The \textit{vav} oncogene antagonises EGFR signalling and regulates adherens junction dynamics during \textit{Drosophila} eye development

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ABSTRACT

Organ shaping and patterning depends on the coordinated regulation of multiple processes. The \textit{Drosophila} compound eye provides an excellent model to study the coordination of cell fate and cell positioning during morphogenesis. Here, we find that loss of \textit{vav} oncogene function during eye development is associated with a disorganised retina characterised by the presence of additional cells of all types. We demonstrate that these defects result from two distinct roles of Vav. First, and in contrast to its well-established role as a positive effector of the EGF receptor (EGFR), we show that readouts of the EGFR pathway are upregulated in \textit{vav} mutant larval eye disc and pupal retina, indicating that Vav antagonises EGFR signalling during eye development. Accordingly, decreasing EGFR signalling in \textit{vav} mutant eyes restores retinal organisation and rescues most \textit{vav} mutant phenotypes. Second, using live imaging in the pupal retina, we observe that \textit{vav} mutant cells do not form stable adherens junctions, causing various defects, such as recruitment of extra primary pigment cells. In agreement with this role in junction dynamics, we observe that these phenotypes can be exacerbated by lowering DE-Cadherin or Cindr levels. Taken together, our findings establish that Vav acts at multiple times during eye development to prevent excessive cell recruitment by limiting EGFR signalling and by regulating junction dynamics to ensure the correct patterning and morphogenesis of the \textit{Drosophila} eye.

KEY WORDS: Vav, EGFR, Adherens junctions, Eye patterning, Morphogenesis

INTRODUCTION

Morphogenesis during animal development relies on cell fate specification together with correct organisation of cells and establishment of cell-cell junctions within epithelia. The coordination of these distinct processes is key to establishing normal cell function in a complex system. The \textit{Drosophila} compound eye is ideally suited for dissection of the genetic and cellular requirements that govern pattern formation at molecular, cellular and tissue levels within an epithelium. The \textit{Drosophila} adult eye is characterised by a very stereotyped organisation. One retina contains around 750 identical visual units called ommatidia, each ommatidium consisting of a precise number of cells that are specified during larval and pupal stages. During the third-instar larval stage, the eight photoreceptors (or R cells; neuronal) and the four lens-secreting epithelial cells (cone cells, CCs) differentiate in the eye imaginal disc, starting with the selection of an initial photoreceptor, the R8, from a pool of progenitor cells. R8 then recruits R2 and R5, which themselves recruit R3 and R4. After the second mitotic wave, R1, R6 and then R7 are added to the ommatidium, rapidly followed by selection of CCs. During pupal life, pigment cells differentiate from a pool of interommatidial pigment cells (IPCs). Two primary pigment cells are added first, enwrapping each cone cell cluster (lying on top of the eight photoreceptors) to form the core ommatidium. Excess interommatidial cells are eliminated by apoptosis, leaving the precise final cell number, and the remaining accessory cells ultimately differentiate as secondary and tertiary pigment cells along with sensory bristles, which completes formation of the hexagonal lattice that surrounds the core ommatidium (reviewed in Kumar, 2012). Different signalling pathways are used in combination to determine the different fates, among which Epidermal Growth Factor Receptor (EGFR) signalling is required for the differentiation of all cell types except R8. Expression of a dominant-negative EGFR completely prevents the formation of the retina (Freeman, 1996). Furthermore, the strength of EGFR signalling has to be tightly regulated by a balance between activators and inhibitors to allow the exact number of each cell type to be recruited to each ommatidium (Shilo, 2005; Yogev et al., 2008). Thus, ommatidium development is a model to understand how EGFR signalling can be modulated during development to allow correct cell fate specification.

Beside cell fate acquisition, cell-cell adhesion and intercalation are crucial for the different cells to be placed and held in their correct positions, representing another fundamental aspect of eye development and function. Several adhesion molecules such as N-Cadherin (Hayashi and Carthew, 2004), DE-Cadherin (Grzeschik and Knust, 2005) and proteins from the Nephrin and Neph1 families (Bao and Cagan, 2005) have been implicated in these cell-sorting processes to regulate accessory cell numbers, shapes and positions. Some regulators of cell-cell junctions in the retina have been identified, such as the CIN85 and CD2AP orthologue Cindr, an adaptor protein that assembles complexes, required to link cell surface adhesion molecules and the actin cytoskeleton to allow cell shape changes (Johnson et al., 2008). However, the mechanisms by which cell-cell junctions are stabilised while allowing cell movements remains elusive, and the identification of new regulators of junction dynamics will help our understanding of how tissue development is achieved.

