based on the phenotype induced by the *step* RNAi, we cannot exclude the possibility that the *step* phenotype is a result of a reduction in Arf1 activity. The lack of redundancy between Arf1 and Arf6 in wing margin patterning, and the presence of distinct phenotypes present in Arf6 mutants and in the *step* knockdown suggests that the Arf6-Step-Arf1 cascade does not play an important role in the *Arf6* wing margin phenotype. However, the possibility that Step acts independently on both Arf1 and Arf6 emphasises the need to disentangle the contribution of Arf6 and Arf1 to margin development.

The presence of the Arf6-GEFs throughout the wing, independently of Wg signalling activity, indicates that the specificity of the Arf6-GEFs will be determined by their subcellular localisation. Knocking-down the individual GEFs in the wing and carrying out Arf6-GTP pull-downs to measure the relative levels of Arf6 in a GTP bound form could provide us with an idea as to the sufficiency for the GEFs to act as Arf6-GEFs in the wing. This would not however tell us which Arf-GEFs are necessary to induce Arf6 activity in the context of Wg signalling. Considering the quantity of tissue required for these assays, and the very low levels of Arf6-GTP at a steady state, this process would also be technically challenging. The presence of multiple Arf6-GEFs in the same cell also suggests there could be a level of functional redundancy between them: simultaneously knocking down the Arf6-GEFs would begin revealing whether Arf6-GEF redundancy plays an important role in wing margin patterning.

Establishing which Arf6-GEF is responsible for Arf6 activity in the wing margin would provide a proximal explanation for the regulation of Arf6, but would not provide an insight into the regulatory processes taking place upstream of the GEF. The requirement for Wg signalling activation would first be tested *in vitro* to overcome the limitations presented by the small amount of tissue in the wing. *Drosophila* cells, such as S2R+ that are responsive to the Wg ligand could be treated with Wg

conditioned medium (Vincent, 2014). The relative level of Arf6-GTP would be monitored following the treatment to see whether inducing Wg signalling is sufficient to induce Arf6 activation. The same experiment could be carried out while simultaneously knocking down components of the signalosome, which could help establish whether Arf6 activation requires a functional signalosome. If Arf6-GTP levels were found to increase in response to exogenous Wg, the cellular model could also provide a means to systematically test the effect of knocking down the putative Arf6-GEFs on the relative levels of Arf6-GTP both in the presence and absence the Wg ligand.

Is Arf6 involved in Notch signalling?

Based on the dominant effect of the loss of *Arf6* on wing margin development, I suspected that the Arf6 could be regulating N signalling. To my knowledge, Arf6 has not previously been implicated in N signalling. The Notch signalling pathway is notable for its sensitivity to gene dosage, which is thought to result from the absence of an amplification step in the pathway, and its dependence on stoichiometric interactions between pathway components (Guruharsha et al., 2012). Previous studies have described crosstalk between Wg and N signalling (Collu et al., 2014; Hayward et al., 2008; Noll et al., 2000) which has been shown to be particularly relevant during wing development (Axelrod et al., 1996; Brennan et al., 1999; De Celis and Bray, 1997; Diaz-Benjumea and Cohen, 1995; Hayward et al., 2005; Hing et al., 1994; Langdon et al., 2006; Micchelli et al., 1997; Muñoz-Descalzo et al., 2010; Rulifson and Blair, 1995; Sanders et al., 2009).

N signalling is required during both the selection and differentiation of sensory organ precursors (SOPs). We therefore began by assessing whether $Arf6^{KO}$ induces bristle development defects beyond those of the wing margin. N signalling plays an essential role in the development and precise patterning of the thoracic bristles (Corson et al., 2017; Couturier et al., 2019). Although the patterning of the rows of thoracic bristles is not noticeably affected in the $Arf6^{KO}$ background (Figure 15A), I noticed an increased frequency of duplicated scutellar bristles in $Arf6^{KO}$ flies (blue arrowhead Figure 15A, quantified in A'), a phenotype associated with Notch signalling defects (Mummery-Widmer et al., 2009; Zhang et al., 2012). Crossing $Arf6^{KO}$ flies with a hypomorphic *N* allele (N^{1}) (Lehmann et al., 1983) dramatically increased the penetrance of the bristle duplications, with 100% of $Arf6^{KO}$ homozygotes in a heterozygous N^{1} background having at least one bristle duplication (Figure 15A'). This dominant genetic interaction is suggestive of Arf6 acting as a positive regulator of N signalling during SOP selection.

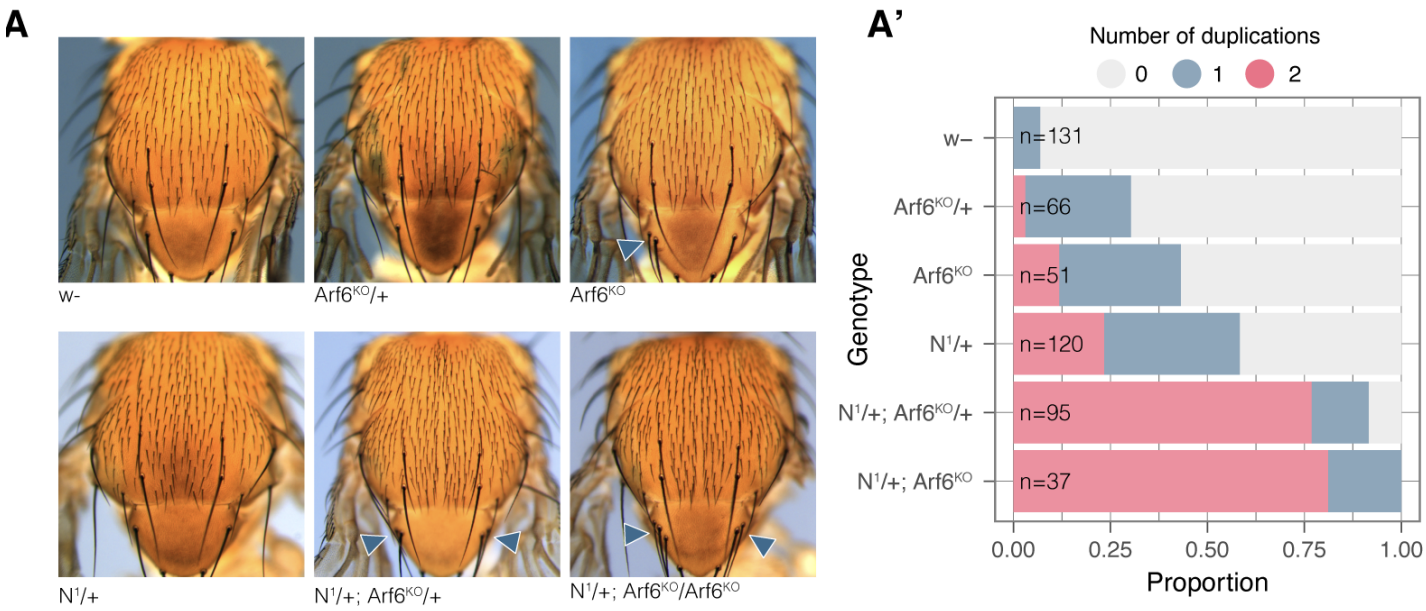


Figure 15 Arf6 genetically interacts with *N*

A The thoracic and scutellar bristles of WT (w-) and heterozygous or homozygous *Arf6^{KO}* flies are presented. *Arf6^{KO}* does not induce noticeable patterning defects in the stripes of thoracic bristles, but does lead to low numbers of scutellar bristle duplications (closed blue arrowheads, quantified in **A'**). The penetrance and expressivity of the scutellar bristle duplications present in heterozygous *N^{1}* flies is strongly enhanced in either heterozygous or homozygous Arf6^{KO}.

N mutant wings are characterised by a thickening of the L3 and L5 wing veins, accompanied by a distal notching of the wing margin. These phenotypes serve as an effective method to identify potential N regulators through genetic modifier screens (Zacharioudaki and Bray, 2014). Although these phenotypes are not present in *Arf6^{KO}* wings, mild ectopic veins are present in homozygous *Arf6* mutant wings, predominantly emanating from the posterior cross vein and the distal end of the L2 longitudinal vein (closed orange arrowhead, Figure 16). The distal ends of the L4 and L5 veins were widened (closed blue arrowheads Figure 16).

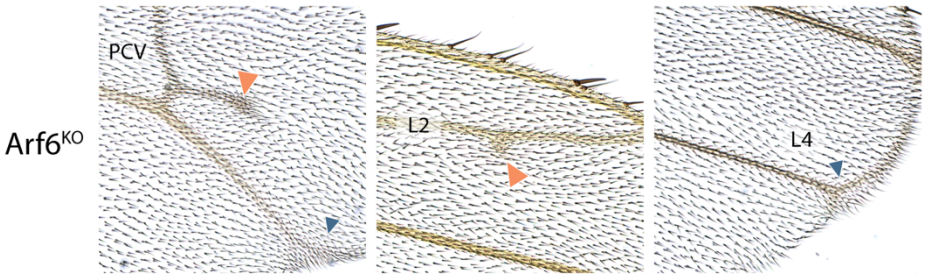


Figure 16 Arf6 mutants induce recessive wing vein defects

Wing vein defects are present in homozygous *Arf6* mutant wings (three independent Arf6^{KO} wings are presented). Examples of the most common defects are shown: ectopic veinlets (closed orange arrowheads) emanating from the posterior cross vein (PCV) and L2 longitudinal vein (L2). The L4 and L5 veins are often observed splitting where they reach the wing margin (closed blue arrowheads).

N[1], *Arf6*[KO], double heterozygotes exhibited a strong enhancement in both the penetrance and expressivity of the *N*[1] notching and wing vein phenotypes (Figure 17A, A', B). The widening of the longitudinal wing veins L3 and LV was particularly prominent in the distal tips of the veins at their convergence with the wing margin (closed blue arrowheads Figure 17A). Although there was a further increase in the strength of the phenotype in homozygous *Arf6*[KO] in the *N*[1] background, the viability of the flies and the lack of a stronger *N* phenotype indicates that Arf6 is not essential in N signalling. Interestingly, despite the dramatic wing phenotypes induced by the genetic interaction between N and Arf6, the number of stout margin bristles were mildly, but statistically significantly increased in in the *N1, Arf6*[KO] double heterozygous wings (Figure 17C). Due to the extensive notching present in the Arf6 homozygotes in the N[1] background, I was unable to reliably quantify the number of stout bristles in the wing margin.

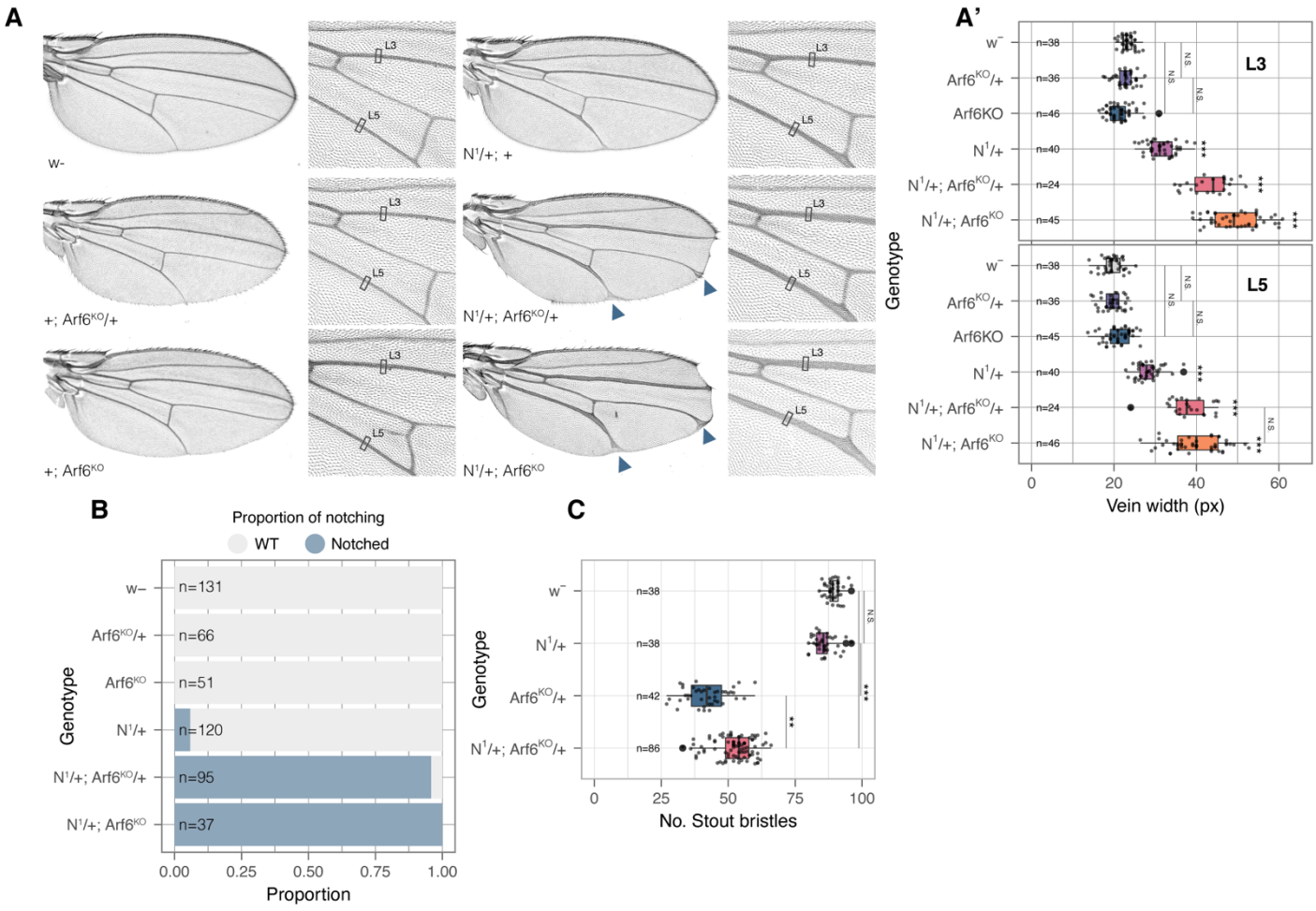


Figure 17 Arf6[KO] enhances N phenotypes in the adult wing

A Whole wings and zooms showing details of the L3 and L5 (labelled on the image) are presented. The black rectangles represent the area in which the wing vein width was measured. The wing vein thickening present in heterozygous *N1* wings is strongly enhanced in a heterozygous or homozygous *Arf6*[KO] background (widths of the L3 and L5 are plotted and analysed independently in **A'**). Wing vein widths were modelled using a generalised linear model (GLM) with gaussian errors. **B** the penetrance of wing notching phenotype present in the *N*[1] background is dramatically increased in either heterozygous or homozygous mutant background. The extent of the notching is also strongly increased. **C** the loss of margin bristles in heterozygous *Arf6*[KO] wings is partially rescued in a heterozygous *N*[1] background.

Taken together, these genetic interactions are suggestive of Arf6 acting as a positive, but non-essential regulator of N signalling. This is particularly apparent through the lack of wing margin notching and vein thickening in the homozygous *Arf6* mutant wings. Strikingly, the haploinsufficiency of *Arf6* in the wing margin bristles that initially prompted us to test for a role for Arf6 in N signalling is mildly rescued in the *N* mutant background, whilst the *N* phenotypes are enhanced (Figure 17C). This would suggest that while Arf6 appears to act as a positive regulator of N signalling, N acts antagonistically to Arf6 activity in the development of wing margin bristles.

Based on the mild rescue of the $Arf6^{KO}$ phenotype in a hypomorphic *N* background, I tested whether increasing the level of N signalling in the wing margin would enhance the *Arf6* phenotype. Previous studies have shown that hyperactivating N signalling by expressing an activated form of Notch (NICD) can induce bristle loss at the wing margin, producing a phenotype similar to that of *Arf6* mutants (Langdon et al., 2006; Sanders et al., 2009). I expressed either full-length, wild-type Notch (N^{FL} (Lawrence et al., 2000a; Seugnet et al., 1997)) or an activated form of notch, Notch Extracellular Truncation lacking the extracellular N domain (NEXT (Vaccari et al., 2008)) to increase Notch activity in the wing margin. Expressing N^{FL} in a wild-type wing led to a mild N gain of function phenotype, manifesting in small numbers of ectopic stout mechanosensory bristles in the wing margin (closed blue arrowheads Figure 18) and mild defects in bristle differentiation (closed orange arrowheads in Figure 18A). Expressing NEXT under the same conditions induced a more pronounced phenotype, with most of the bristles in the anterior wing margin being reduced to sockets (closed orange arrowheads Figure 18A) and curling of the wing blade. These defects are consistent with the previously described phenotypes induced by the N gain of function in the wing margin (Diaz-Benjumea and Cohen, 1995). This phenotype can be explained by an excess of N signalling in the pIIb leading to its conversion to a pIIa. High N signalling in the shaft cell then converts it into a socket cell (Guo et al., 1996). The phenotype therefore represents a late defect in bristle development, downstream of the establishment of the PNCs by Wg signalling, and the subsequent selection of SOPs via lateral inhibition. The clusters of deformed bristle sockets therefore arise from single SOPs and were therefore quantified as single bristles. Expressing N^{FL} in an otherwise WT background did not phenocopy the *Arf6* mutant, with the number of stout bristles remaining broadly unaltered in the wing margin (Figure 18A'). I was unable to count the bristles in the NEXT expressing wings, however the density of the remaining stout bristles combined with the abundance of sockets indicates that NEXT also did not phenocopy the *Arf6* phenotype.

When N^{FL} or NEXT were expressed at the D/V under C96-Gal4 in a heterozygous $Arf6^{KO}$ background, the Arf6 phenotype was dramatically enhanced, resulting in phenotypes comparable to

the homozygous Arf6 mutant (Figure 18A, A'). A portion of the remaining stout bristles displayed the differentiation defect observed when over-expressing N in an otherwise WT background (orange arrowhead Figure 18A).

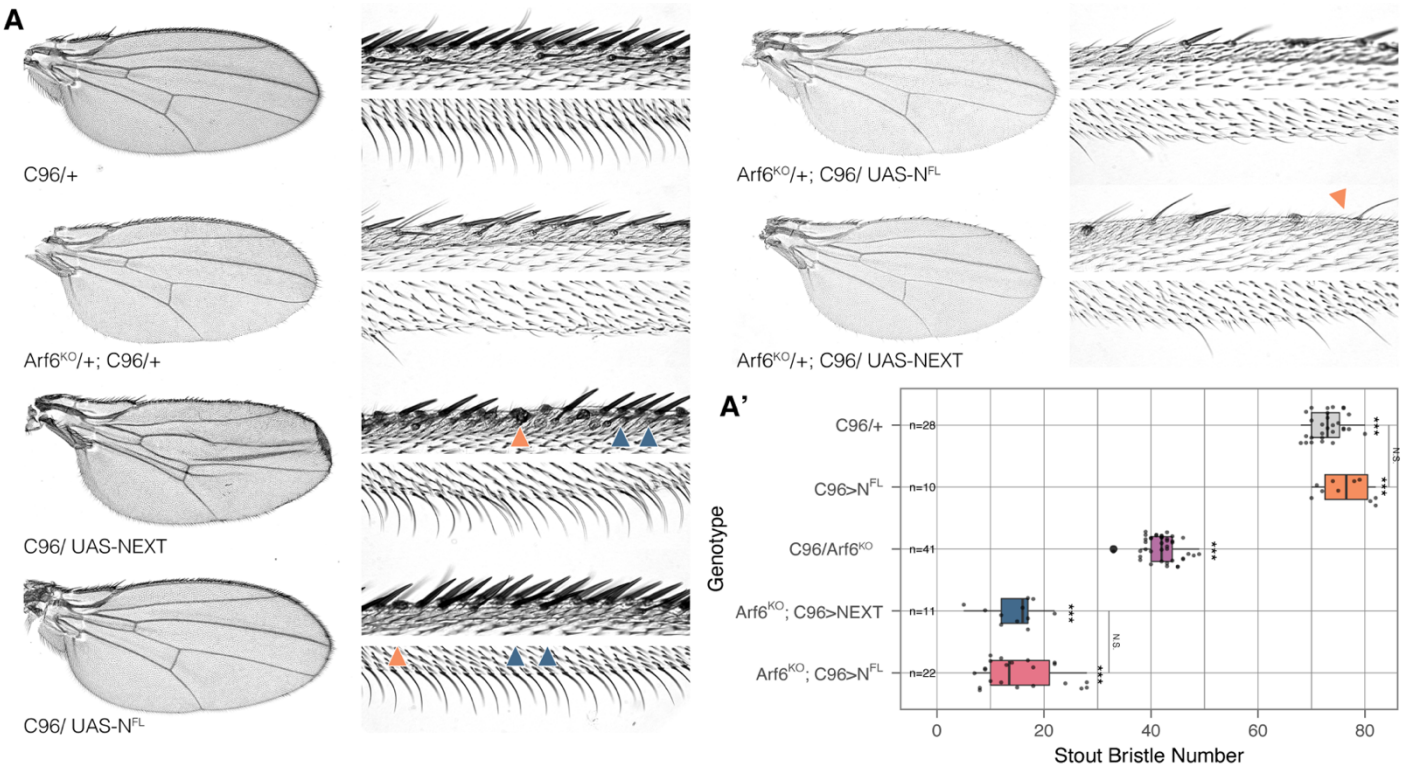


Figure 18 The Arf6 margin phenotype is enhanced by N signalling

A WT wing control is represented by C96-Gal4/+. The number of margin bristles are strongly reduced in the heterozygous *Arf6^{KO}* background (anterior stout mechanosensory bristles are quantified in **A'**). Expressing an activated, truncated form of the N receptor (NEXT) at the D/V with C96-gal4 leads to defects in bristle development, resulting in the replacement of the bristle by socket cells (closed orange arrowheads) and the introduction of ectopic margin bristles (closed blue arrowheads)**.** The number of stout mechanosensory bristles could not be reliably quantified in the C96>NEXT background. Wings expressing full-length WT N (N^{FL}) under the control of C96-gal4 show mild margin bristle defects, including the formation of ectopic margin bristles (closed blue arrowheads) and the conversion of bristles into sockets (closed orange arrowheads). These phenotypes are qualitatively similar to those induced by NEXT expression, but much milder. Expressing either NFL or NEXT in a heterozygous *Arf6^{KO}* leads to a strong enhancement of the *Arf6^{KO}* phenotype without producing wing margin notching. Both NEXT and N^{FL} enhance the *Arf6* phenotype to a similar extent.