One candidate that has been implicated in both mediating the EGFR signalling pathway and regulating cell adhesion is the guanine nucleotide exchange factor (GEF) Vav. The expression of Vav oncogenes in numerous human cancers (Lazer and Katzav, 2011) justifies a better understanding of their roles during tissue development and the characterisation of pathways with which they
interact. Members of the Vav family are activated upon phosphorylation by cell surface receptors, and they can, in turn, activate the Rho GTPases by facilitating the exchange of a GDP for a GTP (Bustelo, 2000).

We previously demonstrated that the unique Drosophila vav gene is required for axon growth and guidance at different developmental stages in Drosophila, including guidance of photoreceptor axons from the larval eye disc into the optic lobe. We showed that vav is ubiquitously expressed in the eye disc, which is consistent with a possible function of Vav during eye development (Malarte et al., 2010).

Interestingly, mammalian Vav proteins are activated by tyrosine phosphorylation in response to EGFR induction, and their SH2 domains associate directly with the receptor both in vitro and in vivo (Bustelo et al., 1992; Margolis et al., 1992; Tamás et al., 2003). In Drosophila, Vav and the EGFR have been also shown to interact physically (Dekel et al., 2000; Sarkar et al., 2007) and genetically (Fernández-Espartero et al., 2013). These interactions and the ubiquitous expression of vav in the larval eye disc prompted us to examine whether Vav was involved in eye development and patterning and its relationship with EGFR signalling.

In this work, we show that Vav plays distinct roles during eye development to limit cell recruitment in the newly assembled ommatidium. Vav acts reiteratively in a GEF-dependent manner to recruit the correct number of photoreceptors, CCs and pigment cells. It does so, on the one hand, by downregulating the EGFR pathway, thus revealing a new interplay between Vav and the EGFR pathway. On the other hand, Vav regulates adhesion and the stability of cell-cell junctions to limit the number of primary pigment cells recruited.

RESULTS

vav mutant retinas display increased cell numbers at the apical surface

To determine whether the absence of vav caused a phenotype in the retina, we dissected vav pupal retinas once all cell recruitment and morphogenesis events are complete. We labelled them with the Discs large (Dlg) seaptate junctional marker, which allows the identification of all different cell types by shape and position (Fig. 1A). We found that vav retinas were disorganised and did not harbour the interweaving hexagonal lattice that characterises the wild-type ommatidia (Fig. 1A,B). A total of 17.85% of ommatidia (n=112 ommatidia from six retinas) were also misoriented along the equatorial-polar axis (Fig. 1C). In addition, many vav ommatidia contained extra cells of all types. To quantify this excess, we counted the number of cells forming the hexagonal lattice, which in wild-type is 18, including the four CCs, the two primary cells and the 12 accessory cells surrounding the ommatidia core (six secondary cells, three tertiary cells and three bristles) that are actually shared with neighbouring ommatidia (Fig. 1D). We found that vav ommatidia displayed an average of three extra cells per ommatidum (n=37 ommatidia, from four retinas). In addition to the excess in interommatidial cells (Fig. 1H), we observed that 32.1% (n=3620 ommatidia from 20 retinas) of vav ommatidia had extra CCs, ranging from five (85.5% of the cases, Fig. 1E) to eight CCs (Fig. 1G), and that 25.3% of vav ommatidia (n=1332 ommatidia from ten retinas) had extra primary...
cells (Fig. 1F,G). Similar results were obtained with other vav alleles (vav1, vav2, vav3 and vav1/2/3) and in clones (Fig. 11 for vav1; supplementary material Fig. S3A for vav1/2/3), confirming that loss of Vav function causes a cell number increase.

vav mutant ommatidia possess extra photoreceptors

Having found that there are more cells than normal in the absence of Vav function at the apical retina, we investigated whether the number of photoreceptors lying underneath accessory cells was affected in vav retinas. To this end, we used an antibody against Elav, a neuronal marker that is expressed in all photoreceptors, and counted numbers of the R1-R7 photoreceptors that were present in the same focal plane and the number of the inner photoreceptor R8 located underneath R7. Seven photoreceptors per ommatidium were present on the same focal plane in wild-type retinas (n=91 ommatidia from four retinas, data not shown), as opposed to in vav mutant ommatidia, where 71.5% contained eight or more photoreceptors (n=233 ommatidia from four retinas, Fig. 2C). This was not due to a mis-localisation of R8s as another R cell per ommatidium was visible more basally in the retina (data not shown). This result shows that vav retinas are characterised by having a majority of ommatidia with at least one extra photoreceptor cell. This prompted us to analyse in more detail how R cells are specified during eye development in the absence of vav.

R7 photoreceptors are recruited in excess and prematurely in vav mutant ommatidia

To investigate whether the extra photoreceptors recruited in vav ommatidia belong to a specific R cell subtype or whether any subtype was recruited in excess, we used markers that are specific to individual R cell subtypes. In wild-type eye imaginal discs (supplementary material Fig. S1A), the presumptive R8 expresses senseless (sens) (Nolo et al., 2000). Occasional ommatidia with extra Sens-positive cells were found in vav eye discs (supplementary material Fig. S1B), although these cells were Elav-negative and excluded from ommatidia (supplementary material Fig. S1B*), suggesting that they failed to differentiate further as neurons. Hence, the extra Elav-positive cells found in 71.5% of vav ommatidia are unlikely to be of the R8 subtype.

In larval eye discs, the position of the extra R cells within each vav clusters suggested that they were R7s (supplementary material Fig. S1C-E). To confirm their identity, we used Prospero (Pros), a marker expressed in R7 (Kauffmann et al., 1996). Two or three Pros-expressing cells per ommatidium instead of one were often present in young vav ommatidia (Fig. 2A,B). These extra R7s were maintained throughout retina development, as pupal vav ommatidia with extra photoreceptors displayed two or more Pros-positive cells (Fig. 2C*). We then compared the timing of recruitment in wild-type and vav retinas. Spalt Major (Salm) and Pros expression in R7 starts in ommatidia rows seven and eight in wild-type larvae (Domingos et al., 2004; Kauffmann et al., 1996) (supplementary material Fig. S1C), and in rows five and six in vav discs (supplementary material Fig. S1D), suggesting that precocious recruitment of R7 takes place in the absence of vav. Taken together, our results show that Vav regulates the number and timing of R cell recruitment by limiting the excessive and premature acquisition of R7 fate.

Vav acts reiteratively during eye formation

The formation of an ommatidium is a stepwise process, starting with the selection of the R8. R cells recruit each other successively and send signals to select the four CCs. Pigment cells are recruited later by signalling emanating from the adjacent CCs (Kumar, 2012). Hence, there are at least two explanations for the presence of extra cells of all types in vav mutants: (1) the presence of extra accessory cells is a secondary consequence of recruiting extra R7s; or (2) Vav is acting reiteratively at each step of ommatidia assembly. To discriminate between these two hypotheses, we examined whether there was a correlation between the numbers of the different cell types observed in vav pupal retinas. Although some ommatidia had the correct number of CCs, primary cells (Fig. 3A) and photoreceptors (Fig. 3A′), others had more of the three cell types (Fig. 3A,A′). We also identified ommatidia with more CCs, although their R cell number was correct (Fig. 3B,B′,C,C′). This result shows that extra CCs can be produced in the absence of extra photoreceptors and therefore argues in favour of a reiterative role of Vav.

Similarly, no correlation was found between the number of CCs and primary cells because the following four types of ommatidia coexisted in vav retinas: those with excess CCs but the correct number of primary cells; those with the normal number of CCs but excess primary cells; those with the correct number of CCs and primary cells; and those with an excess of CCs and primary cells (Fig. 3D). Finally, among the ommatidia with five CCs (n=159 ommatidia from seven retinas), 54% had two primary cells, whereas 46% had three primary cells (Fig. 3E). Hence, an excess of primary cells is not a consequence of the presence of additional CCs. Taken together, our data suggest that Vav acts reiteratively during eye development to ensure that the correct number of each cell type is specified at each stage.