These data show that the *Arf6* phenotype is strongly enhanced in response to even small increases in N activity, as both NEXT and N^{FL} induced a similar phenotype in the *Arf6^{KO}* background, despite the disparity in their intrinsic signalling activity. A loss of bristles in response to increased N signalling can either result from an 'early' or 'late' defect in bristle development. The late phenotype takes place following the selection of SOPs (as was observed in the over-expression of N in the WT background), where the increase in N signalling drives the transformation of the pIIb to pIIa, and the eventual shaft to a socket. The 'early' phenotype can be explained by a failure of SOP selection from within the PNCs; cells destined to become the SOP express high levels of Dl, inducing N signalling in

the surrounding cells, triggering their expression of genes of the *E(spl)-C*, which antagonise the activity and expression of proneural factors. The forced increase in N signalling increases the expression of *E(spl)-C* in all the cells, blocking SOP fate, and returning the cells of the PNC to epidermal identity.

The enhancement of the *Arf6* phenotype by the over-expression of NEXT or N^{FL} is indicative of the 'late' N gain-of-function phenotype being converted into an 'early' phenotype in an *Arf6* background, leading to a further loss of SOPs. The similarity between the effects of expressing NEXT or N^{FL} indicates that the wing margin bristles are particularly sensitive to even mild increases in N signalling. This similarity between the effects of expressing NEXT and N^{FL} also indicates that the extracellular domain of N is not necessary to enhance the *Arf6* phenotype. There are several possible explanations for this phenomenon.

The level of Sens in WT wing margin PNCs is much greater than in the *Arf6* mutant. The low level of Sens is likely to render the PNCs more sensitive to small changes in their regulators, as it would take little to completely abolish *sens* expression. Even mild increases in N signalling in this context is therefore enough to block SOP selection. Although the NECD was not necessary to block SOP selection, it may have participated by contributing to the cis-inhibition of Dl in the PNCs, suppressing lateral inhibition. By increasing N signalling activity in the wing margin, N^{FL} and NEXT could also have cell autonomously increased the expression of E(Spl)C, further suppressing SOP fate. The mild rescue we observed of the *Arf6* phenotype in the heterozygous N^{1} background is consistent with these results: the lower *N* expression will reduce the threshold of Dl required in order for a cell in the PNC to overcome cis-inhibition and be selected as a SOP. If a failure of SOP selection is responsible for the loss of bristles in the N gain of function context, then this would need to be confirmed by staining for Sens in early pupal wings when the mechanosensory SOPs are selected. A failure of SOP establishment would also predict a loss of the neurons that innervate the anterior wing margin. It would therefore be important to specifically label the progeny of the pIIb (the neuron and the sheath cells) to confirm that the apparent SOP loss is not simply a conversion of the pIIa to the 'inner' cell fates that are not readily apparent.

These results are compatible with a model in which Arf6 is required upstream of lateral inhibition, in the establishment of the wing margin PNCs. Although this is typically due to a result of a defect in Wg signalling, I cannot exclude the possibility that the suppression of Sens is a result of precocious E(spl)-C expression, that is enhanced by the increase in N signalling.

Is *E(Spl)-C* expression increased in *Arf6^{KO}* wing margin PNCs?

The anti-neural role of E(spl)-C proteins, combined with the enhancement of the *Arf6* mutant phenotype by both increased Wg and N signalling made me suspect that the Arf6 phenotype could be due to a precocious expression of *E(spl)-C* genes, leading to a suppression of *sens* expression in the wing margin PNCs.

The genes of the *enhancer of split complex (E(spl)-C)* encode proteins belonging to the bHLH and Bearded families of transcriptional regulators (Lai et al., 2000). During wing development, the E(spl)-C incorporate multiple signals, including N signalling and proneural proteins for their expression, and are responsible for the repression of proneural genes during lateral inhibition (Cooper et al., 2000; Lecourtois and Schweisguth, 1995). The ectopic expression of *E(spl)M7* and *E(spl)M8* have previously been shown to lead to a reduction in the number of wing margin bristles (Ligoxygakis et al., 1999) and a direct suppression of Sens expression and activity (Jafar-Nejad et al., 2003), inducing a phenotype similar to those observed in the *Arf6* mutants.

GFP-tagged M7, M8 and Mδ proteins (Couturier et al., 2019) were used to reveal the distribution of the E(Spl)-C proteins in the wing margin. Mδ served as a negative control as its over-expression in the wing was not sufficient to induce a loss of wing margin bristles (Ligoxygakis et al., 1999). All three constructs were enriched in the nuclei of cells in the PNCs of the anterior wing margin bristles (Figure 19A, B, C). Consistent with the suppression of *E(Spl)-C* expression by high levels of Sens (Jafar-Nejad et al., 2003; Nolo et al., 2000), the strongest Sens expressing cells in the anterior margin did not show E(Spl)-C accumulation (pink arrowheads Figure 19A, B and C), and likely represent the SOPs of the presumptive chemosensory bristles that are selected before the SOPs of the stout mechanosensory bristles (Jafar-Nejad et al., 2006). In accordance with the previous descriptions of their expression patterns, none of the E(Spl)-C proteins showed enrichment in the PNCs of the non-innervated posterior margin bristles, with only M8-GFP being restricted to the margin cells (consistent with the regulation of *E(Spl)-C* by N signalling) (closed blue arrows Figure 19B'). Cells could also be observed far from the anterior wing margin PNCs expressing M8-GFP, without expressing Sens (orange arrows Figure 19B) suggesting that M8 expression could be independent of Sens.

Contrary to my original hypothesis, there was a general reduction in the level of the three tested E(Spl)-C proteins in the wing margin PNCs of the *Arf6* mutant alongside the reduction in Sens (Figure 19A', B', C'), and I observed no induction of ectopic E(Spl)-C in the posterior wing margin, which would be necessary to explain the loss of posterior margin bristles and Sens in the *Arf6* mutants.

Despite the decrease in M8-GFP in the *Arf6*^{KO} background, the M8::GFP signal was still present in the margin cells (blue arrowhead Figure 19B and B').

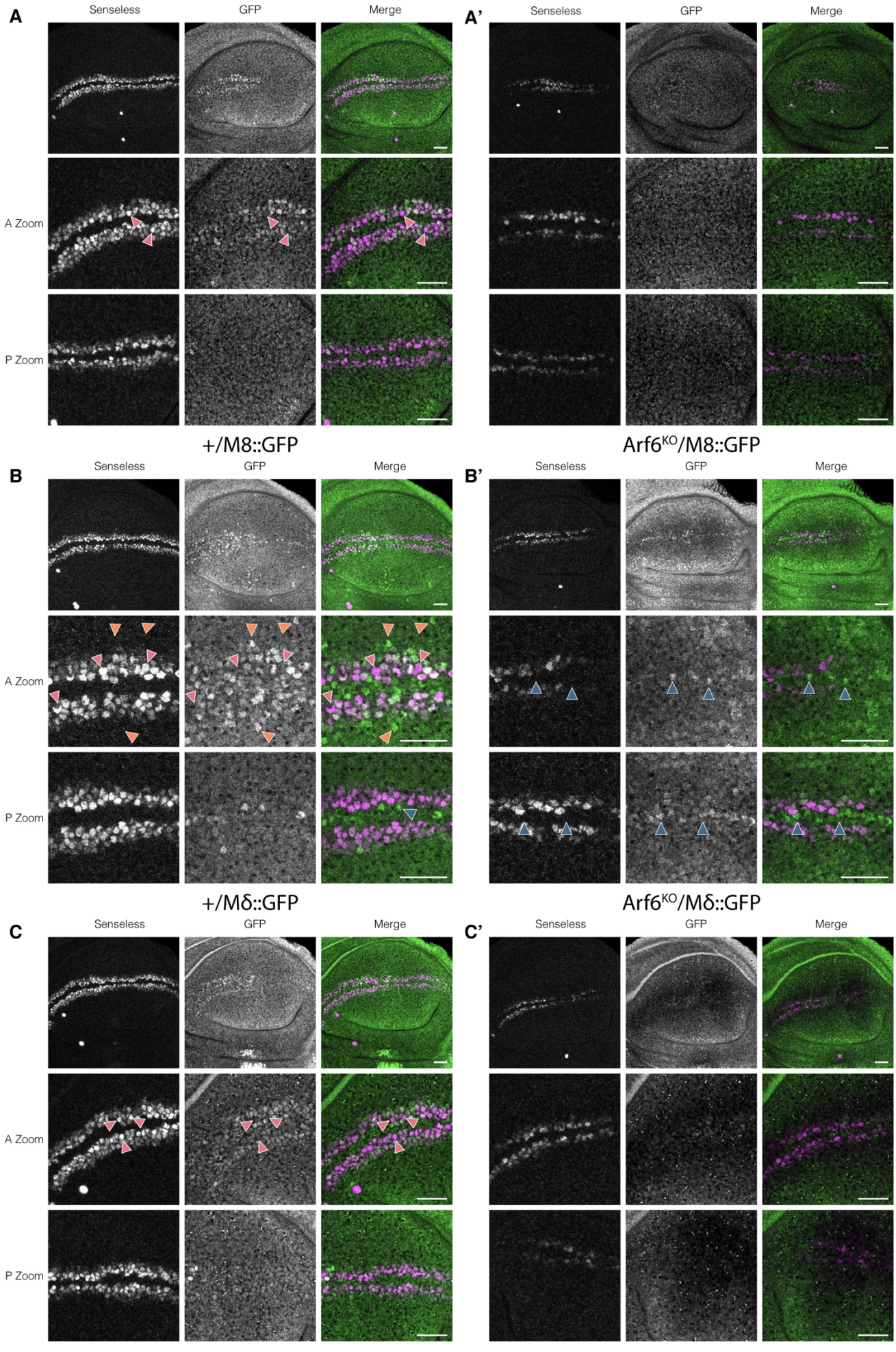

A
Senseless
GFP
Merge
A'
A Zoom
P Zoom
+/M8::GFP
Arf6KO/M8::GFP
B
B'
+/Mδ::GFP
Arf6KO/Mδ::GFP
C
C'

Figure 19 *E(spl)-C* expression is reduced in *Arf6^{KO}* wing imaginal discs

A E(spl)M7 expression in a w- wing imaginal disc. M7 was present in the PNCs of the anterior wing margin (labelled with Sens). M7 did not fully co-localise with Sens, and nuclei with high Sens were often negative for M7 (pink arrowheads). Very few cells were positive for M7 in the posterior wing margin. Anterior and posterior zooms are presented. **A'** M7 expression is strongly reduced in heterozygous Arf6^{KO} flies, this is particularly prominent in the PNCs of the anterior wing margin. Sens is also strongly reduced. **B** M8 showed a similar distribution to that of M7, with numerous nuclei in the anterior of the wing being labelled for M8. M8-positive nuclei are present beyond the domain of Sens expression (closed orange arrowheads). There was also reduced M8 intensity in the nuclei with high Sens (pink arrowheads). Few M8-positive nuclei are present in the posterior of the wing, with the majority localising to the margin cells of the posterior wing margin (closed blue arrowhead). **B'** the expression of M8 is strongly reduced in the anterior wing margin PNCs in the heterozygous Arf6^{KO} wing imaginal disc. Punctuated expression remains in wing margin cells in both the anterior and posterior wing margin (closed blue arrowheads). **C** Mδ is expressed in cells corresponding to the PNCs of the anterior wing margin bristles. Similarly to M7 and M8, high levels of Sens are associated with low levels of Mδ (pink arrowheads). **C'** The expression of Mδ is strongly reduced in the heterozygous Arf6^{KO} background. Scale bars represent 20 µm.

These data suggest that the reduction of Sens in the *Arf6^{KO}* wing margin PNCs takes place independently of a repression of *sens* expression by E(Spl)-C proteins. However, it is not clear whether the observed reduction in *E(spl)-C* expression is a direct consequence of the reduced level of Sens, a defect in N signalling, or a combination of both. I can begin discriminating between these possibilities based on the extensive data available on the regulatory logic governing *E(spl)-C* expression in PNCs and SOPs.

The PNC expression of *E(Spl)-C* in the anterior wing margin requires positive inputs from both the bHLH proneural factors (Ac/Sc) and N signalling (Cooper et al., 2000; De Celis et al., 1996; Kramatschek and Campos-Ortega, 1994; Nellesen et al., 1999; Singson et al., 1994). In *sc$^{10-1}$* mutants in which both *ac* and *sc* are deleted, the SOP and PNC expression of *E(Spl)-C* is lost (Singson et al., 1994). Despite the loss of *ac/sc,* the expression of *sens* is maintained in the wing margin in *sc$^{10-1}$* flies (Jafar-Nejad et al., 2003; Jafar-Nejad et al., 2006; Lai, 2003). Together, these observations would suggest that Sens is not sufficient to induce and maintain *E(spl)-C* expression in the absence of Ac and Sc. Indeed, the ectopic expression of *E(spl)M8* induced by ectopic *sens* expression in a WT background is likely to be a result of an increase in *Dl* and *ac/sc* expression (Nolo et al., 2000). The early PNC expression of *sc* in the anterior wing margin is also maintained in *sens* mutant clones, indicating that Sens is also not necessary for the PNC expression of *ac/sc* (Jafar-Nejad et al., 2003; Nolo et al., 2000).

These observations could be interpreted as Sens not being necessary for the PNC expression of *E(Spl)-C* in the wing margin, however Sens has been shown to directly bind to the proneural bHLH proteins, and act as a transcriptional co-activator of proneural target genes (Acar, 2006). As *ac/sc* directly regulate *E(Spl)-C* genes, Sens is likely to be necessary for the expression of the E(spl)C in the anterior wing margin PNCs. Moreover, low levels of Sens relative to the abundance of the bHLH

proteins leads to Sens acting as a direct transcriptional repressor of *ac* expression, and possibly other proneural genes (Acar, 2006; Jafar-Nejad et al., 2003).

The reduced level of Sens in the wing margin PNCs of *Arf6* mutants could therefore be sufficient to explain the reduced E(spl)C levels I observe, both through a reduced transcriptional activity of the bHLH proteins, and potentially through the remaining low levels of Sens directly repressing the expression of proneural genes. The loss of other bristle classes in the anterior wing margin of $Arf6^{KO}$ flies is also indicative of a loss of *ac/sc* expression, consistent with *ac* and *sc* being expressed downstream of Wg signalling (Rulifson et al., 1996). To test the requirement for Sens for the expression of *E(spl)C* genes, I would knock-down *sens* in the wing margin in a background carrying tagged E(Spl)C proteins. This would remove the PNCs in the wing margin without impacting N or Wg signalling. Conversely, re-expressing *sens* in an *Arf6* mutant wings with tagged E(spl)C proteins would allow us to test whether re-introducing *sens* expression in the *Arf6* mutant wing is sufficient to restore *E(spl)-C* expression.

The most parsimonious explanation for these results is that the reduction of E(spl)-C in the *Arf6* background is a result of the loss of Sens. I cannot however exclude the possibility that a defect in N signalling also independently contributes to the reduction in E(Spl)-C expression. Together, these results reaffirm a model in which Arf6 is necessary for wing margin PNC establishment, and although the Arf6 phenotype is enhanced by increased N signalling in the wing margin, the phenotype itself does not appear to be due to a hyperactivation of canonical N signalling inducing the loss of PNCs.

Does Arf6 directly regulate Notch receptor trafficking?

I wanted to test whether Arf6 could be involved in regulating the trafficking of the N receptor. N signalling is tightly regulated through trafficking of both the N ligands (Serrate and Delta) and the receptor itself (Conner, 2016; Le Borgne et al., 2005). Blocking N trafficking at different stages in the trafficking route can induce both N loss- and gain-of-function phenotypes (Vaccari et al., 2008).

To test whether Arf6 is found in the same compartment as N, I stained for both NICD and NECD in the Arf6::GFP background (Figure 20A, B respectively). Although Arf6, NICD and NECD all show punctate distributions in the disc, I did not observe any significant co-localisation between Arf6 and NICD or NECD (Figure 20A' and B'), suggesting that they are not localised in the same subcellular compartment in a steady state. In contrast to WT Arf6, $Arf6^{Q67L}$::GFP showed a strong co-localisation with both NICD and NECD (Figure 20 C, C', D, D'), however $Arf6^{Q67L}$ expression did not induce a re-

localisation of either epitope, suggesting that although GTP-locked Arf6 localises to the same subcellular compartment as NICD and NECD, it does not participate in N trafficking.

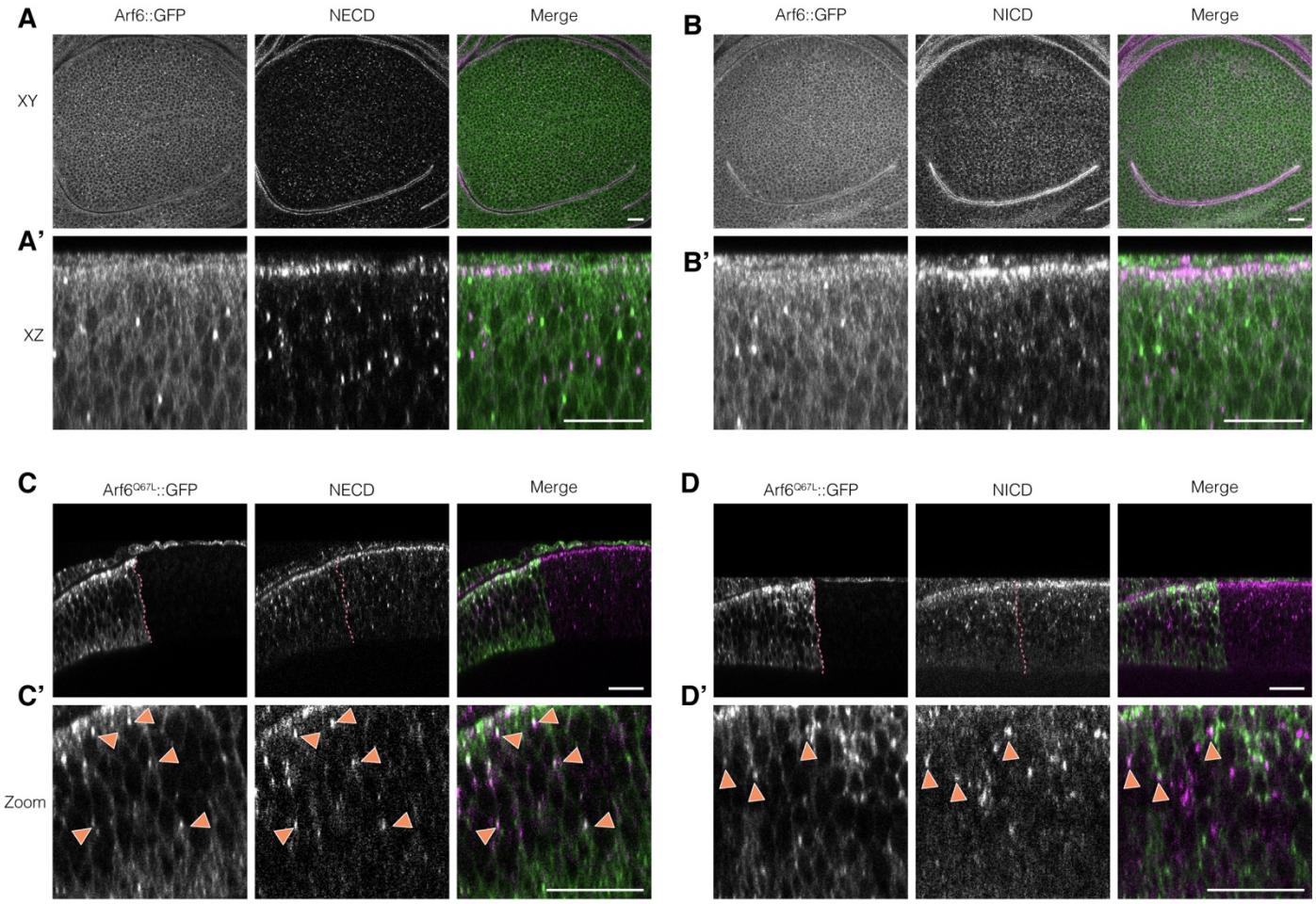


Figure 20 Arf6 does not act directly on the Notch receptor

A staining for NECD and **B** NICD in wing imaginal discs carrying endogenously GFP tagged Arf6 (Arf6::GFP). Both NICD and NECD are concentrated at the apical membrane and in punctae. Neither epitope shows significant co-localisation with Arf6::GFP. Expressing $Arf6^{Q67L}$ in the posterior compartment of the wing with hh-gal4 then staining for **C** NECD and **D** NICD. Strong colocalization is seen between $Arf6^{Q67L}$ and NECD (**C'**) and NICD (**D'**) but neither NICD nor NECD is mis-localised in response to $Arf6^{Q67L}$. Scale bars represent 20 µm.

I next tested whether the localisation of the N receptor was modified by the loss of *Arf6*. Staining for NICD and NECD in homozygous $Arf6^{KO}$ wing imaginal discs (Figure 21A-D') or in $Arf6^{KO}$ clones revealed no apparent change in the localisation of either NECD or NICD (Figure 21 E- F'). Together, these data indicate that Arf6 does not play an essential role in the trafficking of the N receptor. The very subtle putative N phenotypes in the *Arf6* mutant background would suggest that any defect in N localisation is likely to be extremely mild, and likely below the detection threshold.