Fig. 2. Extra photoreceptors in vav eye disc and pupal retina. (A,B) Third-instar larval eye discs are oriented with anterior towards the left and stained with antibodies against Elav (red), Salm (green) and Pros (blue). Wild-type (wt) ommatidia possess a single R7 (arrow) identified by the expression of Pros (A), whereas vav ommatidia display multiple R7s that are recruited precociously (arrow) (B). (C) 48 h APF vav retina stained with antibodies against Elav (red), Pros (orange) and Dlg (green). Shown are projections of confocal sections underneath the apical surface of the pupal retina showing R1-R7 expressing Elav. R7s appear orange as they are positive for both Pros and Elav. Ommatidia with only seven R cells on the same plane possess only one R7 (arrowhead, C), whereas ommatidia with extra R cells have multiple R7s (arrowheads, C′). The number of photoreceptor cells (PR) is indicated.
To investigate this hypothesis, we analysed the expression patterns of several readouts of the EGFR pathway in vav eye discs and retinas. First, we assessed the transcriptional activation of Kekkon, a specific EGFR target gene, which functions as a negative regulator of the pathway (Ghiglione et al., 2003). Higher levels of kekkon-lacZ were detected in vav clones (Fig. 4D,E), implying that EGFR signalling was hyper-activated in the absence of vav. We also used the phosphorylation of MAPK (known as Rolled in Drosophila) as another direct readout of EGFR signalling, which can be detected with the anti-diphosphoERK (diPERK) antibody. vav cells showed very high levels of diPERK compared with control tissue in mosaic pupal retinas (Fig. 4F,G). Interestingly, diPERK levels were increased in a cell-autonomous manner, indicating that EGFR signalling was upregulated autonomously in vav cells. Accordingly, vav mosaic ommatidia with CC excess all contained at least one vav mutant CC (n=62 ommatidia from 15 retinas) (Fig. 1I). Taken together, these results indicate that Vav acts autonomously as a negative regulator of EGFR signalling in the retina.

To further confirm this, we tested whether altering the levels of EGFR signalling had any effect on vav phenotypes. First, we inhibited the EGFR pathway in the eye by overexpressing Argos, a secreted protein that associates with Spitz, the EGFR ligand, therefore decreasing the quantity of ligand available. This resulted in an adult with a smaller eye with a rough aspect (Freeman, 1994) (Fig. 4H,J). This deleterious effect was largely rescued when removing vav (Fig. 4K), demonstrating that loss of vav function has an opposing effect to that of gain of argos. Second, we examined the consequences of halving the dose of Spitz in vav mutants. The vav; spi+ retina organisation was largely rescued (compare Fig. 4M with 4N) confirming that Vav antagonises the EGFR signalling pathway during eye development.

**Vav function in the retina relies on DH domain integrity**

Most characterised Vav proteins’ roles require their GEF function, although they have also been shown to act independently of GEF activity (Bustelo, 2000). To determine whether the new Vav role as an inhibitor of EGFR signalling depends on its GEF activity, and given that it is known that Rac proteins can be activated through the Vav GEF function (Bustelo, 2000; Couceiro et al., 2005), we first examined whether Rac and vav retinas displayed similar phenotypes. As opposed to the vav retina, excess CCs or primary cells were not observed in retina in which 90% of ommatidia were null for the three Drosophila Rac members, Rac1, Rac2 and Mtl (supplementary material Fig. S3B). Occasional excess of interommatidial cells was observed, although the phenotype was milder than in vav retinas (supplementary material Fig. S3B). Given that the vav and Rac phenotypes in the retina are different, as opposed to the similar axon guidance phenotypes they display (Malartre et al., 2010), Rac proteins are unlikely to be the main Vav effectors for regulating cell recruitment in the eye, suggesting that Vav uses distinct effectors to regulate eye patterning and axon targeting.

We next generated new vav alleles using CRISPR/Cas9-mediated mutagenesis to create deletions in the DH domain that mediates GEF activity. Mice expressing enzymatically inactive but normally folded Vav1 protein (Vav1Δκ) have been generated by introducing a point mutation that changed only two amino acids in the DH domain (Bustelo et al., 2009). Based on this work, and because these amino acids are conserved in Drosophila, we targeted the same region to create small deletions in the DH domain that are predicted to abolish GEF function. We obtained a mutant deleted for only one amino acid (vavΔκ) and a mutant deleted for three amino acids (vavΔκMQR, Fig. 5A,B). First, we confirmed that the GEF activity was...
compromised in these new DH point mutation alleles, as they all exhibited phenotypes similar to those of vav and Rac nulls during axonal targeting (Malartre et al., 2010; data not shown). Interestingly, these mutants also exhibited phenotypes similar to those associated with the vav-null alleles in the retina, with lower penetrance only for the single amino acid deletion (Fig. 5C-F). The vavΔAMQ deletion was sufficient to recapitulate the strength of vav-null phenotypes indicating that Vav function in the eye requires its GEF activity. Similarly, we generated mutations in the C-terminal domain of Vav that deleted either both the SH2 and SH3 domains or only the SH3 domain (Fig. 5A). Phenotypic analysis showed that the loss of the SH2 and SH3 domains fully impaired Vav function in the retina, whereas the loss of the SH3 domain alone had no effect (Fig. 5E,F). Taken together, our analyses indicate that both Vav GEF activity and the binding of Vav to the EGFR are necessary to inhibit EGFR signalling. This suggests that both recruitment to the EGFR and downstream signalling activity mediated by activation of GTases are necessary to perform this unexpected inhibitory function of Vav.