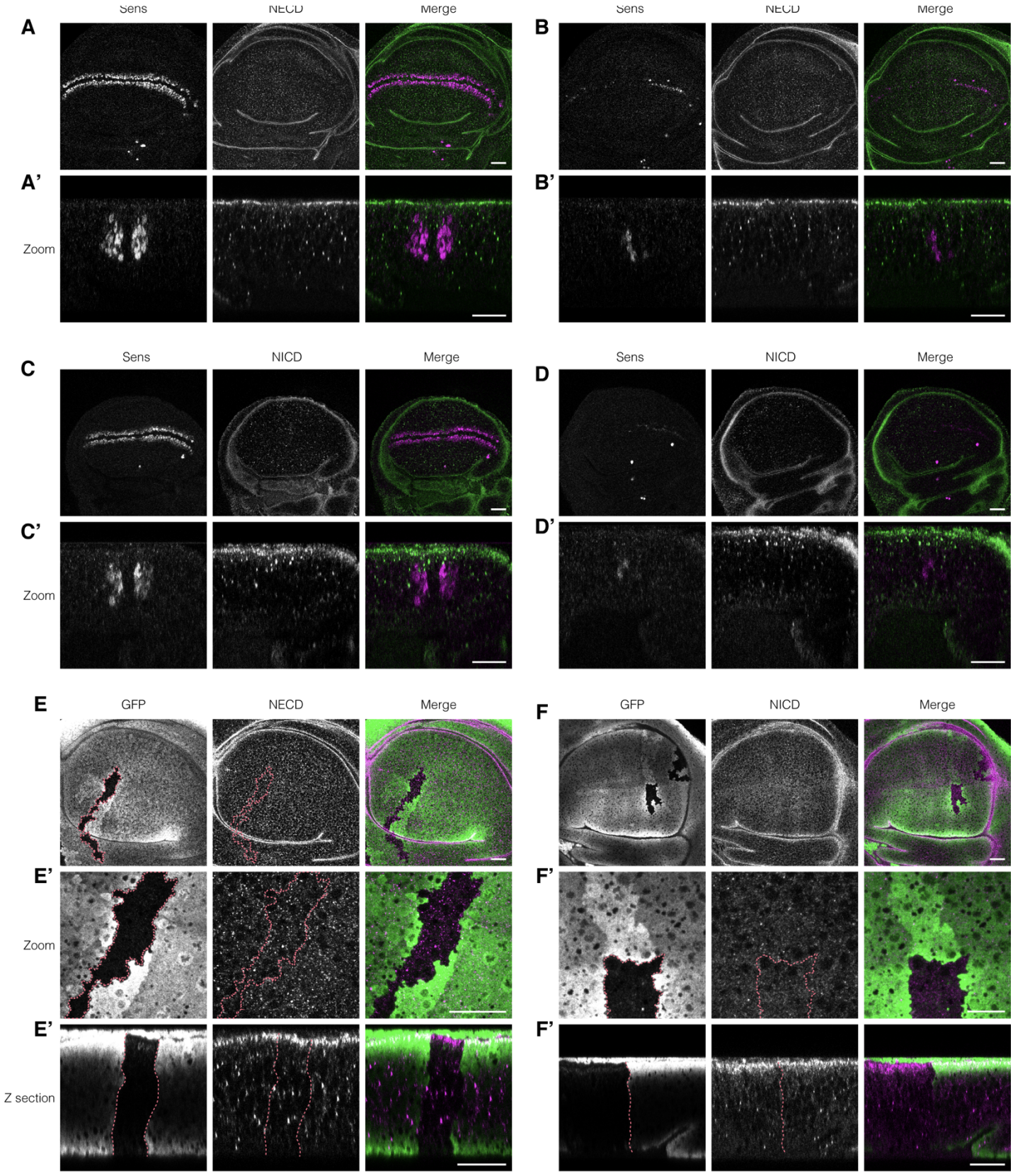


Figure 21 Notch localisation is not affected by *Arf6^{KO}*

A The localisation of NECD in WT (w-) wing imaginal discs. **A'** Z sections through the D/V border are shown. **B** There are no apparent defects in NECD localisation in homozygous *Arf6^{KO}* discs. **B'** A Z-section through the anterior D/V border is shown. **C** NECD was stained in the same conditions as NECD. **D** No apparent NICD localisation defects were present in the homozygous *Arf6^{KO}* wing imaginal discs. Z-sections are presented for both WT and *Arf6^{KO}* discs **C'** and **D'** respectively. Arf6^{KO} and WT discs were dissected, stained, mounted, and imaged simultaneously to avoid variation. Sens staining was used to distinguish between WT and Arf6^{KO} discs. NECD and NICD staining in homozygous Arf6^{KO} clones (**E** and **F** respectively). No apparent defects were present for either NICD or NECD. Zooms of the clones are presented in **E'** and **F'** and Z-sections through the clones are presented in **E''** and **F''.** The clone boundaries are represented as pink dashed lines. Scale bars represent 20 µm.

Is the distribution of Delta affected in the Arf6 mutant?

The lack of a noticeable effect of the loss of *Arf6* on the distribution of the N receptor suggests that the genetic interaction between *N* and *Arf6* could be due to Arf6 regulating N regulators. The wing vein thickening resulting from the genetic interaction between the $Arf6^{KO}$ and N^{1} was particularly pronounced at the convergence of longitudinal wing veins with the wing margin (Figure 17A) which could be indicative of a defect in Dl activity at the wing margin. Dl is predominantly localised in punctae that were particularly numerous and intense around the D/V boundary (Figure 22)**.** Dl is also concentrated at the apical membrane (labelled with aPKC) of the cells flanking the wing margin (closed orange arrowheads Figure 22A). Staining for Dl in homozygous $Arf6^{KO}$ mutant wing imaginal discs revealed a reduction in both the number and the intensity of Dl punctae in the Arf6 mutant wing and in the intensity of Dl at the apical membrane at the D/V boundary (Figure 22A-C). Consistent with a role of Arf6 in regulating high threshold Wg signalling, there was also a significant reduction in the intensity of Dl at the apical membrane in the cells flanking the D/V boundary (closed blue arrowheads figure 13A, C).

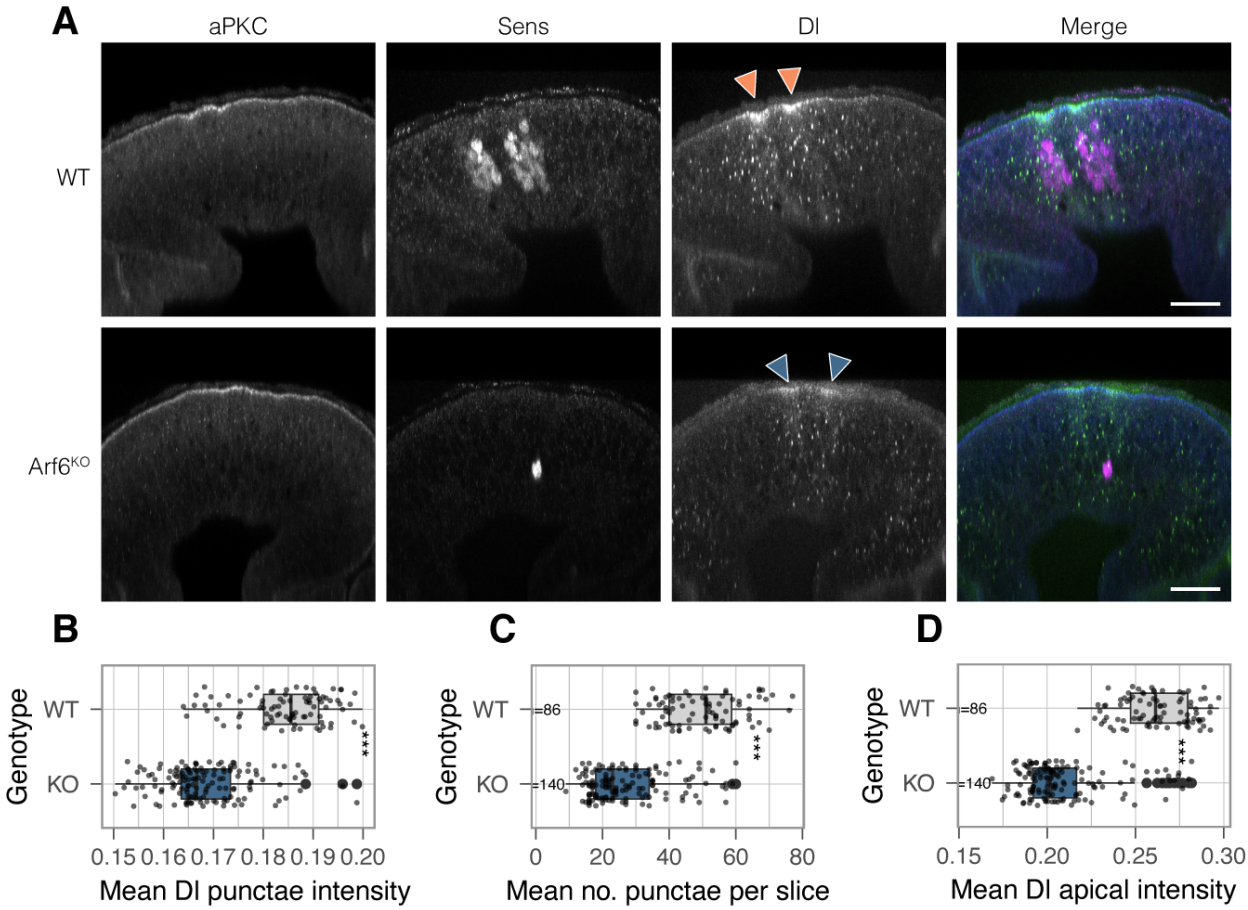


Figure 22 Delta levels are reduced in *Arf6^{KO}* wing imaginal discs

A WT and homozygous Arf6^{KO} wing imaginal discs stained for endogenous Dl protein. aPKC is used to mark the apical domain, and Sens to distinguish between WT and Arf6^{KO} discs. A decrease in both the intensity **B** and number **C** of Dl punctae was observed in Arf6^{KO} discs. The stripes of Dl flanking the D/V boundary (closed orange arrowheads in **A**) showed a reduced intensity in Arf6^{KO} wing imaginal discs (closed blue arrowheads in **A**). Quantified in **D**. Scale bars represent 20 µm.

These preliminary results suggest that Arf6 could be required in regulating the levels of *Dl* in the wing. The decrease in Dl at the D/V could be explained by the defect in high level Wg signalling we observe in the *Arf6* mutant wing, as *Dl* expression is regulated by *Wg* signalling in late L3 wing imaginal discs (De Celis and Bray, 1997). The defect in Wg signalling would not explain the more general defect in Dl levels further into the dorsal and ventral compartments of the wing. This would therefore suggest that the effect of *Arf6^{KO}* on Dl could be a compound effect of a defect in Wg signalling, coupled with an independent, more general effect on Dl levels.

Aspects of the defects observed in heterozygous *Dl* mutant wings are consistent with what I observe in *Arf6* mutant wings, such as the formation of ectopic veins and distal widening of the longitudinal veins (De Celis, 1998; Huppert et al., 1997). The role of Dl as a N ligand therefore makes

it tempting to speculate that this defect in Dl levels is responsible for the enhancement of the *N* phenotype I observe in double heterozygous N^{1}, $Arf6^{KO}$ flies. However, the stoichiometry of the interactions between N and Dl means that removing a single copy of *Dl* in a *N* mutant background can suppress both the *N* and the *Dl* phenotypes, including wing notching (De Celis and Bray, 2000; Fostier et al., 1998; Vassin et al., 1985). This contrasts with what we observe in the genetic interaction between *N* and *Arf6,* and suggests that the defect in Dl levels may not be sufficient to explain the interactions we observe. This could be tested by increasing the gene dosage of *Dl* under its endogenous regulation in an *Arf6, N* double mutant background (LeBon et al., 2014). If Arf6 activity simply regulates Dl levels, increasing Dl levels should rescue the N phenotype by re-balancing the levels of Dl and N. However, if Arf6 is also required in another step in N signalling, the phenotype will not be rescued.

The genetic interactions we have described between *N* and *Arf6* are indicative of a non-essential role of Arf6 in N signalling. Further work will be required to dissect the relative importance of the role of Arf6 in Wg and N signalling to the establishment of the wing margin patterning. An initial test would be whether re-introducing Dl, once again using a *Dl* duplication, in an $Arf6^{KO}$ background would be sufficient to rescue the *Arf6* margin phenotype. In a complementary experiment, the effect of reducing *Dl* dosage on the *Arf6* margin and wing vein phenotypes should allow the two phenotypes to be decoupled, the expectation being that the wing vein phenotype will be enhanced, while the wing margin phenotype will not be affected. I believe that to fully understand the *Arf6* phenotype, and how its cellular function is related to the observed phenotypes, I will need to look more broadly at the effect of removing *Arf6* on cell physiology, such as membrane composition, trafficking pathways, and cytoskeleton dynamics. Once again drawing upon the principle of parsimony, the phenotypes I observe are likely to be a result of complex, subtle interactions between two signalling pathways, however the ultimate cause for these defects is most likely common to both pathways.

Methods

The majority of the methods are presented in the manuscript. In this section I have presented the additional materials and methods used during my thesis.

Generating the protein-protein interaction network

A list of proteins known to be involved in the wingless signalling pathway was generated using FlyBase pathways (FBgg0000889). I carried out a literature review to identify proteins known to suppress the signalling activity of Arm^{S10}. This was not limited to suppression of Arm^{S10} in the context of wing development. The protein-protein interaction network (PPI) was generated using MIST, showing only interactions within the group of proteins. Low confidence interactions were excluded. PPI was plotted used Cytoscape V3.8.0 (Shannon, 2003). Node colours were mapped based on whether the protein gain-of-function or loss of function could suppress Arm^{S10} activity.

Quantification of punctae

The quantification of the number and intensity of punctae was carried out using a custom Cell Profiler pipeline based on the default speckle counting pipeline (McQuin et al., 2018). Counts were normalised to the measurement area.

Fly lines

The following stocks were obtained from Bloomington Stock centre: UAS-NEXT::HA (#63220), UAS-N^{FL} (#26820). Mutant alleles used: N^{1} (#6873), pbl^{2} (#9120), pbl^{3} (#9358), $Rho1^{720}$ (#7325) , tum^{DH15} (#8687), tum^{AR2} (#8686). Tagged proteins: EFA6::GFP (#60587), Siz::GFP (#60287).

E(spl)-C expression was revealed using E(spl)m8-HLH::GFP (a endogenous CRISPR knock-in), and E(spl)m7-HLH::GFP and E(spl)mδ-HLH::GFP (both GFP tagged proteins derived from a BAC (Couturier et al., 2019)) (kind gifts from François Schweisguth). UAS-$Arf6^{Q67L}$::GFP was a kind gift from Suzanne Önel.

The following RNAi lines were used: EFA6-RNAi (VDRC #GD42321), step-RNAi (#KK109449 and #GD32374), siz-RNAi (Bloomington #39060).

Antibodies

Rabbit anti-Pavarotti 1:250 (Lie-Jensen et al., 2019) (a kind gift from Kaisa Haglund), mouse anti-Dl (DSHB C594.9B), mouse anti-NICD (DSHB C17.9C6), mouse anti-NECD (DSHB C458.2H).

Mounting nota

Nota from flies fixed in 70% ethanol were dissected and mounted on silicon dissection plates under 70% ethanol with dissection pins. Samples were imaged using a Leica M205 with an attached Leica DFC7000T.

Discussion

Many of the mechanisms and components of the core signalling pathways that orchestrate metazoan development were initially described through studies carried out in the *Drosophila* model. The Notch and Wingless/Wnt signalling pathways are no exception. The requirement for a wide range of signalling pathways and morphogenic processes to establish the final size, shape and pattern of the adult wing makes the wing a powerful model in which to dissect the mechanisms of signalling pathways. During my research I have worked to understand the roles of the highly conserved small GTP-binding protein Arf6 in wing development. *Arf6* mutant wings displayed a defect that is typically associated with defects in Wg signalling. My objective was to understand the processes underlying this defect. I established that Arf6 has a specific role in the establishment of the proneural clusters that line the wing margin, downstream of high level Wg signalling, but that it is dispensable in the activation of low-level wingless signalling. I demonstrated that Arf6 acts genetically downstream of the stabilisation of Arm, but upstream or at the level of Pangolin activity.

Based on the dominant nature of the *Arf6* phenotype, I tested for a role of Arf6 in the N signalling pathway and found that the *Arf6* mutants strongly enhance *N* mutant phenotypes. Conversely, increasing N signalling in the *Arf6* mutant strongly enhanced the *Arf6* phenotype. The *Arf6* mutant phenotype was also associated with a reduction in the levels of the N ligand, Dl.

Together, my data implicate Arf6 as a novel regulator of Wg signalling and are suggestive of a role for Arf6 in N signalling. The mechanistic mechanisms underlying the roles for Arf6 in these signalling pathways remain to be established, and this represents the focus of ongoing work. In the following discussion, I have explored models that could help explain the potential mechanisms through which Arf6 could regulate Wg signalling, drawing heavily upon mammalian literature, and critically evaluating the models in the light of our findings. I will then discuss the next steps that will help discriminate between these proposed models.

Understanding the Arf6 phenotype

The loss of wing margin bristles, combined with the reduced *sens* expression observed in the Arf6 mutant, are characteristic of a defect in canonical Wg signalling late in wing development resulting in a failure of PNC establishment at the wing margin (Couso et al., 1994; Diaz-Benjumea and Cohen, 1995; Jafar-Nejad et al., 2006; Lawrence et al., 2000b; Phillips and Whittle, 1993; Vincent, 2014). The *Arf6* phenotype is particularly striking due to *Arf6* being haploinsufficient in wing margin

development. Dominant phenotypes are very rare in diploid organisms, and are typically explained by either the level of the gene product being close to the minimum threshold required for wild-type function, or the breaking of the stoichiometry of protein complexes (Kondrashov and Koonin, 2004; Ohnuki and Ohya, 2018; Wilkie, 1994). Interestingly, genes encoding proteins involved in molecular signalling networks, or incorporated into macromolecular complexes are predicted to be more sensitive to gene dosage (Veitia and Potier, 2015). The dominance of the *Arf6* phenotype has intrigued me throughout my thesis. This is primarily due to the apparent paradox between the *Arf6* phenotype dominantly causing the loss of *sens* and margin bristles, and the lack of a more general Wg phenotype in the wing in homozygous *Arf6* mutants. This contrasts with the phenotypes of mutations in core components of the Wg pathway, which typically show the inverse relationship, with an absence of Wg defects in heterozygotes, and a more general, dramatic phenotype in homozygous mutants (Couso et al., 1994). This trend extends to *sens* mutants, which do not show defects in bristle differentiation in heterozygotes, but a total loss of external sensory bristles in homozygotes, including those of the wing margin (Jafar-Nejad et al., 2006; Nolo et al., 2000).

A general, moderate reduction in Wg signalling activity can produce phenotypes superficially similar to the *Arf6* mutant without impacting wing growth (Baena-Lopez et al., 2009; Couso et al., 1994; Pai et al., 1997; Phillips and Whittle, 1993). Importantly, this suggests that the level of Wg signalling necessary to activate low threshold targets 'saturates' before *sens* begins to be expressed (Figure 23). Although this is a huge oversimplification of the transduction of Wg signalling, it represents a theoretical context in which defects in Wg signalling may appear to be specific to low-, or high-level signalling.

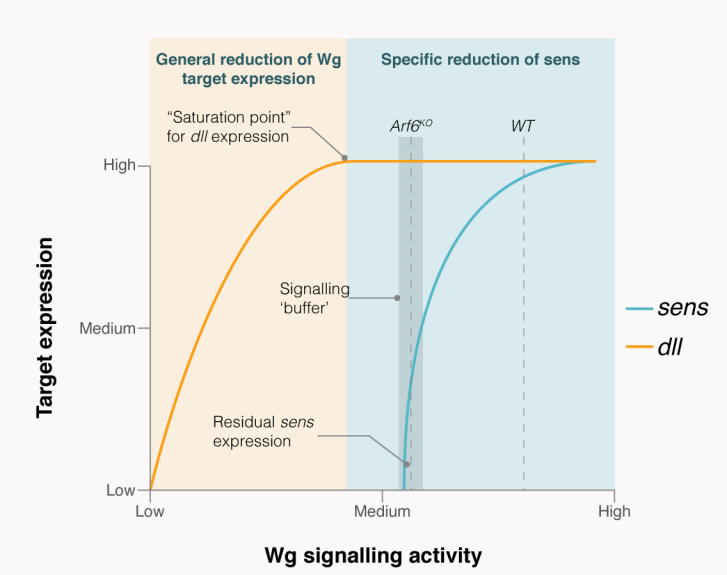


Figure 23 The relationship between low- and high-level Wg signalling

A simplified representation of a theoretical relationship between low- and high- level Wg signalling. This model can explain how a general reduction in Wg signalling can specifically affect the expression of high-level signalling, without impacting low threshold targets. This model depends on the level of low threshold target expression saturating before high threshold targets begin to be expressed. A buffering effect would be required to prevent stabilised Arm from re-introducing *sens* expression in an *Arf6* mutant background.

A model in which the removal of *Arf6* simply induces a general reduction in the transduction of Wg signalling would therefore explain the phenotype we observe in the *Arf6* mutant under otherwise physiological conditions. However, this model would predict that simply increasing Wg signalling activity (such as through the expression of Arm^{S10}), would re-introduce Sens and margin bristles into *Arf6* mutant wings. This contrasts with what we observe in the *Arf6* mutants, in which the ability of constitutively active Arm (Arm^{NDel} and Arm^{S10}) to induce *sens* is dominantly suppressed without blocking its ability to induce the low threshold target *Dll*. A further model parameter is therefore necessary, that dictates that the reduction in Wg signal transduction in the *Arf6* mutant background is buffered against the activity of Arm necessary to trigger *sens* expression. This buffering effect must also already be present in the heterozygous *Arf6* mutant in order to explain the dominant suppression of Arm^{S10} and Arm^{NDel}.

There are very few examples of such Wg regulators in the literature. However, the most phenotypically similar mutants are those of the neural selector gene *cut (ct)*, as some *ct* alleles lead to a dominant loss of margin bristles throughout the wing margin, and margin notching (Jack et al.,

1991; Krupp et al., 2005; Micchelli et al., 1997). As I discussed in the introduction, *ct* is expressed in response to N signalling, and is necessary to prevent autocrine Wg signalling activity in the wing margin cells (Micchelli et al., 1997). Similarly to the *Arf6* mutants, increasing Wg signalling in *cut* mutants does not rescue the wing margin bristles; however Sens is not abolished in *ct* mutant clones, indicating that Ct is not required for Wg transduction (Buceta et al., 2007; Krupp et al., 2005). The loss of wing margins in *ct* mutants is likely to be a result of a late developmental defect in the wing margin bristles, downstream of SOP establishment, and not a failure of PNC establishment as we observe in the *Arf6* mutants.

The lack of described Wg signalling regulators that give rise to comparable phenotypes to those induced by the loss of *Arf6* hint at the possibility that Arf6 could be necessary in a novel regulatory step in Wg signalling. I could not establish the mechanism through which Arf6 acts During my research, however the requirement of Arf6 genetically downstream of Arm stabilisation and upstream or at the level of Pan activity restricts the possible steps of the Wg pathway that could be affected. With this in mind, I will describe the potential links between Arf6 and Wg signalling in the following sections, and critically evaluate each step. These models will later serve as the basis on which the experiments to determine the function of Arf6 in Wg signalling will be designed.

How could Arf6 act downstream of Arm stabilisation?

Arm is the key effector for the activation of Wg signalling, and alongside Pan, is necessary for the activation of all described Wg signalling targets (Franz et al., 2017). Blocking the access of Arm to the nucleus will therefore lead to a complete loss of Wg signalling activity. Although there is a strong reduction in Sens in the wing margin PNCs of *Arf6* mutants, *sens* expression is not fully abolished. This indicates that Arf6 contributes to the establishment of the wing margin PNCs, but is not a core, general regulator of Wg signalling transduction. This is supported by the lack of effect of the loss of *Arf6* on the expression of the low level Wg signalling target, *Dll,* and the absence of wing notching in the *Arf6* mutant background.

A model in which Arf6 plays a role in regulating the signalling output of stabilised Arm would need to take into consideration the specificity of the *Arf6* phenotype to high level signalling, and the lack of rescue by exogenous constitutively active Arm. One possibility is that in the *Arf6* background, Arm stabilised downstream of Wg activation is sequestered in a compartment, restricting its access to the nucleus. One of the proposed molecular sinks for cytoplasmic Arm is the adherens junctions (AJ) (Bienz, 2005; Heuberger and Birchmeier, 2010; Valenta et al., 2012). Although the role for Arm from

the AJs in activating Wg signalling is still debated, it has been shown that over-expressing the intra-cellular domain of E-Cadherin is sufficient to suppress Wg signalling, and that removing a single copy of E-Cadherin can rescue a strong a*rm* hypomorph (Cox et al., 1996; Sanson et al., 1996). This suggests that although Arm from the adherens junctions may not directly promote Wg signalling, the AJ can sequester Arm, stabilised following the deactivation of the destruction complex.

Regulation of Arm sequestration by Arf6?

A recent study proposed that actomyosin contractility might provide a mechanism through which cytoplasmic Arm could be sequestered to the adherens junctions (Hall et al., 2019). The authors found that strongly increasing non-muscular myosin NMII activity could induce wing margin notching, and a reduction in Dll staining (Hall et al., 2019). Importantly, increased actomyosin activity could also suppress the ability of Arm^{S10} to induce *dll* expression (Hall et al., 2019). The authors suggested that the increase in actomyosin activity induced the stabilisation of the adherens junctions, sequestering cytoplasmic Arm, and reducing Wg signalling. The effect of these treatments on *sens* expression was not presented.

Actomyosin contractility is promoted by the localised activity of the Rho family of small GTPases, comprising Rho, Rac and cdc42 (Arnold et al., 2017). Arf6 has been shown to regulate actin cytoskeleton dynamics by indirectly modulating the activity of the Rho GTPases (Singh et al., 2019). Arf6 can activate Rho-GTPase activity by promoting phosphoinositide synthesis at the plasma membrane, that can in turn increase the membrane localisation of Rho-GEFs (Singh et al., 2019). Arf6 can also negatively regulate Rac1 activity by recruiting the Rac1-GEF inhibitor, NM23-H1, to sites of cell-cell contact, locally reducing Rac1 activity (Palacios et al., 2002). The modulation of Rho-GTPase activity by Arf6 raises the possibility that Arf6 modulates Wg signalling by regulating actin cytoskeleton dynamics.

In a model in which the *Arf6* margin phenotype is a result of increased actomyosin contractility, Arf6 would be predicted to act as a negative regulator of actomyosin activity. We did not observe any of the characteristic morphological defects of increased contraction in *Arf6* mutant clones, including smooth clone boundaries, and changes in the intensity and subcellular localisation of actin, E-Cadherin and Arm. Beyond the loss of margin bristles, *Arf6* mutant wings were broadly morphologically WT, showing no curling or distortion or PCP defects. Although this suggests that the *Arf6* phenotype is not a result of increased contraction, more subtle, localised changes, or changes in actomyosin dynamics could be easily overlooked.

I initially identified Pav as potentially linking Arf6 to Wg signalling, due to its described non-canonical role as a negative nuclear regulator of Wg signalling. I hypothesised that Arf6 could be responsible for restricting Pav activity in the wing, preventing it from suppressing high level Wg signalling. This model was supported by the partial rescue of the Arf6 phenotype in a *pav* mutant background. However, dominant enhancement of the Arf6 phenotype in the *tum* mutant background, and lack of observable change in endogenous Pav localisation in *Arf6* mutant tissue are less convincing. In the original publication describing the role of Pav in Wg signalling, the authors found that Tum appeared to act in a partially redundant manner with Pav, with both proteins requiring functional nuclear localisation signals to reduce Wg signalling (Jones et al., 2010). I would have therefore expected *tum* mutants to have a similar effect to *pav* mutants in their ability to rescue *Arf6.*

The conflicting effects of *pav* and *tum* alleles in the *Arf6* background may be explained by a recent study describing another non-canonical role of *pav* and *tum* in wound healing in *Drosophila* (Nakamura et al., 2020). This is perhaps unsurprising considering the analogy between cytokinesis and wound healing, with both processes requiring the precisely coordinated formation and contraction of an actomyosin ring and rapid membrane turnover (Abreu-Blanco et al., 2014; Darenfed and Mandato, 2005). Pav was shown to directly bind to, and induce the bundling of fibrous actin (Nakamura et al., 2020). Consistently, *pav* mutant embryos showed defects in wound healing, characterised by a reduced rate of actin accumulation, a narrower actin ring, and slower wound closure. In contrast, wound closure was accelerated in *tum* mutant embryos, accompanied by increased wound expansion and a wider actin ring (Nakamura et al., 2020). Following this logic, the rescue of the *Arf6* phenotype could be explained by reduced actin bundling and contractility in the *pav* mutant background, and enhanced by the additive effect of increased contractility in the *tum* background due to increased Rho activity (Nakamura et al., 2020). This is supported by the enhancement of the *Arf6* phenotype in the *rho1* mutant background and the partial rescue in the *pbl* mutant background. Pbl has also been shown to act as a negative regulator of Wg signalling during embryonic development, likely through its activity as a Rho-GEF, although a mechanism was not described (Greer et al., 2013). Importantly, Arf6 has been shown to accumulate at the wound margin in *Drosophila* embryos, suggesting a potential involvement for Arf6 during this process (Hunter et al., 2015). Although these data may provide a potential resolution to the conflicting phenotypes I observed in the genetic interactions between *tum, pav* and *Arf6*, I cannot exclude the possibility that the *Arf6* mutant background is simply sensitised to modifications of the actin cytoskeleton.

The activation of high level canonical Wg signalling has been correlated with an increase in NMII- and Rho1-dependent apical actomyosin contractility in the *Drosophila* wing (Zimmerman et al.,

2010). This could help to explain the enhancement of the *Arf6* margin phenotype by Arm^{S10}, Arm^{NDel} and Dsh, as the Wg-signalling-induced increase in actomyosin contractility could act synergistically with the *Arf6* mutant phenotype. The activation of Arf6 could be coupled with increased actomyosin contractility, as the Arf6-GEF, Step has been shown to be recruited to sites of high actomyosin activity such as cell-cell junctions, the cleavage furrow and the periphery of wounds (West et al., 2017). Step was then shown to inhibit actomyosin contractility, potentially through the activation of Arf6 and Arf1 (West et al., 2017). This is consistent with our observation that knocking-down *step* phenocopies aspects of the *Arf6* margin phenotype. The planar cell polarity defects present in the *step* knockdown may be due to a more general defect in Rho-GTPase activity, or a reduction in Arf1 activity (Carvajal-Gonzalez et al., 2015; Eaton et al., 1996).

Reduced cdc42 activity has previously been shown to induce a loss of mechanosensory bristles in the anterior wing margin, without a loss of Wg expression (Baron et al., 2000). Similar to *Arf6,* the dominant negative cdc42 phenotype was sensitive to *N* dosage: the removal of a single copy of *N* (using deficiency Df(1)N-54l9), partially rescued the loss of wing margin bristles, whereas increasing N signalling using a hypermorphic *N* allele ($N^{Ax\text{-}E2}$) enhanced the loss of wing margin bristles (Baron et al., 2000).

Taken together, these studies provide evidence for the co-option of a series of proteins involved in regulating actomyosin contractility during cytokinesis and wound healing as regulators canonical Wg signalling. Although many of the functional links I have presented have been experimentally tested, the integration of these links into a wider regulatory network have not. An overview of a theoretical model is presented below in Figure 24

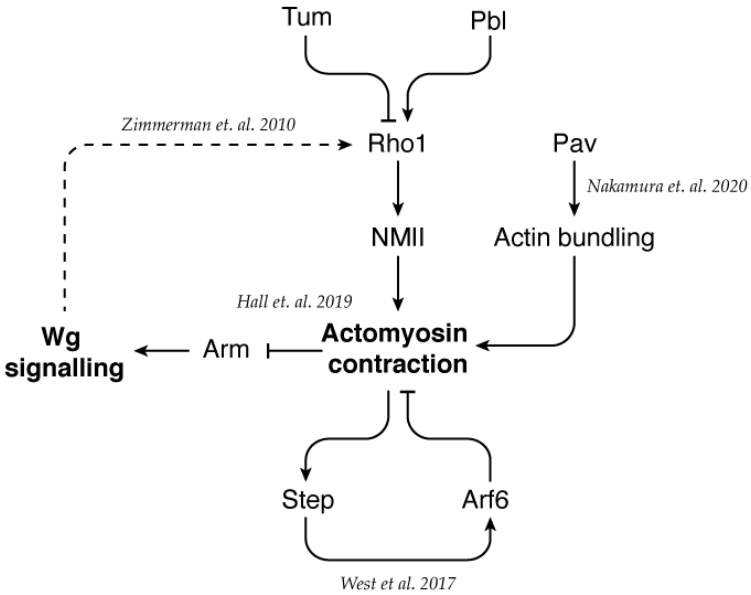


Figure 24 Theoretically linking Arf6, actomyosin dynamics and Wg signalling

A hypothetical model through which Arf6 could regulate Wg signalling through the modulation of actomyosin contractility. The corresponding studies describing the individual links of the model are shown.

Could Arf6 regulate Arm signalling activity?

Rac1 has been shown to be activated downstream of Wnt signalling, promoting the JNK2 kinase-mediated phosphorylation of β-catenin at serines 191 and 605 (Wu et al., 2008). S191 and S605 phosphorylation was said to promote the interaction between β-catenin and LEF-1 in the nucleus, increasing Wnt signalling activity (Jamieson et al., 2015). Although Rac1-GTP associated with β-catenin was predominantly found in the nucleus, Rac1 activation was suggested to take place at the plasma membrane, and to depend on LRP5/6, DVL, and $G\alpha_q/11\beta\gamma$ (Wu et al., 2008). Arf6 has been suggested to activate Rac1/RhoA downstream of $G\alpha_q$, and to itself be activated downstream of Wnt signalling, in an LRP6, DVL and FZD dependent process (Grossmann et al., 2013; Kim et al., 2013; Pellon-Cardenas et al., 2013; Yoo et al., 2016). Could Arf6 therefore mediate the activation of Rac1 necessary for the phosphorylation of β-catenin, promoting its signalling activity? Serine 191 is conserved between Arm and β-catenin: the role of this phospho-site has not however been assessed in Wg signalling. From the data presented, the loss of Rac1 appears to induce a complete loss of Wnt3a signalling activity *in vitro*, indicating that these modifications are indispensable for Wnt signalling. Reduced Rac1 activity in the

drosophila wing leads to PCP defects, but does not lead to canonical Wg signalling phenotypes, indicating that the role of Rac1 is not conserved in Wg signalling (Eaton et al., 1995; Eaton et al., 1996).

Could Arf6 regulate nuclear Wg signalling

The process through which Arm/β-catenin enter the nucleus is not fully understood (Fagotto, 2013), but since Arf6 has not previously been implicated in protein nuclear import, it is unlikely to directly regulate the nuclear import of Arm/β-catenin. I have found evidence that the *Arf6* phenotype could be the result of a change in the non-canonical activity of Pav as a negative regulator of Wg signalling during wing development. The mechanism through which Pav regulates Wg signalling is unknown, but it has been shown to rely on the nuclear localisation of Pav, and to act at the level of Pan/TCF activity (Jones et al., 2010). MKLP1 has also been shown to regulate Wnt signalling; however MKLP1 appears to act as a positive regulator of Wnt signalling, suggesting that the regulatory mechanisms are not conserved (Ji et al., 2021; Liu et al., 2020). The requirement for both Pav and Tum to localise to the nucleus suggests they could regulate a nuclear regulator of Wg signalling, although its identity is not known.

Staining for Pav in $Arf6^{KO}$ wing imaginal discs did not reveal a noticeable change in Pav localisation, and the nuclear localisation of Pav was not readily apparent. This is perhaps not surprising considering the dispensability for Arf6 in somatic cytokinesis, suggesting that Arf6 is not an essential regulator of Pav in most contexts (Dyer et al., 2007). Furthermore, expressing either WT *pav* or *tum* in the developing *Drosophila* embryo led to epidermal defects associated with a suppression of Wg signalling, while expressing them during wing development did not phenocopy the *Arf6* phenotype (Jones et al., 2010). This indicates that simply increasing the expression of *pav* or *tum* in the developing wing is not sufficient to suppress Wg signalling, and suggests that Pav and Tum may not regulate Wg signalling in the same manner during embryonic and larval development.

I have discussed the possibility that the genetic interaction we observe between *Arf6* and *pav* could be the result of a recently described role for Pav in the regulation actin bundling, but I cannot exclude the possibility that it is due to the nuclear role of Pav (Nakamura et al., 2020). It is not clear how Arf6 could regulate Pav activity; however the direct binding of Pav and Arf6 is likely to contribute (Dyer et al., 2007; Makyio et al., 2012). When in complex with MKLP1, Arf6's myristoylated N-terminal amphipathic helix is not blocked by the binding of the two proteins (Makyio et al., 2012). The myristoylate and the N-terminal helix both contribute to the membrane binding ability of Arf6 and impart Arf6 with a high affinity for membranes (D'Souza-Schorey and Stahl, 1995). Based on the high

level of sequence conservation of both Pav/MKLP1 and Arf6, these structural properties are also likely to be conserved in *Drosophila*. Arf6-GTP could therefore recruit Pav to endosomal membranes, limiting its nuclear entry and de-repressing Wg signalling. The inhibition of Pav nuclear localisation could also take place independently of direct interactions with Arf6. This could be mediated by the recruitment of Pav to sites of increased actin activity (Nakamura et al., 2020). This would suggest that activated Arf6 would need to promote actin polymerisation, sequestering Pav. It is not clear how $Arf6^{Q67L}$ reduces the subcellular levels of Pav, but a more detailed analysis would be required, to establish whether it was a result of Pav becoming more diffuse, or whether it was truly a result of reduced Pav levels.

Is it really a Wg phenotype?

Reduced Sens staining in the wing margin, and loss of margin bristles in the adult wing are often used as readouts for defects in canonical Wg signalling (Alexandre et al., 2013; Baena-Lopez et al., 2009; Schweizer et al., 2003; Thompson et al., 2002; Vincent, 2014). I have focused on the Wg signalling pathway to explain the *Arf6* mutant phenotype; however, other factors contribute to the expression of *sens* in the wing margin. There is therefore the possibility that the *Arf6* phenotype is not a direct result of reduced Wg signalling, and is instead due to Arf6 being required in a parallel process necessary for *sens* induction by Wg. This is of importance due to the apparent specificity of the *Arf6* phenotype to *sens,* as this places more emphasis on the regulatory nuances associated with the specific target, rather than the defects in the upstream signalling pathway.

The use of Sens as a readout for Wg signalling is fundamentally based on Wg activation being both necessary and sufficient to induce sens expression in the developing wing (Jafar-Nejad et al., 2006). However, the wing pouch of the imaginal disc is the only known context in which *sens* is downstream of Wg signalling (Jafar-Nejad et al., 2006; Nolo et al., 2000; Vincent, 2014). This is exemplified by the sufficiency of Arm^{S10} to induce *sens* expression within the wing pouch, but not in the notum (Sanders et al., 2009). The context specific activation of *sens* by Wg signalling indicates that although Wg signalling contributes to inducing *sens* expression, there is likely to be a greater level of complexity underlying *sens* regulation. The *sens* locus is not exceptional in this regard, as *Ser* and *Dl* expression is also induced by Wg signalling in the wing pouch, but not in the thorax (De Celis and Bray, 2000). This specific activity could be explained by the requirement of wing margin specific co-factors,

or permissive signals that would be required alongside Pan/Arm, or to act in parallel to Wg for the activation of *sens* expression (Figure 25).

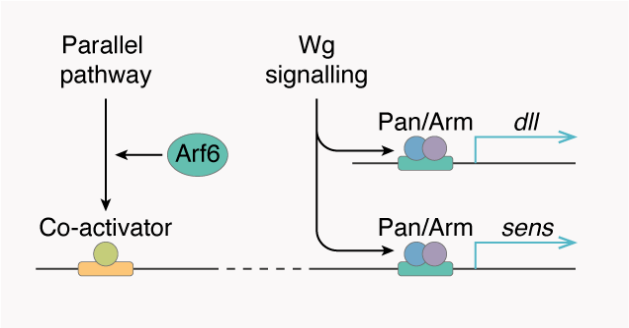


Figure 25 Is Arf6 regulating Wg signalling?

A schematic representation of a model in which Arf6 is required in a parallel pathway to Wg signalling necessary to co-regulate the expression of *sens* but not *dll*. The parallel pathway would not necessarily directly regulate the *sens* locus (represented by the dashed line).

The bHLH proneural transcription factors of the *achaete-scute* complex are the core regulators of *sens* expression in the fly; could the *Arf6* phenotype therefore be explained by a defect in the activity of Ac and Sc? The interactions between Ac, Sc and Sens are complex, both at a molecular and genetic level (I have outlined these interactions in my introduction), however there are several lines of evidence that indicate that the *Arf6* phenotype is independent of a role of Arf6 in the regulation of the bHLH regulatory network. Firstly, while flies that lack both *ac* and *sc* (using the $sc^{10\text{-}1}$ deficiency (Garcia-Bellido, 1979)) lose almost all the bristles on the whole body, the stout margin bristles and non-innervated posterior margin bristles remain (García-Bellido and De Celis, 2009; Jack et al., 1991; Jafar-Nejad et al., 2006). Importantly, Sens is lost from the nota of $sc^{10\text{-}1}$ flies, while remaining in both the anterior and posterior wing margin PNCs (Jafar-Nejad et al., 2006; Lai, 2003). The lack of requirement for the bHLH proneural proteins is particularly apparent in the posterior wing margin bristles, in which no known bHLH proneural factors are expressed, and which are lost in the *Arf6* mutant (Jack et al., 1991; Jafar-Nejad et al., 2003; Nolo et al., 2000). Together, these data indicate that the expression of *sens* in the wing margin, and the formation of stout mechanosensory and non-innervated posterior bristles does not require the activity of the bHLH transcription factors. Thus, If Arf6 were required for the induction or maintenance of *sens* expression downstream of the bHLH proneural proteins, we would have expected the opposite phenotype to the one we observe in the *Arf6* mutants: Sens would have been lost in all SOPs apart from those of the wing margin, resulting in a subsequent loss of all external sensory organs, leaving only the stout and non-innervated margin bristles. Furthermore, Sens is required in the development of all external sensory organs, Arf6 cannot therefore be a general regulator of Sens expression or activity, as we would otherwise expect the loss of all the external sensory organs in an *Arf6* mutant (Jafar-Nejad et al., 2003; Nolo et al., 2000). The

specific loss of Sens in the wing margin of *Arf6* mutants therefore indicates that a Wg-related process is most likely affected.

Beyond Wg signalling, the factors required in the induction of sens expression in the wing margin are not fully understood. Mad (an orthologue of Smad1) and Medea (an orthologue of Smad4) are known for their roles in the transduction of the Decapentaplegic (Dpp, a Drosophila BMP orthologue) pathway, however they have been suggested to play a role in the patterning of the wing margin (Eivers et al., 2011; Quijano et al., 2011; Yang et al., 2013; Zeng et al., 2008a). The balance between the role of Mad in Dpp and Wg signalling was suggested to be mediated by the Sgg-mediated phosphorylation of Mad (Eivers et al., 2009; Yang et al., 2013; Zeng et al., 2008a). In the absence of Wg, Sgg phosphorylated Mad, leading to its degradation, Mad was stabilised by the suppression of Sgg by Wg and Dsh (Eivers et al., 2009; Sekelsky et al., 1995). Expressing a form of Mad lacking the Sgg phosphorylation sites induced the formation of ectopic wing margin bristles, and a mild increase in the level of Sens in the anterior margin.

Mad and Medea were suggested to directly bind to the complex of Pan and Arm, enhancing their signalling activity (Eivers et al., 2011). However, the requirement for Mad does not appear to be specific to the activation of *sens* expression, as Mad RNAi reduced the expression of both *dll* and *sens* (Eivers et al., 2011). This is consistent with the authors suggesting that the complex of Pan/Arm and Mad binds to canonical wingless responsive elements (Eivers et al., 2011). This would therefore indicate that Mad would be a general regulator of Wg signalling, rather than specifically promoting the expression of a subset of Wg targets as we observe with Arf6.

Based on the proposed models of Mad activity in Wg signalling, Mad would only contribute to Wg signalling in the absence of Sgg activity (Eivers et al., 2011; Yang et al., 2013; Zeng et al., 2008a). In contrast to *Arf6* mutants, loss of Mad activity alone would therefore not be expected to suppress signalling by Arm^{S10}. This would however need to be tested due to the potential for Arm^{S10} to bind to components of the degradosome complex, suppressing its activity (Pai et al., 1997; Tolwinski and Wieschaus, 2001).

A number of nuclear Wg regulators have been shown to be required for the activation of Wg signalling through the regulation of the Pan enhanceosome activity (Gammons and Bienz, 2018). A range of cofactors are recruited by the c-terminal of Arm and act to modify chromatin structure (Städeli et al., 2006). The accessibility of the regulatory elements of specific Wg targets to transcriptional regulators could provide a potential mechanism for the decoupling the expression of high and low threshold Wg target genes. Components of the nucleosome remodelling factor complex (NURF) have been shown to be required for Wg signalling to fully induce *sens* expression at the wing

margin (Song et al., 2009). Intriguingly, mutants for a core ATPase of the NURF complex, ISWI, led to a partial suppression of *sens* expression without affecting the expression of *dll* (Song et al., 2009). ISWI was found to bind to Arm, and to be recruited to Wg responsive enhancers. ISWI represents one of the few described factors in the literature that is specifically required for the enhancement of high level Wg signalling (Song et al., 2009). Although Arf6 is unlikely to directly regulate chromatin modelling factors, Pav and Tum have been shown to coimmunoprecipitate with a series of chromatin modelling factors including CAF1-55, a component of the NURF complex, providing a speculative link (Moshkin et al., 2009).

There are likely to be other pathways that contribute to the establishment of the pattern of Sens in the wing margin, but the specificity of the loss of Sens to the wing margin, the lack of dramatic morphological defects in the wing, and the ability of Pan^{VP16} to rescue the Sens in the wing margin make Wg the most likely pathway to explain the *Arf6* phenotype. Nevertheless, I cannot rule out the possibility that Arf6 acts in a process that is in parallel to the Wg signalling pathway, and required for Wg signalling to reach its full potential. Further work will need to be carried out to establish whether this is the case. An initial step would be to carry out RNA sequencing of *WT*, $Arf6^{KO}$ wing imaginal disc, with and without Arm^{S10} expression. The relative expression levels of known targets of Wg signalling could be used to allow us to test for 'cryptic' *wg* phenotypes associated with the loss of *Arf6* (Franz et al., 2017).

Perspectives

What are the next steps?

The data I have presented in my thesis provide evidence of a novel role for Arf6 in developmental patterning through Wg signalling. Although we were able to establish that Arf6 acts genetically downstream of the stabilisation of Arm, but upstream or at the level of the Wg enhanceosome complex, the mechanism through which Arf6 achieves its function in this context remains elusive. In my discussion I have presented several models that could provide the link between Arf6 and Wg signalling based on the data I have presented. I have outlined the next steps of this research under two broad sections: upstream of Arf6, and downstream of Arf6. Upstream refers to the processes governing Arf6 activity in Wg signalling, while downstream refers to the ways in which Arf6 regulates Wg signalling.

Upstream of Arf6

Identifying the Arf6 regulators, and the processes determining their localisation and activity in Wg signalling would help us understand the regulatory framework that surrounds Arf6 function during wing development. The process of identifying the Arf6-GEFs is complicated by the potential for multiple GEFs redundantly regulating Arf6 activation, as all the putative *Drosophila* Arf6-GEFs, Siz, Step and EFA6 are present in the cells of the wing margin cells. Furthermore, care will need to be taken to control for the exchange activity of Step and Siz on Arf1. This can in part be achieved by knocking-down the GEFs in a homozygous *Arf6* mutant background, any enhancement of the phenotype in this background being independent of the Arf6-GEF activity.

Our preliminary data suggest that Step is responsible for the activation of Arf6 during wing margin development. This is based on a partial phenocopy of the *Arf6* phenotype by the knock-down of *step,* and the enhancement of the Arf6 phenotype by *step* RNAi. These findings will need to be complemented with biochemical assays showing both the sufficiency and necessity of Step for exchange activity for Arf6. This would ideally be carried out *in vivo* or in *in vitro* cell assays, as all the putative *Drosophila* Arf6-GEFs appear to contain functional Sec7 domains, making it likely that they are all able to activate Arf6 in biochemical assays. Following the identification of the Arf6-GEF, the key question is how the GEF is regulated in the context of wing margin development, and more specifically whether Arf6 activation is a direct consequence of Wg activation.

Our observations provide indirect evidence that Arf6 activation is unlikely to be directly coupled with Wg activation: knocking down components of the signalosome components *arrow* or

dishevelled did not block the signalling activity of Arm^{NDel}. Furthermore, the activation of *sens* expression Arm^{NDel} was suppressed even far into the dorsal and ventral wing compartments in which there are low levels of endogenous Wg. Arf6 activation may therefore be entirely independent of Wg signalling, or could integrate multiple inputs to refine the output of Wg signalling. These inputs may be intrinsic cellular processes such as the cell cycle. This possibility is especially pertinent, due to multiple studies reporting cell cycle and cytokinesis regulators influencing Wg signalling (Greaves et al., 1999; Greer et al., 2013; Jones et al., 2010; Krupp et al., 2005; Marygold and Vincent, 2003).

Very little is known about the *Drosophila* Arf6-GAPs; however there is growing evidence that the Arf-GAPs constitute the largest family of Arf effectors (East and Kahn, 2011). The role and regulation of Arf6-GAPs has not been tested in Wg/Wnt signalling. Relevant Arf6-GAPs could ideally be identified based on the ability of their knock-down to rescue the heterozygous but not homozygous *Arf6* mutant. phenotype. Conversely, over-expressing Arf6-GAPs would be expected to recapitulate the Arf6 wing phenotype. Beyond providing us with a proximal mechanistic understanding of the regulation of Arf6 in Wg signalling, these data could provide valuable insights into how Arf6 activity is controlled in cells simultaneously expressing multiple Arf6-GEFs and GAPs (Sztul et al., 2019).

Downstream of Arf6

I have described several models through which Arf6 could regulate the downstream steps of Wg signalling. One of the main distinctions between these models is whether Arm localisation is affected in the absence of Arf6. We did not observe changes in Arm levels in *Arf6* mutant clones, but the lack of a general, dramatic loss of Wg signalling in *Arf6* mutant would suggest that any change in the subcellular localisation or levels of Arm is likely to be subtle. Importantly, the *Arf6* phenotype is far from representing a complete Wg loss-of-function, indicating that Arm must still be present in the nucleus. It is difficult to observe nuclear Arm in the wing imaginal discs, even in conditions in which Arm is over-expressed (Somorjai and Martinez-Arias, 2008; Vuong et al., 2018). Although nuclear export inhibitors such as the CRM1 inhibitor, Leptomyosin B have been used in the study of Arm/β-catenin localisation, they can lead to misleading results due to the nuclear sequestration of β-catenin binding proteins such as APC and Axin, artificially increasing the nuclear retention of β-catenin (Fagotto, 2013). Measuring nuclear Arm levels in a steady state in the wing is unlikely to provide the resolution necessary to explain the Arf6 phenotype.

Tracing the fate of Arm released from the adherens junctions, and directly attributing changes in Wg signalling to changes in Arm levels in the adherens junctions poses a further challenge:

fundamentally, we do not know how changes in the level of cytoplasmic or junctional Arm/β-catenin translate into changes in Wg signalling. This represents one of the core issues in the ongoing debate regarding the role of a functional exchange of β-catenin/Arm in the activation of Wnt/Wg signalling (Van Der Wal and Van Amerongen, 2020). The tools do not yet exist to selectively label Arm molecules at the adherens junctions and to track their fate. Many studies therefore rely on the correlation between increases in the expression of Wg signalling reporters, and changes in the subapical and membrane-associated pool of Arm.

An effective strategy to begin understanding whether the retention of stabilised Arm plays a role in the *Arf6* phenotype would need to remain agnostic as to the identity of a potential Arm sink. I would begin by using the *Arf6* mutant background to carry out a biased modifier screen of cytoskeleton regulators, including the Rho family regulators, to identify the potential factors involved in the *Arf6* phenotype. The pleiotropic effects of constitutively activating actomyosin constriction, Rho-GTPases or disrupting the adherens junctions mean that experiments would ideally be carried out in a highly temporally controlled manner, or with very mild treatments. A screening background could be generated in which Arm^{S10} or Arm^{NDel} are expressed in a heterozygous *Arf6* mutant. This would provide a genetic background sensitised to small increases in the signalling capacity of the stabilised Arm. This background would also allow the role of N signalling regulators to be tested, as in this background, the major source of Wg signalling would be independent of a need for endogenous N-mediated *wg* expression.

Looking beyond wing development

We identified the *Arf6* phenotype due to the external morphological defects present in the *Arf6* mutant wing. The involvement of Wg and N signalling in a wide range of developmental and homeostatic processes means that we have likely overlooked other, less apparent processes involving Arf6 activity. Establishing that Arf6 acts on Wg, and potentially N signalling will help focus the search for processes in which Arf6 is required, ultimately leading to a more profound understanding of the developmental roles of Arf6 and its regulators.

It remains to be determined whether the role we have described for Arf6 in Wg signalling is conserved in Wnt signalling. Wnt conditioned medium has previously served as the only method used to activate Wnt signalling in studies of the role of Arf6 in Wnt signalling (Grossmann et al., 2013; Pellon-Cardenas et al., 2013). Under these experimental conditions, an upstream requirement for Arf6 will be mask a potential downstream function, possibly leading to a secondary role being missed.

Experiments in which Wnt signalling is activated either through the expression of stabilised β-catenin, or through the suppression of the β-catenin destruction complex would reveal whether this is the case. If Arf6 were found to act at multiple levels in Wnt signalling (signalosome activity, adherens junction stability and downstream of β-catenin stabilisation), then Arf6 could represent a valuable therapeutic target. The majority of colorectal cancers are caused by mutations in APC, leading to a suppression of the activity of the destruction complex and accumulation of β-catenin, constitutively activating ligand-independent Wnt signalling (Nusse and Clevers, 2017; Wood et al., 2007). Similarly, inactivating mutations of Axin and hyperactivating mutations in β-catenin itself have also been identified (Nusse and Clevers, 2017). A role for Arf6 downstream of the stabilised β-catenin would therefore makes Arf6 a potentially effective target with which to cell autonomously target Wnt signalling. Drugs that efficiently suppress Arf6 activity are available, having been approved for clinical use (Macia et al., 2021).

The level of conservation combined with the restricted number of Arf6 family proteins in *Drosophila*, and the ease of phenotypic scoring, make the wing margin an ideal context in which to further investigate the role of Arf6, and other Arf family GTP binding proteins and their regulators, in Wnt/Wg signalling. The dominance of the Arf6 phenotype is indicative of a lack of functional redundancy between Arf6 and other Arfs in the wing margin, and highlights a distinct role for Arf6 in this context, which could provide a model in which to study the activity and regulation of Arf6 beyond Wg signalling pathway.

Annex

During my thesis I was involved in writing a review entitled *Functions of Wnt and Hedgehog-containing extracellular vesicles in development and disease* discussing our current understanding of the role of extracellular vesicles in Wnt and Hh signalling. I also contributed to a spotlight article entitled *Circulating Hedgehog: a fresh view of a classic morphogen* describing and discussing Suzanne Eaton's extensive contributions to our understanding of Hh transport and signalling. Both of these articles are presented in annexes.

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REVIEW

Functions of Wnt and Hedgehog-containing extracellular vesicles in development and disease

Tamás Matusek, Julien Marcetteau and Pascal P. Thérond*

ABSTRACT

Secreted morphogens play a major role in the intercellular communication necessary for animal development. It was initially thought that, in order to organize tissue morphogenesis and control cell fate and proliferation, morphogens diffused freely in the extracellular space. This view has since changed following the discovery that morphogens of the Wnt and Hedgehog (Hh) families are modified by various lipid adducts during their biosynthesis, providing them with high affinity for the membrane bilayer. Recent work performed in model organisms suggests that Wnt and Hh proteins are carried on extracellular vesicles. In this Review, we provide our perspectives on the mechanisms of formation of Wnt- and Hh-containing extracellular vesicles, and discuss their functions during animal development, as well as in various human physiopathologies.

KEY WORDS: Hedgehog, Wnt, Extracellular vesicles, Morphogen

Introduction

Intercellular communication is crucial for tissue homeostasis under both physiological and aberrant conditions. Paracrine signaling, in which a ligand is released from its site of production and travels through the extracellular environment, plays an instructive role during morphogenesis (Wolpert, 2016). Paracrine signaling is also implicated in pathological disorders, including cancer progression, the regulation of cancer stem cell homeostasis and in passing directive cues to the surrounding stromal cells (Hanahan and Weinberg, 2011). Under both normal conditions and during cancer development, cell-fate decisions and tissue homeostasis require precise input of combinatorial information received from the extracellular space.

Secreted morphogens act in a paracrine manner during embryogenesis (Wolpert, 2016). These molecules define cellular fate and organize tissues over both short and long ranges by establishing a functional gradient of activity through a variety of transportation mechanisms. Failures in secretion, extracellular transport or transduction of these morphogenetic signals lead to severe developmental disorders and can induce tumorigenesis (Beachy et al., 2004; Briscoe and Thérond, 2013; Nusse and Clevers, 2017).

The Hedgehog (Hh) and Wnt families of secreted molecules are classic morphogens originally identified in *Drosophila*. Only one *Hh* gene is present in *Drosophila*, whereas vertebrates express three paralogues, i.e. sonic hedgehog (*Shh*), indian hedgehog (*Ihh*) and desert hedgehog (*Dhh*). Wingless (*Wg*) is the main Wnt gene expressed in *Drosophila*, whereas 19 Wnt homologs have been identified in vertebrates (Nusse and Clevers, 2017). Hh and Wg proteins are responsible for directing cell-fate decisions and patterning processes during embryonic segmentation, and the development of the *Drosophila* larval imaginal discs (Lee et al., 2016; Swarup and Verheyen, 2012). Their vertebrate orthologs also fulfill essential roles in developmental patterning, such as during limb development and morphogenesis of the neural tube (Briscoe and Small, 2015; Briscoe and Thérond, 2013). Furthermore, they are essential for stem-cell maintenance in different tissues, including the adult intestine, epidermis and brain (Álvarez-Buylla and Ihrie, 2014; Nusse and Clevers, 2017; Shyer et al., 2015; Strzyz, 2016).

In addition to its various morphogenetic roles, the deregulated secretion of Hh family proteins drives the proliferation of many cancer types, including breast, prostate, pancreatic, lung, hepatocellular and digestive tract cancers (Beachy et al., 2004; Briscoe and Thérond, 2013). Hh production also influences the tumor microenvironment in a paracrine manner by signaling to the stroma, which then communicates back to the tumor, promoting its growth (Rubin and de Sauvage, 2006; Yauch et al., 2008). Wnt signaling is well known for its prominent role in colorectal cancer but is also important in various other cancers (Clevers et al., 2014; Nusse and Clevers, 2017). Since these morphogen-mediated signals influence tumor formation, to understand how they are transported from cell to cell will improve our knowledge of these deleterious pathologies.

During their biosynthesis, the Hh and Wnt protein families are modified by lipid adducts, providing them with a high affinity for biological membranes and cell surface proteins. The mature Hh peptide has a cholesterol moiety covalently attached to its C-terminus and a palmitic acid (saturated fatty acid) on its N-terminus (Pepinsky et al., 1998; Porter et al., 1996). By contrast, Wnt proteins are lipidated with the monounsaturated fatty acid palmeolic acid (Takada et al., 2006; Willert et al., 2003). Hh molecules also appear to exist in many different post-translationally modified forms *in vivo* and have been found to have more than one type of fatty-acid attachment (Long et al., 2015). These modifications could greatly influence the affinity or localization of morphogens to specific membrane microdomains, which raises the possibility that various pools of morphogens with different activity are present in the same tissue (Palm et al., 2013).

Paradoxically, lipid modifications of Hh and/or Wnt do not restrict their extracellular diffusion and, instead, promote controlled long-range signaling activity (Callejo et al., 2006; Gallet et al., 2006; Lewis et al., 2001; McGough and Vincent, 2016). This phenomenon prompted research into alternative transport mechanisms to free diffusion. Both Hh and Wnt proteins bind to heparan sulfate proteoglycans (HSPGs) with high affinity at the cell surface (Yan and Lin, 2009). HSPGs prevent their rapid, unregulated dilution in the extracellular space, maintaining the local high concentration that is necessary for signal activation, while still promoting their spread along the tissue surface (Baeg et al., 2001; Gallet et al., 2006; Han et al., 2004a,b). A recent study demonstrated that the core protein of a *Drosophila* HSPG, Dally-like protein (Dlp), acts as a Wg chaperone, shielding the lipid moiety (McGough et al., 2020). The authors proposed a model in which Wg spreads by sequentially passing between the heparan sulfate chains to the protein core of Dlp

Université Côte d'Azur, CNRS, INSERM, Institut de Biologie Valrose (iBV), Parc Valrose, 06108 Nice Cedex 2, France.

*Author for correspondence (therond@unice.fr)

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Box 1. Alternative carriers of Hh and Wnt morphogens

Several alternative mechanistic models have been proposed for the extracellular transport of Hh and Wnt. Secreted lipid-modified Hh is found in soluble multimers, in which the lipids could be shielded from the hydrophilic environment (Zeng et al., 2001). It has also been proposed that Hh and Wnts associate with lipoprotein particles, a carrier of circulating lipids including cholesterols, whose association with morphogens is promoted by cell-surface glypicans (Eugster et al., 2007; Panáková et al., 2005; Kaiser et al., 2019). Both morphogens may be anchored in the phospholipid monolayer of lipoprotein particles through lipid or sterol anchors. In the mouse hindbrain choroid plexus, for example, Wnt5A is expressed and secreted into the cerebrospinal fluid mainly on lipoprotein particles. Immunofluorescence quantification experiments have shown that this secretion mechanism accounts for 20–30% of extracellular Wnt5A produced in this tissue, whereas exovesicle-associated Wnt5A represents only 3–7% (Kaiser et al., 2019). Strikingly, primary choroid plexus cell culture supernatant contains Wnt5A exclusively associated to lipoproteins and not extracellular vesicles (EVs) (Kaiser et al., 2019).

Moreover, extracellular, soluble, monomeric Hh and Wnt have also been observed. For instance, the lipid adduct of monomeric Wnt associates with the soluble lipocalin family member secreted Wg-interacting molecule (SWIM) in *Drosophila* (Mulligan et al., 2012). Similarly, soluble monomeric Shh is associated with the vertebrate-specific secreted glycoprotein Scube2 (Creanga et al., 2012; Tukachinsky et al., 2012). Similarly, extracellular *Drosophila* Hh associates with the soluble protein shifted (shf) (Glise et al., 2005; Gorfinkiel et al., 2005; Hsieh et al., 1999). In each of these examples, the lipid and cholesterol anchors are shielded from the aqueous environment, resulting in morphogen solubilization and extracellular spreading. These different carriers provide alternative extracellular transport mechanisms for morphogens, independently of EVs.

molecules to eventually reach its receptor. Several other mechanisms that allow extracellular transport by shielding the Hh and Wnt lipid anchors from the aqueous environment have been proposed (Box 1). However, a number of independent studies have suggested that Hh and Wnt are secreted on extracellular vesicles (EVs) in order to exert their long-range signaling function (see Fig. 1 and Box 2 for mechanisms of EVs formation), in animal model systems and in human cerebrospinal fluid (Beckett et al., 2013; Coulter et al., 2018; Gradilla et al., 2014; Gross et al., 2012; Koles et al., 2012; Korkut et al., 2009; Liégeois et al., 2006; Matusek et al., 2014; Parchure et al., 2015; Tanaka et al., 2005; Vyas et al., 2014).

Owing to space constraints, in this Review we will focus on four main questions related to these new discoveries. First, how does intracellular trafficking contribute to the generation of functional EVs carrying the Hh and Wnt morphogens? Second, which EV-generating mechanisms are involved in the establishment of morphogen gradients? Third, how are Hh- and Wnt- containing EVs linked to human disease models? Last, what are the main technical hurdles for the functional study of EVs *in vivo*?

Hh and Wnt trafficking in secreting cells – primary and secondary secretion

Growth and patterning of the *Drosophila* wing imaginal disc relies on the activity of a range of morphogens, including Hh and Wg, and impaired secretion of these proteins results in strong tissue morphological defects (Box 3). After their secretion, Hh and Wg induce the expression of target genes in a concentration-dependent manner within responsive cells. Several lines of evidence suggest that, following their maturation inside conventional secretory compartments, Hh and Wg reach the extracellular leaflet of the apical plasma membrane within the subset of cells that produce Hh and Wg (hereafter referred to as producing cells; Fig. 2, route 1, Box 3). From there, they are then re-internalized and follow a secondary secretion to form a functional extracellular gradient of activity (Fig. 2, route 2). We present and discuss experimental evidence, and possible routes following endocytosis of Hh and Wg.

By overexpressing Hh (tagged with GFP or CFP) in *Drosophila* wing discs, it was shown that Hh traffics to the plasma membrane of producing cells where it forms nanoclusters (Vyas et al., 2008). Tagged Hh is then re-internalized by producing cells (Callejo et al., 2011; D'Angelo et al., 2015; route 2 in Fig. 2A). Consistently, endogenous Hh accumulates at the surface of producing cells when endocytosis was blocked by using a dominant-negative form of dynamin (Ayers et al., 2010; Callejo et al., 2011). Hh was also found in the early endocytic and recycling compartments (marked by Rab5 and Rab4, respectively) of producing cells (D'Angelo et al., 2015). Accordingly, expressing a dominant-negative form of Rab5 inhibits Hh endocytosis, trapping it inside producing cells and resulting a reduction in long-range Hh activity (Callejo et al., 2011; D'Angelo et al., 2015; Eugster et al., 2007). Moreover, interfering with the recycling machinery by using RNA interference (RNAi) against Rab4 also reduced long-range signalling activity and target-gene expression (D'Angelo et al., 2015). Altogether, these data suggest that perturbing Hh endocytosis leads to a defect in its long-range activity, and that the recycled pool of endocytosed Hh (Fig. 2A, routes 2 and 3) contributes to the activity of the extracellular pool of Hh.

Interestingly, tagged-Hh expressed in cells was observed by immunoelectron microscopy to localize to intraluminal vesicles (ILVs) of multivesicular bodies (MVBs), both *in vitro and in vivo* (Gradilla et al., 2014; Parchure et al., 2015). Moreover, endogenous Hh was observed in enlarged defective endosomes positive for the endosomal sorting complex required for transport (ESCRT)-0 subunit Hrs and Rab7 as well as on ILV membranes (Parchure et al., 2015). This suggests that, after its internalization, Hh follows an endocytic route destined for degradative MVBs (Fig. 2A, routes 4 and 6). This was tested *in vivo* by using a loss-of-function mutation in the *Drosophila* class C vacuolar sorting protein deep orange (dor), which impairs lysosomal degradation, resulting in accumulation of polyubiquitylated proteins that are no longer degraded. In the absence of deep orange, the level of endogenous Hg in producing cells is not increased (Callejo et al., 2011; D'Angelo et al., 2015). Altogether, these data suggest that endocytosed Hh is not targeted for degradation in MVBs but follows an MVB-dependent secretion route after it has been re-internalized by producing cells (Fig. 2A, routes 4 and 5).

Wnt proteins also undergo extensive trafficking before being released into the extracellular milieu. During their primary secretion, Wnts are first palmitoylated in the endoplasmic reticulum (ER) and are then introduced to the apical surface of the plasma membrane (Fig. 2B, route 1; Langton et al., 2016). This trafficking process requires the activity of the shuttling Wnt-binding protein wntless (Wls, also known as Evi or Sprinter) and the retromer complex (Franch-Marro et al., 2008; Port et al., 2008). Similar to Hh, *Drosophila* Wg is then endocytosed from the apical membrane in a dynamin-dependent manner (Fig. 2B, route 2) and transcytosed to the basal side of the epithelium (Fig. 2B, route 2b). In *Drosophila*, this process is mediated by a membrane-anchored E3 ubiquitin ligase of the ring finger (RNF) protein family, called Godzilla (gzl) (Yamazaki et al., 2016). Inactivation of Wg on the basal surface of the Wg-producing epithelium by ectopic expression of Notum – an enzyme that removes palmitoleate and, therefore, inactivates Wg – greatly abrogates the expression of the Wg target gene *senseless* in receiving cells. This suggests that the basal release of Wg is necessary to establish a functional Wg-signaling response in the receiving tissue (Yamazaki

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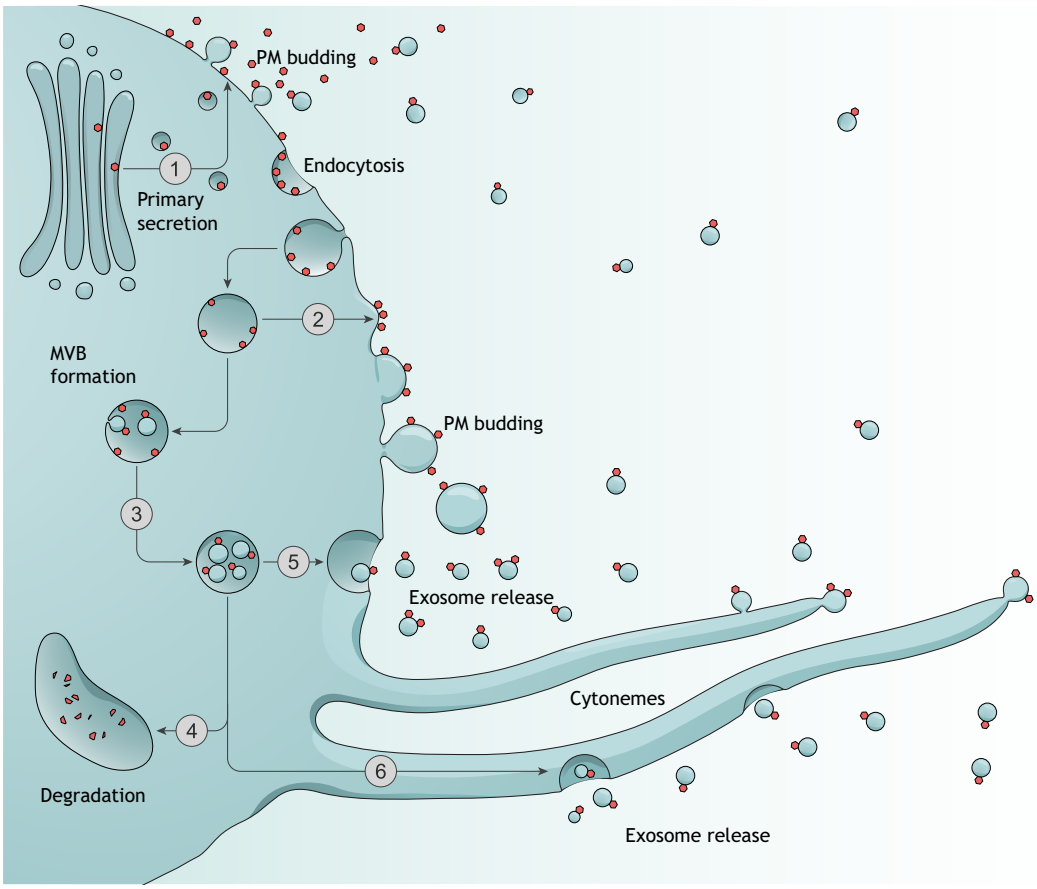

Fig. 1. Schematic representation of the mechanisms for EV generation. Following primary secretion (1), cargoes (red) are loaded onto plasma membrane buds or follow endocytic routes (2–6). ESCRT-dependent plasma membrane budding takes place directly following primary secretion (1); alternatively, signaling molecules can be re-endocytosed and recycled back to the plasma membrane for release (2). Intraluminal vesicles (ILVs) bud inwardly from the limiting membrane of endosomes (3), resulting in the formation of multivesicular bodies (MVBs) that, typically, culminate in cargo degradation (4). MVBs can also function as secretory compartments (5) by fusing their limiting membrane to the plasma membrane; this releases ILVs and their cargos into the extracellular space. Both MVB fusion and plasma membrane budding can also take place along or at the tips of filopodial extensions, also referred to as cytonemes (6).

et al., 2016). However, it appears that active Wnt proteins can also be released apically (Yamamoto et al., 2013). In 2D or 3D cultures of Madin-Darby canine kidney (MDCK) cells, different Wnt proteins follow different routes. Whereas Wnt11 is preferentially secreted from the apical surface, Wnt3a is predominantly secreted basally (Yamamoto et al., 2013). Importantly, these different trafficking pathways coincide with the selective loading of Wnt11 and Wnt3a into distinct EV populations *in vitro* (Chen et al., 2016). Basolaterally secreted Wnt3a co-fractionates with ESCRT proteins at a density that is typical for exosomes (see Box 4). By contrast, apically secreted Wnt11 and Wnt3a co-fractionate with CD63 and Hsp70 on EVs of different densities. Additionally, lipidation of Wnt3a also influences its trafficking, as removal of the lipid adduct specifically perturbs its secretion on EVs originating from the basal surface (Chen et al., 2016).

Whether recycling endosomes are involved in Wg trafficking has not yet been tested (Fig. 2B, route 3), but intracellular Wg has been shown to be present in MVBs (Fig. 2B, route 4), suggesting that, instead of being degraded (Fig. 2B, route 6), Wg follows MVB-dependent secretion after its internalization in producing cells (Fig. 2B, routes 4 and 5; Gross et al., 2012). Taken together, these findings suggest that the formation and function of extracellular Hh and Wg gradients depend not only on primary secretion but require secondary secretion after re-internalization.

Secretion of Wnt and Hh on EVs

In *Drosophila*, the need for Hh and Wg to be endocytosed in producing cells and then re-secreted into the extracellular space is still not fully understood. One possibility is that, following their primary secretion, morphogens require further packaging into a final form that is responsible for their long-range signaling activity. EVs containing Hh and Wg were first identified following the biochemical fractionation of conditioned medium of naïve or Hh- and Wg-expressing insect cultured cells. The proteomic analysis of these vesicles identified known regulators of EVs formation, such as members of the ESCRT machinery (Box 2) and the small GTPase Rab superfamily (Beckett et al., 2013; Gradilla et al., 2014; Gross et al., 2012; Matusek et al., 2014; Parchure et al., 2015; Vyas et al., 2014). Depleting these proteins in cultured cells led to a decrease in morphogen secretion on EVs (Beckett et al., 2013; Gross et al., 2012; Parchure et al., 2015). Importantly, purified Hh- and Wg-containing EVs share the biophysical characteristics of exosomes, such as their size and density (see Box 4). It was concluded that overexpressed Hh and Wg are secreted on EVs in cultured cells (Beckett et al., 2013; Gradilla et al., 2014; Gross et al., 2012; Matusek et al., 2014; Parchure et al., 2015; Vyas et al., 2014).

In the *Drosophila* wing imaginal disc, secreted Hh and Wg were both observed far away from their sites of production, and

Box 2. Mechanisms of EV formation

Extracellular vesicles (EVs) form and can be secreted through several alternative mechanisms (see also Fig. 1). EVs can be released through budding of the plasma membrane, an ESCRT-dependent process, similar to the mechanism of retrovirus budding from infected cells (Fig. 1, route 1) (Christ et al., 2017). The cargos present on these EVs originate from primary secretion at the plasma membrane or from recycling following endocytosis (Fig. 1, route 2). An alternative route involves the formation of intraluminal vesicles (ILVs) in the lumen of endosomes, which results in multivesicular bodies (MVBs) (Fig. 1, route 3). Some of these MVBs are degradative (Fig. 1, route 4), whereas others behave as secretory compartments by fusing their limiting membrane with the plasma membrane (Fig. 1, route 5); this releases the ILVs and associated cargoes into the extracellular space. Once in the extracellular space, secreted ILVs are termed exosomes (Yáñez-Mó et al., 2015). The intracellular sorting of the cargoes and formation of the inward bud at the endosome-limiting membrane requires the activity of endosomal sorting complex required for transport proteins (ESCRTs) 0 to III (ESCRT0-III). We do not aim to provide a comprehensive description of ESCRTs here, as their function has been summarized in many excellent reviews (see, for example, Stoten et al., 2018; Radulovic and Stenmark, 2018; Vietri et al., 2020). We would like to emphasize, however, that ESCRT function is highly pleiotropic. ESCRTs are required in numerous processes involving membrane deformation, constriction and scission, such as the final step of cytokinesis, membrane repair and nuclear membrane fusion during cell division (Campsteijn et al., 2016; Vietri et al., 2020. Interestingly, MVBs can also be generated independently of ESCRT activity through sphingolipid ceramides (Trajkovic et al., 2008). Both budding of the plasma membrane or fusion of MVB with the plasma membrane can also occur along or at the tip of long filopodia extensions, also called cytonemes (Fig. 1, route 6). After membrane scission (inward or outward), ESCRTs become trapped inside the generated EVs and can, therefore, be used as protein markers (see Box 4).

Box 3. *Drosophila* imaginal disc epithelium as model tissue to study morphogen activity

Most functional studies describing the roles of Hh and Wg extracellular vesicles (EVs) have been carried out in the *Drosophila* wing imaginal disc (Beckett et al., 2013; Gradilla et al., 2014; Gross et al., 2012; Matusek et al., 2014; Parchure et al., 2015). In this tissue, Hh and Wg are produced by a subset of cells (producing cells), and trigger the fate of receiving cells that participate in the control of tissue patterning. Cells close to the source of Hh and Wg morphogen respond to high morphogen levels, whereas cells that are further away are able to respond to intermediate and low levels. These graded levels of activation, which specify different cell fates, led to the proposal that a gradient of secreted morphogen is responsible for the overall patterning of the tissue (Wolpert, 2016). Impaired secretion, spreading or transduction of one of these morphogens results in strong morphological tissue defects, such as the lack of wings in the mutant animals (hence the name *wingless*), or outgrown and/or duplicated wings in case of increased or ectopic signaling activity. Although work carried out in *C. elegans* – in which the Wg orthologue EGL-20 had been endogenously fluorescently tagged – provided evidence that Wnt forms an extracellular gradient (Pani and Goldstein, 2018), it has recently been suggested that Wg secretion is not essential for wing disc patterning in *Drosophila* (Alexandre et al., 2014). Indeed, replacing the endogenous gene with one that resulted in Wg being tethered to the plasma membrane was sufficient to rescue most developmental defects of Wg loss-of-function, including patterning, but not full growth of the wing (Alexandre et al., 2014). On the basis of this study, it was proposed that Wg is, indeed, secreted but that its patterning activity does not depend on its secretion and long-range activity. In response to these findings, a more-recent study demonstrated that the membrane-tethered Wg protein appears to have greater stability than its wild-type counterpart, resulting in membrane-tethered Wg being present in cells that otherwise do not express Wg (Chaudhary et al., 2019), suggesting that this property of membrane-bound Wg protein explains its ability to rescue wing patterning (Chaudhary et al., 2019).

associated with exosome markers – Hh with ESCRTs (Matusek et al., 2014) or the tetraspanin CD63-GFP (Gradilla et al., 2014) and Wg with CD63-GFP and Rab4 (Gross et al., 2012). Further *in vivo* analysis showed that endogenous Hh secretion depends on ESCRT function (Gradilla et al., 2014; Matusek et al., 2014; Parchure et al., 2015). To challenge their role *in vivo* and to avoid pleiotropic functions of ESCRTs – as complete loss of ESCRT function strongly affects cell architecture and viability (see Box 2) – mild, spatially and temporally controlled depletion of ESCRT was used in Hh-producing cells, which resulted in a retention of Hh at the external cell surface and a concomitant reduction in long-range Hh activity (Gradilla et al., 2014; Matusek et al., 2014; Parchure et al., 2015). In addition, the number of Hh-containing endocytic structures within Hh-receiving cells was reduced (Gradilla et al., 2014). Interestingly, endogenous secreted Hh and ESCRT subunits, such as charged multivesicular body protein 1 (Chmp1) were observed at the surface of Hh-receiving cells, confirming that these proteins travel together to their target cells (Matusek et al., 2014). However, the role of ESCRTs in Wg secretion is yet to be assessed *in vivo*.

How morphogens transported on EVs interact with their receptors is not known, nor do we know whether morphogen EVs need to be endocytosed to trigger signaling. However, we believe that Hh present on EVs is accessible to its receptor Patched (Ptc) at the surface of receiving cells for two reasons. First, non-permeabilized purified EVs revealed that Hh is associated to the surface of EVs in *Drosophila* (Matusek et al., 2014). Second, the forced expression of a mutant form of Ptc that cannot be endocytosed in the wing disc, lead to the trapping of Hh associated to ESCRT Chmp1 particles, suggesting that Ptc interacts with Hh-EVs and sequesters them (Matusek et al., 2014).

Although multiple studies have addressed the processes necessary for the production and secretion of EV-associated Wnts (Parchure et al., 2018), in both *Drosophila* and in vertebrates, little is known regarding the mechanism through which these EVs transduce Wnt signaling in receiving cells. The Wnt palmitoleate moiety contributes to the binding of Wnts to FZD receptors (DeBruine et al., 2017), suggesting that Wnts must be extracted from EV membranes to signal; yet, how this process would be mediated is unknown. Alternatively, active Wnts could be cleaved from the surface of EVs; a recent study suggested that Wg is cleaved by the ADAMTS family protein Sol narae (Sona), resulting in the formation of an active C-terminal Wg fragment (Won et al., 2019). Expression of this fragment in the *Drosophila* wing imaginal disc is sufficient to activate the expression of low, but not high, threshold Wg targets, suggesting that other processes need to act in tandem. Interestingly, the authors showed that Sona is secreted on EVs. Hh and Wg dispersion through EVs might also be regulated by long dynamic cellular extensions, called cytonemes (Bischoff et al., 2013; Gradilla et al., 2014; Rojas-Ríos et al., 2012; Sanders et al., 2013; Stanganello et al., 2015). Such cytonemes could specifically transport Wg- or Hh-containing EVs. Indeed, there is evidence suggesting that EVs originating from MVBs are released from cytonemes or bud from their tips (González-Méndez et al., 2017).

Studies of cytonemes have provided complementary information regarding the interaction of Hh with Ptc (Kornberg, 2017). In the wing imaginal disc, Ptc was found to be present at the surface of receiving cells on cytonemes that are directed towards Hh-containing EVs (Chen et al., 2017; González-Méndez et al., 2017, 2020). Interestingly, it has been shown that, *in vitro*, filopodia

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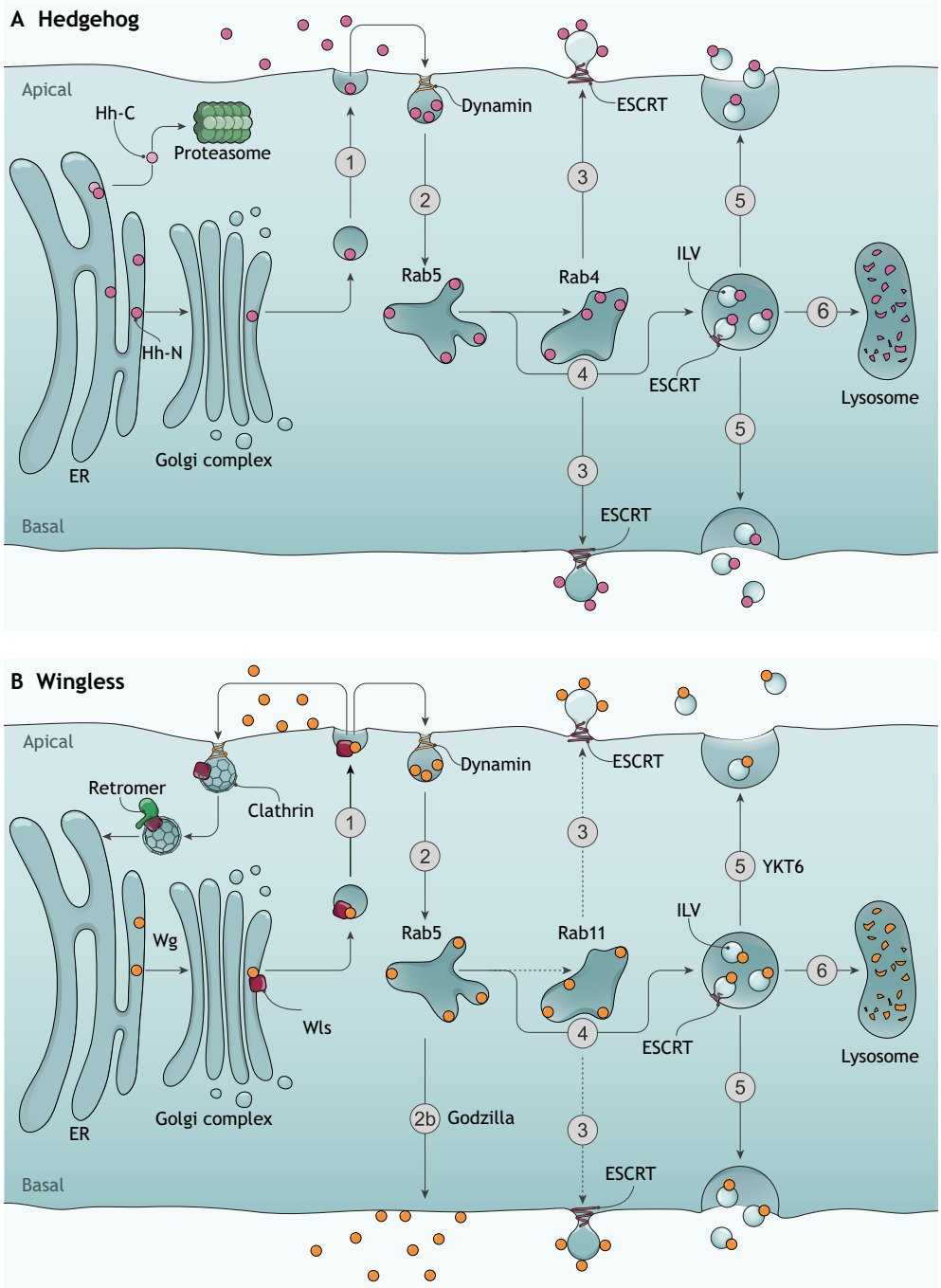

Fig. 2. Summary of Hh and Wg trafficking routes in *Drosophila*. (A,B) Hh (A) and Wg (B) proteins undergo signal sequence cleavage and enter the secretory pathway. Subsequently, Hh and Wg are lipid-modified (see main text). Autoproteolytic cleavage of the Hh precursor protein occurs in the endoplasmic reticulum (ER), resulting in two peptides, C–terminal Hh peptide (Hh-C) that translocates out of the ER and undergoes degradation through the activity of the proteasome, and an N–terminal Hh peptide (Hh-N) that is covalently attached to cholesterol. Following maturation, Hh (A) and Wg (B) are both trafficked to the extracellular leaflet of the apical plasma membrane within the Hh- and Wg-producing cell, where their soluble forms are secreted (1). Primary secretion of Wg is mediated by the shuttling protein Wntless (Wls), which is then endocytosed via a clathrin-dependent mechanism and recycled back to the Golgi through the activity of the retromer complex. Subsequently, both morphogens can be re-internalized, upon which they are found in Rab5-positive early endosomes (2). From there – via recycling endosomes positive for Rab4 in the case of Hh (A) or positive for Rab11 in the case of Wg (B) – they can be recycled to the plasma membrane before undergoing secondary secretion after plasma-membrane budding (3). However, Wg can also be transcytosed via Rab5 from the apical to the basal membrane (2 and 2b), a process dependent on the activity of the E3 ubiquitin ligase Godzilla. In addition, both Wg and Hh can pursue an alternative endocytic route via multivesicular bodies (4), followed by either apical or basal release on EVs (5), or become degraded in lysosomes (6). Note that the apical secretion of Wg requires the SNARE protein YKT6 (5).

can be hot-spots for the endocytosis of EVs (Heusermann et al., 2016). It is, therefore, possible that Hh-containing EVs are taken up by Ptc-containing cytonemes present on target cells. This interesting possibility implies that Hh-EVs are not simply dispersed into the extracellular space but transit from one filopodia to another through direct contact. Whether, in this context, Hh-containing EVs mediate the entire patterning activity of Hh or only a subset of it will be an interesting challenge to resolve.

Taken together, there is increasing evidence that Hh and Wg are secreted through a secondary secretory pathway that leads to their secretion on EVs; the latter might correspond to exosomes or microvesicles (Box 4) but their function still warrants further investigation.

Biogenesis of functional Wnt- and Hh-containing EVs

The biogenesis of morphogen-containing EVs is still poorly understood. As discussed above, there is a clear link between Hh and Wg endocytosis, and their extracellular gradient formation, but what is the evidence that modifying the endocytosis of Hh or Wg influences their release on EVs?

In the case of Hh, blocking endocytosis by depleting dynamin and Rab5 in Schneider insect (S2R+) cells by using RNAi leads to a reduction in the release of Hh-containing EVs into the cell-culture medium (Parchure et al., 2015). Interestingly, mutant variants of Hh that are unable to nanocluster are internalized and secreted in EVs to a lesser extent *in vitro*, and display decreased long-range activity *in vivo* (Parchure et al., 2015; Vyas et al., 2008).

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Box 4. EV purification protocols and protein markers

Extracellular vesicles (EVs) can be further classified into small and large EVs based on their size, physical characteristics (floating density), isolation properties (centrifugation speed/force) and biogenesis. Small EVs, commonly referred to as exosomes have a diameter of 30–150 nm and a density of 1.13–1.19 g/ml (Théry et al., 2018). Small EVs are, typically, pelleted at higher centrifugation forces (100,000–200,000 ***g***; Théry et al., 2006); they are formed in cells as ILVs within MVBs, and are released following the fusion of MVBs with the plasma membrane. Small EVs are often associated with tetraspanins, such as CD63, CD9 and CD81, as well as heat shock proteins and ESCRT components, including Alix and TSG101 (Théry et al., 2018).

Large EVs (also termed microvesicles or ectosomes), are between 200 nm and 2 µm in diameter, and are generated by budding and/or blebbing from the plasma membrane (Mathieu et al., 2019). Large EVs can be pelleted at a much lower centrifugation force, i.e. 10,000–20,000 ***g***, but are also present in high-speed fractions (Mathieu et al., 2019). However, there is no clear consensus in the EV community regarding the floating density of large EVs, and precise size-based classification of microvesicles can be quite heterogenous (Théry et al., 2018).

It is worth emphasizing that the origin of the different types of EVs cannot always be deducted from their size or density. For example, small EVs shed from the plasma membrane can overlap in their physical properties with those of exosomes (Hara et al., 2010; Maguire et al., 2015; McConnell et al., 2009; Thouverey et al., 2009).

Importantly, EV preparations can also include other particles of similar size and density, such as enveloped viruses or high-density lipoproteins and exomeres, i.e. non-membranous nanoparticles <50 nm (Zhang et al., 2018). Most EV preparations are, therefore, likely to consist of a mixture of different types of small EVs and of non-EVs. Therefore, deciphering the differences in the biogenesis of EV subtypes could permit exosomes to be distinguished from plasma membrane-derived EVs owing to the presence of distinct markers, but such an approach has to be taken very cautiously. For further details, we refer the reader to the guidelines published by the International Society of Extracellular Vesicles (ISEV), which provides a regularly updated, curated resource for the isolation and classification of EVs (Théry et al., 2018).

Furthermore, the requirement for both Rab4 and ESCRTs for long-range Hh activity raises the possibility that, following endocytosis from the early endosomal compartment, Hh can take two routes to be released. It can either be recycled back to the plasma membrane in Rab4-containing vesicles that then bud off from the membrane, or it can be incorporated into secretory MVBs – together with other regulatory components for subsequent release on exosomes (Fig. 2). Our data support the first route (Matusek et al., 2014). First, under conditions of impaired MVB activity, long-range Hh activity is not affected. However, although weak depletion of the *Drosophila* ESCRT proteins Alix (PDCD6IP in humans) or Vps32 (officially known as Shrub; CHMP4A, CHMP4B and CHMP4 in humans) does not affect MVB homeostasis, it leads to the accumulation of the Hh at the plasma membrane and defects in long-range Hh activity (Matusek et al., 2014). However, there is no apparent correlation between the loss of Hh activity and defects in MVB formation, suggesting that ESCRT-mediated regulation on Hh is independent of MVBs (Matusek et al., 2014).

Another pertinent argument relates to the differential requirement of ESCRT sub-complexes for Hh secretion. Depletion of ESCRT-I and ESCRT-III but not ESCRT-II subunits affects Hh secretion and its long-range activity (Matusek et al., 2014). This is of interest because, although ESCRT-II subunits are necessary for endosomal sorting, only the ESCRT-II complex is dispensable in budding events at the plasma membrane during retrovirus release (Martin-Serrano and Neil, 2011). Interestingly, the ESCRT-III component Vps32 has been detected in Hh-containing EVs *in vivo* and *in vitro*, whereas the secreted ESCRT-II component Vps36 has not been associated with extracellular Hh (Matusek et al., 2014). Furthermore, ESCRT-II proteins were not observed in Hh-containing EVs purified from cultured cells (Matusek et al., 2014).

Together, these findings support a model in which Hh is released through budding of Hh-containing vesicles from the plasma membrane in a manner that is independent of secretory MVBs.

Research dissecting the roles of EV-bound members of the Wnt morphogen family have also provided conflicting results. Although numerous studies have identified new function for EVs containing Wnt proteins (see below), in *Drosophila* the contribution of EV-bound Wg to patterning events is less clear. Although several studies suggested that, *in vitro*, Wg being present on EVs, no agreement was found regarding their functional relevance (Beckett et al., 2013; Gross et al., 2012). Endogenously secreted Wg colocalizes with exosomal markers, such as Rab4 and GFP-tagged CD63 in the extracellular space of the wing disc (Gross et al., 2012). This, together with the fact that intracellular Wg colocalizes with GFP-tagged MVB markers, such as CD63 and Lamp1, when overexpressed in wing discs, led to the proposal that, *in vivo*, Wg is secreted on exosomes (Gross et al., 2012). Gross and colleagues also reported that only 8% of extracellular Wg colocalizes with secreted CD63-GFP. This, if representative of the proportion of exosome-association, raises the question as to how the overall patterning activity of secreted Wg is affected when the exosomal secretion of Wg is blocked. Moreover, depletion of the SNARE protein YKT6 in Wg-producing cells reduces the short-range activity of Wg (Gross et al., 2012). As SNARE proteins promote the fusion between vesicles and membranes, it is possible that they are required for the fusion of the MVB-limiting membrane with the plasma membrane in order to release ILVs (Fig. 2B, route 5). However, as SNAREs are involved in all steps of membrane fusion during secretory and endocytic processes, such a specific role in exosome production still needs to be confirmed.

In summary, although there is good evidence that Hh-containing EVs participate in Hh-dependent patterning of the wing disc in *Drosophila*, the requirement of EV-mediated transport in Wg in patterning is less well understood.

Hh and Wnt EVs in development and human pathologies

In this section, we present more examples illustrating the context-dependent function for EVs containing Hh and Wnt. These two morphogens have been found to be secreted on different types of EV, the origins of which are not always known. For example, Shh has been observed in EVs derived from apical microvilli budding at the surface of the mouse ventral node during development and was initially thought to be necessary to break symmetry during embryonic development (Tanaka et al., 2005). Interestingly, exosomes can also be carriers of Hh-related peptides that are required for cuticle formation in the nematode *Caenorhabditis elegans*. Indeed, the membrane-bound V0 sector of the vacuolar H^+ ATPase (V-ATPase), an integral membrane component of MVBs, is required for the apical secretion of exosomes containing Hh-related peptides in nematodes (Liégeois et al., 2006).

Shh-containing EVs have been intensively studied owing to their roles in various human pathologies (Table S1). EVs carrying Shh are released into circulation following apoptosis and/or T-cell stimulation (Martínez et al., 2006), and similar EVs have a protective effect by activating the nitric oxide (NO) pathway following endothelial injury (Agouni et al., 2007; Bueno-Betí et al., 2019). Shh-containing EVs also promote angiogenesis by stimulating the expression of

pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) (Soleti et al., 2009). Shh has been observed on liver cell-derived EVs, and a role for Shh-EVs in vascular remodeling during cirrhosis has been suggested (Witek et al., 2009). Evidence for protective and angiogenic effects of Shh-EVs was also provided by using mouse models of ischemic injury and angiotensin II-induced hypertension, respectively (Benameur et al., 2010; Marrachelli et al., 2013). However, it is worth noting that, in most of these studies, the effect of Shh-EVs was compared to that of soluble recombinant Shh that, unlike endogenous Shh, is not cholesterol modified. Therefore, activities of Shh-EVs with that of physiological secreted, lipidated form of Shh – which is soluble but lipidated – were not compared in these studies.

More surprisingly, Shh produced in epithelial hair follicle cells mediates the development of the embryonic dentate gyrus in the mouse hippocampus (Choe et al., 2015). Here, the uncleaved Shh precursor molecule is packaged into EVs within platelets and delivered to the dentate gyrus by crossing the semipermeable blood brain barrier (Choe et al., 2015). However, whether platelets release active mature Shh on EVs in this case remains to be answered. Further examples of the functions of Shh EVs are presented in Table S1.

Various Wnt ligands are also secreted on EVs (Table S1). For instance, in *Caenorhabditis elegans,* EVs carrying Wnt are released from the cilia of neurons, and disruption of this process leads to behavioral disorders (Wang et al., 2015). In *Drosophila* neuromuscular junctions, EVs carrying Wg are responsible for tuning the level of postsynaptic sensitivity (Kerr et al., 2014). One example in vertebrates is Wnt3a, a hematopoietic growth factor that stimulates acute myeloid leukemia progression (van den Berg et al., 1998; Reya et al., 2003; Valencia et al., 2009). EV-bound Wnt3a also stimulates clonogenic growth of the so-called side population (SP) of B-cell lymphoma cells, which are considered to have cancer stem cell-like properties (Koch et al., 2014). In this scenario, Wnt3a-expressing SP cells provide the ligand to surrounding stromal-like non-SP cells, promoting growth of the entire cell population (Koch et al., 2014). Wnt3a is also secreted on EVs derived from activated platelets upon co-culture with HT29 colon carcinoma cells and is able to mediate their growth (Dovizio et al., 2015). Moreover, the self-renewal properties of mammary stem or progenitor cells (MaSCs) are dependent on Wnt signaling and can be improved upon treatment with MaSC-derived EVs that contain Wnt3a and Wnt1 (Bussche et al., 2016). Moreover, these EVs have an even greater capacity to promote growth than recombinant Wnt proteins alone, highlighting that Wnt-containing EVs have enhanced signaling activity in this system (Bussche et al., 2016). Wnts are also known to contribute to fibrotic changes within cardiac tissue, induced by disruptions to the heart morphology, through the activation of cardiac fibroblasts (Działo et al., 2019). It has recently been demonstrated that exogenous Wnt3a-containing EVs purified from L-cells, i.e. Wnt-expressing fibroblasts of mouse subcutaneous connective tissue can trigger the canonical Wnt pathway in cultured human cardiac fibroblasts (Działo et al., 2019). However, the *in vivo* contribution of Wnts transported on EVs to sites of cardiac fibrosis remains unclear.

The Wnt4 ligand was also found on EVs in several studies. In human umbilical cord mesenchymal stem cells, Wnt4-containing EVs mediate the activation of β-catenin in the surrounding endothelial cells and display proangiogenic effects, thereby establishing a paradigm for cutaneous wound healing (Zhang et al., 2015a). In a rat skin-burn model, such Wnt4-containing EVs induce an accelerated rate of re-epithelialization *in vivo* (Zhang et al., 2015b). Furthermore, Wnt4 secreted on EVs from colorectal cancer cells under hypoxic conditions is important in the promotion of angiogenesis and enhanced tumor growth (Huang and Feng, 2016).

In a mouse radiation-injury model, other EVs containing macrophage-derived Wnts (Wnt5a, Wnt6, Wnt9a) were identified to be responsible for the maintenance of the intestinal epithelium upon irradiation (Saha et al., 2016). The role of EV-associated Wnt5b *in vitro* was recently investigated in cancer cell migration and proliferation (Harada et al., 2017). EVs purified from the conditioned medium of cultured lung adenocarcinoma or Wnt5b-expressing L-cells efficiently activate Wnt signaling in Chinese hamster ovary cells, and enhance the migration and proliferation of A549 lung adenocarcinoma cells (Harada et al., 2017).

Collectively, these lines of evidence confirm that Shh- and Wnt-containing EVs are involved not only in developmental processes in animal models but also in several human physiopathologies, including cancers.

Components of morphogen signal transduction pathways in EVs

An interesting new development comes from studies showing that EVs not only carry Hh and Wnts but also the corresponding signal transduction pathway components that modulate their activities. For example, the Shh antagonist hedgehog-interacting protein (HHIP, hereafter referred to as HIP), directly binds to Shh (Chuang and McMahon, 1999). In mice, HIP is released on EVs from damaged endothelial cells during acute graft-versus-host disease (aGVHD) (Nie et al., 2016). HIP binds to Shh on circulating EVs, blocking the protective effect of Shh, NO release and the subsequent decrease of reactive oxygen species during aGVHD (Nie et al., 2016).

Other Hh pathway components have also been found on EVs *in vitro*. Human cervical cancer cells release not only Shh on EVs but also its receptor patched homolog 1 (PTCH1) and, further downstream, the positive regulator and G protein-coupled receptor smoothened homolog (SMO) (Bhat et al., 2018). In *Drosophila*, the polarized basal transport and the release of Ptc on EVs has been implicated in shaping the Hh gradient in the wing imaginal disc epithelium (González-Méndez et al., 2020). Another interesting finding is related to the primary cilium, which is necessary for activation of Shh signaling in vertebrates (Nager et al., 2017). Binding of Shh to its receptor PTCH1 at the cilium triggers the trafficking of the G-protein-coupled receptor GPR161, a negative regulator of the Hh pathway, from the cilium back into the cell (Nager et al., 2017). Alternatively, transfer of GPR161 from cilia to EVs has also been observed under pathological conditions. Transfer of GPR161 to EVs is regulated through activation of Shh at the primary cilium, suggesting that signal-dependent EVs remove activated signaling molecules, such as GPCRs, from the cilium (Nager et al., 2017).

EVs have also been shown to contain other components and regulators of the Wnt-signaling pathway, including β-catenin and frizzled-10 (FZD10, also known as FZ10) (Chairoungdua et al., 2010; Kalra et al., 2019; Scavo et al., 2019). β-Catenin, carrying several activating mutations, was observed in EVs released from colorectal cancer cells, and β-catenin from EVs purified from the human colon cancer cell line LIM1215 was able to transfer to the cytoplasm and nucleus of human colon carcinoma cells (Kalra et al., 2019). An increase in the expression of Wnt target-genes was also observed (Kalra et al., 2019). In a different study, the negative regulator of Wnt signaling proline-rich protein 7 (PRR7), was found to be secreted in EVs from rat hippocampal neurons (Lee et al., 2018). Overexpression of PRR7 in these neurons was sufficient to block the synaptogenic role

of Wnt5a and Wnt7a in a non-cell autonomous manner. Furthermore, the RNAi-mediated knockdown of *Prr7* led to an increase in the levels of Wnt5a and Wnt7a in the exosomal fraction secreted from cultured rat hippocampal neurons, whereas overexpression of PRR7 in human embryonic kidney 293 cells decreased the secretion of Wnt7a (Lee et al., 2018). Interestingly, increased Wnt signaling activity resulted in reduced levels of PRR7 on EVs, suggesting a Wnt -signaling-dependent feedback mechanism that controls the activity and secretion of Wnts on EVs. (Lee et al., 2018). Similarly, activating *Apc* mutations in mouse colorectal cancer cell organoids led to increased secretion of Wnt3a-containing EVs (Szvicsek et al., 2019).

The Wnt-binding protein Wls is also secreted in EVs at glutamatergic synapses in *Drosophila* motorneurons from where it is, subsequently, transferred to postsynaptic cells (Korkut et al., 2009; Koles et al., 2012). Although the conserved role of Wls in the trafficking of Wnts from the Golgi to the plasma membrane is well established (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006; Port et al., 2008), the requirement for secreted Wls remains unclear and technically challenging to test (Beckett et al., 2013). A model has been proposed, in which Wnt-associated Wls helps to target Wnts to secretory MVBs, and Wnt-free Wls is recycled back to the Golgi (Beckett et al., 2013; Gross et al., 2012). The functional relevance of the presence of Wls on extracellular EVs is not yet clear (Beckett et al., 2013) but the presence of Wls in only a small subset of Wg EVs might modulate the signal carried by Wg EVs (Gross et al., 2012) – although, this model remains to be tested. Altogether, these findings suggest the existence of additional levels regarding the regulation of morphogen signaling, mediated by EVs.

Technical limitations to the characterization of EVs *in vivo*

Our current understanding of EV biology relies heavily on a range of isolation techniques that, although they can provide insights into EV morphology and composition, offer little insight into their functional relevance. Below, we outline some of the technical limitations currently restricting our understanding of how Hh- and Wnt-containing EVs function *in vivo*.

The need for new morphogen-carrying EV markers

The presence of certain surface-protein markers as well as the fractionation of EV pools using sizing columns and ultracentrifugation are typically used to differentiate between exosomes of probable MVB origin and EVs originating from the plasma membrane (Théry et al., 2018). These techniques are based on the physical but not biological properties of EV pools. Nevertheless, until now, no specific protein marker has been identified that definitively allows the origin and nature of EVs to be determined (Box 4). Moreover, the density and size of EVs of different origins can be very similar or even overlapping (see Box 4 and references therein).

One of the most frequently used protein markers to identify secreted exosomes, i.e. small EVs released from secretory MVBs (Box 4), in *Drosophila* is the human tetraspanin CD63 antigen (CD63) a protein without a fly homolog, which was originally identified as a late endosomal/lysosomal protein (Pols and Klumperman, 2009). In *Drosophila*, the use of CD63 for the purpose of exosome purification is based on the assumption that it behaves similarly in *Drosophila* and human cell cultures, and accumulates in exosome fractions. Unfortunately, the colocalization of CD63 with Hh and Wg morphogens depends on the level of forced CD63 expression, which gives rise to conflicting conclusions (see above; Beckett et al., 2013; Gradilla et al., 2014; Gross et al., 2012; Panáková et al., 2005).

It is, therefore, important to identify protein signatures of EVs pools and identify specific markers in experimental model animals. This knowledge would help in the characterization of EVs not only based on size and composition, but also on function.

Standardization of differential purification and classification methods

Biochemical and physical characterizations of purified EVs still need to be improved, as different EV populations appear to have distinct biological activities. For example, ultracentrifugation of Shh-containing EVs secreted from cultured human embryonic kidney (HEK293T) cells results in two distinct EV pools that vary in size, protein and miRNA composition as well as signaling activity (Vyas et al., 2014). Incubation of mouse embryonic stem cells with purified Shh-EVs induced Shh gene targets and the subsequent differentiation of ventral neural progenitors. However, only Shh-containing EV fractions obtained from centrifugation at 150,000 ***g***, which are also enriched in β1-integrins – but not those obtained from centrifugation at 250,000 ***g*** – displayed such activity (Vyas et al., 2014). Interestingly, β1-integrins are necessary for this activation, suggesting that other proteins present at the surface of EVs participate in morphogen activity (Vyas et al., 2014). Similarly, Shh EVs purified from a human fetal glial cell line or cerebrospinal fluid have unique protein signatures that differ from the classic CD63-containing exosome populations (Coulter et al., 2018). This observation might be of great importance for future EV characterization studies, as the presence of Shh on EVs does not necessarily result in canonical Shh activity. For example, Shh-EVs inhibit adipocyte differentiation via a non-canonical Shh signaling route, i.e. via Smo, STK11 and PRKAA2 (also known as the Smo–LKB1–AMPK pathway) (Fleury et al., 2016). Similarly, when investigating the morphogenetic role of Shh-EVs in developing rat brains, no measurable canonical Shh activity was found in EVs purified from the medium of cultured hippocampal neurons (Eitan et al., 2016).

Further technical advances regarding the characterization of the molecular signatures of EVs and their physical properties are, therefore, of primary importance. Nanoflow cytometry coupled with structured illumination and atomic force microscopy studies (Choi et al., 2019; Yokota et al., 2019), as well as label-free identification of EVs on nanoparticle substrates by using surface-enhanced Raman scattering (Fraire et al., 2019), represent significant innovations. Great effort is made to further develop such methods by using fluorescently tagged EV-specific membrane proteins; here, the molecular signature of the combined fluorescent tags can be analyzed using fluorescence correlation spectroscopy at the level of individual EVs (Corso et al., 2019). EVs of different cellular origin and function could, thus, be distinguished with high specificity by using a combination of these methods.

Development of *in vivo* purification methods optimized for small-sample volumes

Current EV purification methods to isolate EVs with high purity, i.e. ultracentrifugation and sucrose density gradient purification, require large amounts of initial material, complicating the analysis of *in vivo* EV function in animals with limited extracellular space and body fluids. Indeed, morphogen-containing EVs are commonly purified from the conditioned medium of cultured cells overexpressing Wg or Hh. Attempts to purify Shh-containing EVs from *ex vivo* cultured primary notochord cells derived from early chick embryos, does not yield sufficient quantities of pure EVs with which to perform functional assays (Vyas et al., 2014). To tackle this issue, EV isolation methods tailored to the analysis of small volumes of

biological fluids are currently under development (Shi et al., 2019). As Hh and Wnt morphogens also use alternative carriers, such as lipoproteins that fractionate together with EVs (see Box 1 and Box 4), methods to distinguish these different carriers are also very important. This would greatly facilitate the analysis of biofluids, such as blood or *Drosophila* haemolymph, which contain a mixture of these morphogen carriers. Assuming that sufficient material is available, purification from small-volume biofluids will allow analysis of the proteomic, lipidomic, DNA and miRNA composition of EVs - and, ultimately, their function.

Conclusions and perspectives

Although there is a growing number of *in vitro* examples that illustrate the roles of EVs carrying Wnt and Hh morphogens in different cell types, there is still a lack of functional assays to assess the physiological roles of these EVs. This is, in part, due to our limited understanding of EV biogenesis, their efficacy in signal transduction, how they travel within tissues (i.e. stability, dynamics and turnover) and how their transported signals are processed in receiving cells. Specific regulators of each EV class (Box 4) will need to be identified as, *in vivo*, distinct mechanisms of EV biogenesis might simultaneously operate in the same producing cell. New tools are, therefore, needed to determine the contribution of each distinct EV pool to morphogen secretion and signaling. Further work focusing on the isolation of Hh- and Wg-containing EVs from *in vivo* sources will help to clarify how the protein composition of EVs influences their signaling activity. Understanding the biogenesis of these EVs is also important for the development of therapeutic strategies targeting the (patho) physiological consequences of their dysregulation. A step in the right direction is the high-throughput testing of drugs to identify specific regulators of EV release. Screening of pharmacologically active molecules for modifiers of GFP-tagged CD63 exosome release in a stable C4-2B cell line, has already identified 22 compounds with a significant effect on EV release *in vitro* (Datta et al., 2018). This study is pioneering work of high value and highlights the need for further testing of new and existing drug libraries. However, *in vivo* validation of these compounds is also necessary, as the ultimate goal is to find specific inhibitors of EV release and to develop methods to deliver them to a certain population of cells, such as cancer cells, tissues or organs. To understand the mechanisms through which EVs carry and transmit morphogenetic signals is, therefore, crucial, not only from the perspective of basic science but also for the development of applied therapeutics that use EVs for targeted drug delivery.

Competing interests

The authors declare no competing or financial interests.

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Supplementary information

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Table S1. Summary of the functions of Hh/Wg-containing EVs in vertebrate pathologies and developmental models

Morphogen	Origin	Cell/tissue type/model	Effect	Reference
Shh	3T3-L1 adipocyte cell line	Type II diabetes	Stimulation of macrophage activation	Song et al., 2018
Shh	Peripheral blood; human lymphoid CEM T cell line; lymphocytes	Apoptosis/T-cell stimulation	Release into the circulation through microvesicles	Martinez et al., 2006
Shh	Human lymphoid CEM T cell line	Endothelial injury	Protective effect (through the NO pathway)	Agouni et al., 2007
		Endothelial injury	Promotion of angiogenesis	Soleti et al., 2009
		ischemic injury	Protective and angiogenic effect	Benameur et al., 2010
		Angiotensin-II induced hypertension	Protective and angiogenic effect	Marrachelli et al., 2013
		Adipocyte differentiation	Inhibition through non-canonical Hh signaling	Fleury et al., 2016
Shh	Peripherial blood	Acute myocardial infraction	Improving vasculogenic capacity of endothelial progenitor cells	Bueno-Beti et al., 2019
Shh	Liver	bile duct ligation	Vascular remodeling during cirrhosis	Witek et al., 2009
Shh	Primary hippocampal neuron culture	Developing rat brain	(no functional effect described)	Eitan et al., 2016
HIP	Blood plasma	aGVHD	inhibition of NO release	Nie et al., 2016
Shh precursor	Hair follicle cells/platelets	Dentate gyrus, from hair follicle cells and packaged into platelets	Stimulation of dentate gyrus development	Choe et al., 2015
Wnt3a	SP cells purified from DLBCL	DLBCL stromal cells	Stimulation of clonogenic growth	Koch et al., 2014

Wnt3a	Activated platelets	HT29 colon carcinoma cells	Growth stimulation by upregulating COX-2	Dovizio et al., 2015
Wnt3a, Wnt1	eMaSC	cMaSC culture	Enhancing cell renewal properties	Bussche et al., 2016
Wnt4	hucMSC	Endothelial cells	Activation of β-catenin, proangiogenic effects	Zhang et al., 2015a
Wnt4	hucMSC	Rat skin burning model	Acceleration of reephitelialization	Zhang et al., 2015b
Wnt4	Colorectal cancer cells	Hypoxia	Promotion of angiogenesis and tumor growth	Huang et al., 2016
Wnt5a,Wnt6, Wnt9	Bone marrow macrophages	Irradiation induced intestinal damage	Suppression of irradiation defects	Saha et al., 2016
Wnt5b	PANC-1 and Caco-2 cells	CHO cells and A549 lung adenocarcinoma cells	Enhanced migration and proliferation	Harada et al., 2017

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SPOTLIGHT

Circulating Hedgehog: a fresh view of a classic morphogen

Elodie Prince, Julien Marcetteau and Pascal P. Thérond*

ABSTRACT

Members of the Hedgehog family of morphogens mediate the intercellular communication necessary for the organisation and development of many animal tissues. They are modified by various lipid adducts, rendering them insoluble in hydrophilic environments and leading to the contentious question of how these molecules travel in the aqueous extracellular space. Seminal work carried out by Suzanne Eaton and her colleagues has shed light on how these morphogens can spread over long distances through their association with lipoprotein particles. In this Spotlight article, we discuss Suzanne's pioneering work and her contribution to our understanding of the transport and activity of morphogens, in particular Hedgehog. We also describe two other essential aspects of her work: the discovery and characterisation of endogenously present Hedgehog variants, as well as her proposition that, in addition to its role as a morphogen, Hedgehog acts as an endocrine hormone.

KEY WORDS: Endocrine hormone, Hedgehog/Wingless, Lipoproteins, Morphogens, Signalling, Endocannabinoids

Introduction

Secreted morphogens are classically thought to diffuse from their source, forming an extracellular gradient through which they communicate spatial information to surrounding tissues (Wolpert, 2016). The local morphogen concentration is then interpreted by receptive cells, triggering changes in cell fate and tissue patterning. Morphogens typically signal in a paracrine manner, acting over both short and long ranges (Ashe and Briscoe, 2006). This raises the question of how morphogens travel from their source to receiving cells, and how a functional morphogen gradient is established and maintained. Answering these questions has long presented both intellectual and technical challenges.

Hedgehog (Hh) family secreted proteins are classical morphogens that were originally identified in *Drosophila*. In *Drosophila*, Hh is responsible for patterning processes during embryonic segmentation and the development of the larval imaginal discs (Lee et al., 2016). Its close vertebrate orthologue, sonic hedgehog (Shh), is also essential for developmental patterning, for example during limb development and neural tube morphogenesis (Briscoe and Thérond, 2013). Hh family proteins are modified by lipid adducts: a palmitic acid (saturated fatty acid) on their amino terminus and a cholesterol moiety covalently attached to their carboxy terminus (Pepinsky et al., 1998; Porter et al., 1996). These lipid modifications render Hh proteins hydrophobic, providing them with a high affinity for cell membranes. These characteristics would seem somewhat paradoxical for extracellular signalling molecules, and suggest the existence of mechanisms to transport Hh proteins in the aqueous extracellular space.

As we highlight below, Suzanne Eaton (1959-2019) addressed this paradox during her career, approaching it using a variety of interdisciplinary approaches and yielding fundamental insights into our understanding of Hh release, spread and activity.

Lipoprotein particles as morphogen carriers

Suzanne's group initially proposed that Hh spreads in association with extracellular lipidic particles (Greco et al., 2001), which they later identified as lipoprotein particles (Panáková et al., 2005). Lipoprotein particles allow Hh to spread by providing a carrier to which Hh can bind, shielding its lipid hydrophobic moieties. This transport mechanism came as a surprise, as lipoproteins had primarily been studied for their roles in the transport of dietary and endogenous lipids between tissues.

In *Drosophila*, the major lipoprotein Lipophorin (Lpp; also known as Apolipophorin) is mainly produced in the fat body (Palm et al., 2012; Prasad et al., 1986), an energy storage and endocrine organ that controls nutrient response, before being released into the blood (haemolymph). By biochemically fractionating the wing imaginal disc, a classical model of Hh signalling, Suzanne and co-workers demonstrated that a portion of Hh could be isolated from the supernatant in association with lipoprotein particles (Panáková et al., 2005). Reducing the total level of circulating Lpp in larvae by specifically depleting Lpp expression in the fat body led to a decrease in the intercellular movement of Hh. This treatment did not cause a general suppression of Hh signalling; instead, only long-range Hh targets were reduced. This may have been the consequence of complementary mechanisms acting in parallel. Indeed, it has since been shown that Hh can also be secreted on extracellular vesicles (EVs), or transported along cellular extensions, called cytonemes, in order to carry out its patterning function (Bischoff et al., 2013; Gradilla et al., 2014; Kornberg, 2017; Matusek et al., 2014; Parchure et al., 2018). The association of Hh with these various carriers could fulfil distinct but complementary signalling roles, which together could make up the functional morphogen gradient. The molecular machinery responsible for selectively loading Hh onto its carriers is still poorly understood but is likely to be cell or tissue dependent and remains an important question in the field.

In order to test whether these findings could be generalised to other morphogens, Suzanne's group also looked at the impact of disrupting Lpp expression on another lipid-modified morphogen, Wingless (Wg) – a *Drosophila* Wnt family protein (Takada et al., 2006; Willert et al., 2003). They found that Wg was associated with lipoproteins, and that a reduction in Lpp was associated with a reduced range of Wg activity in the wing imaginal disc (Panáková et al., 2005). Since then, vertebrate Wnts have also been found to be associated with lipoprotein particles in different fluids, including the cerebrospinal fluid (Kaiser et al., 2019; Neumann et al., 2009).

In linking the fields of lipoprotein metabolism and morphogen signalling, this work provided a first glimpse at a new mechanism regulating the spread of morphogens. Moreover, as Lpp is not

Université Côte d'Azur, CNRS, INSERM, iBV, Parc Valrose, 06108 Nice Cedex 2, France.

*Author for correspondence (therond@unice.fr)

P.P.T., 0000-0003-0434-2334

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expressed in the wing imaginal disc, this study also highlighted for the first time that morphogen patterning activity, previously thought to be autonomously controlled within a tissue, could in fact be remotely regulated by another peripheral tissue source. It also provided evidence for a tight link between lipoprotein metabolism and animal development.

How Hedgehog and lipoproteins meet

As mentioned above, Lpp is not produced in the same tissue as Hh, naturally raising the question of how these molecules meet. Suzanne's group hypothesised that Lpp might regulate membrane-bound molecules involved in Hh spreading and signalling. Around that time, heparan sulphate proteoglycans (HSPGs) had been shown to bind lipidated Hh at the cell surface, and to be essential for both morphogen spreading and signalling (Yan and Lin, 2009), making them likely candidates. Suzanne's group demonstrated that the membrane-associated HSPGs Dally and Dally-like protein (Dlp) recruit circulating Lpp particles to the wing imaginal discs via their heparan sulphate moieties (Eugster et al., 2007). Importantly, the HSPGs remain associated with Lpp particles even upon their release from the membrane. The released form of Dally associates not only with Lpp but also with Hh in the same particles, and is found in endosomes with the Hh receptor Patched (Ptc). Suzanne's group found that released Dally increases the signalling activity of Hh on lipoprotein particles without affecting its spread or stability (Eugster et al., 2007). Soluble heparin is known to induce the dimerisation of Interference Hedgehog (Ihog), a Hh co-receptor, and increase Ihog-Hh binding *in vitro* (McLellan et al., 2006). Thus, the presence of Dally on lipoprotein-associated Hh could bridge heparin sulphate, Hh and Ihog, thereby promoting signalling.

Identification of Hh variants

Through detailed analyses of *Drosophila* tissues and mammalian cell lines by biochemical fractionation (Palm et al., 2013), Suzanne's group also uncovered additional, conserved forms of Hh/Shh: one sterol-modified, lipoprotein-associated variant and a second, lipoprotein-free form that is not sterol modified. Understanding how these different variants of Hh act together *in vivo* is a pressing question in the field.

In order to assess the functional relevance of these two secreted Hh/Shh variants, the Eaton group employed a signalling assay both in mammalian cells and in *Drosophila*. They found that the two released forms of Hh have complementary signalling activities, acting synergistically to fully activate the Hh pathway. Although the lipoprotein-associated Hh could induce the first step in signal transduction, it was not sufficient to activate downstream target genes. However, by adding low levels of the second, lipoprotein-free Hh in addition to the lipoprotein-associated Hh, the pathway could be fully activated (Palm et al., 2013). Although morphogen gradients had historically been considered to consist of a gradient of a single protein, these findings introduced the possibility that the Hh morphogen gradient is heterogeneous, integrating the activities of distinct Hh variants. These variants may differ in their ability to spread in tissues, or in their interaction with receptors and other regulators. The conclusions of these studies are based heavily on extensive *in vitro* and biochemical characterisations, and testing their observations in a tissue-specific, *in vivo* context has been restrained by technical limitations. Ongoing developments in high-resolution imaging and proteomics technologies will hopefully provide a means to gain a more fundamental understanding of these processes.

The role of lipoprotein-derived lipids in the Hh signalling pathway

A central process regulating the Hh signalling cascade is the inactivation of the transmembrane protein Smoothened (Smo), a G protein-coupled receptor, by the Hh receptor Ptc (Briscoe and Thérond, 2013). In the absence of Hh, Ptc enzymatically inhibits Smo activity and induces its degradation (Taipale et al., 2002). By contrast, the binding of Hh to Ptc inactivates Ptc, resulting in the stabilisation and activation of Smo. In *Drosophila*, Smo activation prevents proteolysis of the transcriptional mediator of the Hh pathway, Cubitus interruptus [Ci, a member of the GLI protein family, simultaneously cloned by Suzanne and Kornberg and by Orenic and colleagues (Eaton and Kornberg, 1990; Orenic et al., 1990)], promoting the conversion of Ci from a transcriptional repressor to an activator.

Ptc is a member of the resistance-nodulation-division (RND) protein family and contains a sterol-sensing domain essential for Smo repression. Based on comparative structural analyses, it has been proposed that Ptc transports sterol or other small lipidic molecules (Chen et al., 2002; Frank-Kamenetsky et al., 2002; Kowatsch et al., 2019), and can act as a lipoprotein receptor in addition to being a Hh receptor (Callejo et al., 2008). Interestingly, several small lipidic molecules that could act as Smo agonists or antagonists were identified in chemical screens (reviewed by Peer et al., 2019). Although this suggested that lipid transport could be involved in the regulation of Smo by Ptc, the molecules mediating this process remained elusive.

Suzanne's group became interested in whether the sterol or lipid components of lipoprotein particles could act in the Ptc-mediated inhibition of Smo in the absence of Hh. Their experiments revealed that Ptc could sequester Lpp into an endosomal compartment (Eugster et al., 2007; Khaliullina et al., 2009) and that this process depends on the Ptc sterol-sensing domain. Moreover, when they mutated this domain, they observed an accumulation of lipids and Smo in Ptc-containing endosomes, suggesting that by trafficking through this compartment, Smo could be exposed to the lipids mobilised by Ptc (Khaliullina et al., 2009). The Eaton group also showed that reducing the levels of circulating Lpp in *Drosophila* larvae led to the accumulation of Smo and the stabilisation of both Smo and Ci, but was insufficient for Hh pathway target gene activation (Khaliullina et al., 2009), suggesting that Smo and Ci stabilisation can be regulated by Lpp-derived lipids but that their full-strength activation requires an additional level of regulation. This inhibitory function of lipoprotein-derived lipids could be blocked by the presence of Hh on lipoprotein particles (Palm et al., 2013). This led Suzanne's group to propose a model in which Ptc mediates Smo activity through lipoprotein-derived lipids, tuning the balance between the degradation and recycling of Smo (Khaliullina et al., 2009).

Lipoproteins therefore have two contrasting roles in Hh signalling: they help mobilise Hh ligands from the cell membrane, promoting their spread, but they also provide the lipids necessary for Ptc activity. In this model, the presence of Hh on lipoprotein particles acts as a switch, blocking the Ptc-mediated mobilisation of lipids from the lipoprotein particles, and inhibiting the Ptc-dependent repression of Smo.

Having established that lipoprotein-derived lipids could negatively regulate Hh signalling, Suzanne's group then worked to identify the inhibitory lipids using biochemical fractionation and mass spectrometry on human very-low-density lipoprotein particles (Khaliullina et al., 2015). In doing so, they identified lipoprotein-derived endocannabinoids as novel endogenous Smo inhibitors. As

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endocannabinoids are present in the circulation, the link that Suzanne established suggested that systemic metabolism could directly influence development and tissue homeostasis. Endocannabinoids are unlikely to be the only endogenous Smo regulators, as recent structural and biochemical studies suggest that a sterol lipid, probably cholesterol, is one of the second positive messengers that communicate signals between Ptc and Smo (Kowatsch et al., 2019).

Hh can act as an endocrine hormone

Morphogens are typically thought to act in a paracrine manner, travelling a relatively short distance from their source cells to form a gradient that induces signalling in the receiving tissue. In contrast to the traditional model of morphogen signalling, Suzanne's group found Hh circulating in the haemolymph in association with Lpp (Palm et al., 2013; Panáková et al., 2005). By depleting Hh in a tissue-specific manner, they identified midgut enterocytes (ECs) as a major source of circulating Hh (Rodenfels et al., 2014). In this context, Hh is produced by ECs, secreted, and packaged with lipoproteins. Suzanne's group also showed that circulating Hh did not regulate tissue patterning but instead coordinated larval growth and pupariation timing by acting on two different tissues, the fat body and the prothoracic gland, respectively (Rodenfels et al., 2014). Specifically, Hh signalling in the fat body leads to the release of stored triacylglycerides (TAGs) and to a reduction in larval growth rate, whereas Hh signalling in the prothoracic gland inhibits production of the ecdysone hormone involved in pupariation timing, thereby increasing the duration of growth.

Previous studies have shown that growth and developmental timing are intrinsically linked to nutrient availability. The role of the gut in nutrient absorption hinted that midgut Hh could respond to the nutrient status of the animal, coupling it with growth and development. Interestingly, Suzanne's group showed that Hh production in the midgut, and consequently its secretion into the haemolymph, is physiologically increased upon protein and sugar starvation (Rodenfels et al., 2014). This elevated circulating Hh is essential for normal survival under starvation conditions, and thus provides a protective function against nutrient shortage by extending the larval growth period.

This pioneering work broke away from the established model of morphogen signalling, identifying not only a new function for Hh in the control of developmental timing but, more fundamentally, a new mode of action for Hh as an endocrine metabolic hormone able to facilitate inter-organ communication. This work also introduced Hh into a network that couples larval growth with developmental timing (Boulan et al., 2015). Importantly, the loading of Hh onto lipoprotein particles was also observed in mammalian tissues (Palm et al., 2013), suggesting that the endocrine function of Hh is conserved (Matz-Soja et al., 2014; Song et al., 2018). Suzanne was keen to expand on these findings, exploring a new role for circulating Shh and establishing a project analysing the role of Shh as a regulator of adult adrenal gland homeostasis (Swierczynska et al., 2013).

Conclusions

Together with her lab and collaborators, Suzanne catalysed the bridging of fields – from nutrition, lipidomics and metabolism, to biochemistry, cell biology and developmental biology – with the aim of understanding how morphogens, notably Hh, are transported and signal. Her group's studies, integrating lipoprotein particles into the Hh signalling pathway, formed the basis for a series of discoveries revealing novel mechanisms of Hh function. First, they demonstrated that Lpp could regulate the spread and activity of Hh non-autonomously. Second, their work revealed that the Hh morphogen gradient consists of functionally distinct forms of Hh: one sterol modified and Lpp dependent, and another non-sterol modified and Lpp independent. Finally, contrary to the previously described models of morphogen activity, Suzanne and colleagues discovered that Lpp-associated Hh could act systemically as an endocrine factor, regulating organismal development. These innovative, thought-provoking studies added another level of complexity into the Hh field, pushing our thinking further and motivating the community to adopt novel, more integrative approaches.

Final remarks

'What a powerful thing it is to take two supposedly separate fields – cell biology and developmental biology – and wear both hats at the same time.'

The above phrase, taken from a 2013 interview with Suzanne (Sedwick, 2013), succinctly describes her approach to research. In this Spotlight, we have discussed representative work carried out at the interface of disparate fields, which yielded results that often provided answers to questions that had long remained enigmatic in the field, or renewed our understanding of classically established concepts. These answers were invariably accompanied by many more questions, but they laid the foundation for ongoing projects across many research groups.

Through the examples we have provided, we hope to convey the level of originality present in Suzanne's approaches to understanding the mechanisms of morphogen signalling. She was one of the first to embrace and link fields such as developmental biology, cell biology, organismal growth control and metabolism. Through her work on morphogen regulation, she successfully integrated different scales of biological organisation from the molecular, to the organismal.

We have had the privilege of being associated with Suzanne's career for a long time, and to work on similar questions regarding Hh regulation. Her sudden disappearance was a shock to us all. We will never forget our lively and passionate discussions during international meetings and PhD jury committees. Just knowing that Suzanne would be present at a conference provided an incentive to attend and we would encourage students and colleagues to seek her input on their own projects. Despite our often differing views on the ways in which Hh is transported, and how the different vehicles cooperate, our discussions invariably remained respectful and, above all, inspiring. We shared in her desire to confront dogmas and established concepts in the field. Suzanne's passion for scientific discussion transcended scientific rivalry; she amplified our own excitement, opened our eyes to new ideas and, always positively, encouraged us to explore new avenues. We deeply appreciated her endless, contagious curiosity and drive to solve scientific puzzles, without inflating her own ego, simply to share the joy of contributing her reflections and inspire research. Discussions with Suzanne were not limited to scientific exchanges but embraced subjects ranging from art and literature to more personal exchanges on personal and family lives, subjects on which she was also a source of inspiration. This combination of rare qualities made her an exceptional friend and colleague whose spirit will stay with us forever.

This article is part of a collection that commemorates the work of Suzanne Eaton. See also Dahmann and Classen (2020), Mlodzik (2020), and Palm and Rodenfels (2020) in this issue.

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Competing interests
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