**vav mutant retinas display mis-patterning phenotypes that account for junction or adhesion defects**

To investigate whether all vav eye phenotypes were rescued by decreasing EGFR levels, we compared the numbers of the different cell types in vav and vav; spi/+ retinas. We found that the extra CCs phenotype was almost completely rescued as only 4.3% of vav; spi/+ ommatidia displayed extra CCs (n=2253 ommatidia from eight retinas), compared with the 32.1% found in vav retinas (Fig. 4O). In sharp contrast, the extent of the rescue was different for the primary cells: as the proportion of ommatidia with extra primary cells was only slightly lower in vav; spi/+ retinas (18.1%, n=1114 ommatidia from four retinas) as compared with vav (25.3%, see Fig. 4O). This result supports our earlier conclusion that the recruitment of extra primary cells in vav retinas is not a consequence of extra CCs. Furthermore, it points to the possibility that some extra primary cells observed in vav retinas are due to a Vav function that is independent of its effect on EGFR signalling.

In response to EGFR signalling, the CCs produce the Delta ligand in response to EGFR signalling, the CCs produce the Delta ligand. This ligand is a key component of Delta-Notch signalling from the CC to the primary precursor. This ligand is a key component of Delta-Notch signalling from the CC to the primary precursor. In response to Delta-Notch signalling, the primary cells can either be inhibited from differentiating or be induced to differentiate. In response to Delta-Notch signalling, the primary cells can either be inhibited from differentiating or be induced to differentiate. Extra primary cells can also be caused by defective cell movements (Johnson et al., 2008; Johnson and Cagan, 2009). To determine whether cell-cell junction stability or adhesion could be affected in vav retinas, we assessed the ommatidial mis-patterning score (OMS), a...
means of scoring a broad range of specific traits (Johnson and Cagan, 2009). We found that vav retina scored nine errors per ommatidium on average (n=43 from six retinas). Among these errors, the following can result from junction or adhesion defects: incorrect orientation of CC junctions (supplementary material Fig. S4B), energetically unstable CCs arrangement (supplementary material Fig. S4C), non-equivalent primary cell sizes (supplementary material Fig. S4D), and non-equivalent primary-primary cell junction sizes, with extreme cases where one of the two junctions was completely lost (supplementary material Fig. S4F). Taken together, this suggests that Vav could be involved in cell rearrangement, by forming or stabilising CC and primary-primary cell junctions or by controlling cell movements during pupal eye morphogenesis.

**vav mutant cells are excessively dynamic causing extra primary cell recruitment and patterning delay**

To further analyse the hypothesis that Vav is involved in junction stability or remodelling, we compared the dynamics of adherens junction rearrangements by time-lapse microscopy in wild-type and vav pupae from the time of primary cell recruitment (between 16 h and 19 h APF). Live imaging was performed by visualising the vav junction rearrangements by time-lapse microscopy in wild-type and vav mutant cells are excessively dynamic causing extra primary cell recruitment and patterning delay. Amino acid sequence in the DH domain is given for wild-type Drosophila Vav (dVav) and deletion mutants, together with the corresponding sequence for mice Vav1 (mVav1) and Vav1AA. (C-F) Apical views of 50 h APF retinas stained with anti-Dig antibody showing that, like in vav-null mutants, excess CCs and primary cells are found in vavΔvav, vavΔMQR and vavΔSH2-SH3 mutants, but not in vavΔSH2 mutants. Extra CCs and extra primary cells are indicated by white and pink asterisks, respectively. (G) Quantification of the CC and primary (1°) numbers from the different genotypes. For CCs, n=314 ommatidia from one retina (vavΔvav), 1641 ommatidia from seven retinas (vavΔMQR), 687 ommatidia from three retinas (vavΔSH2-SH3) and 912 ommatidia from two retinas (vavΔSH3).

For primary cells, n=233 ommatidia from one retina (vavΔvav), 1355 ommatidia from seven retinas (vavΔMQR), 660 ommatidia from three retinas (vavΔSH2-SH3) and 912 ommatidia from two retinas (vavΔSH3).

Second, we found that the defects in junction dynamics correlated with the recruitment of extra primary cells. Indeed, we observed cases (n=6 movies out of nine) where two proto-primary cells got in contact several times to enwrap the CC cluster, before failing to establish a stable junction and maintain their niches, allowing a third cell to intercalate and adopt the typical primary cell shape (Fig. 6B; supplementary material Movie 4).

Finally, we observed cases where the two primary cells either failed to establish a stable junction after several attempts in making contact or had already established junctions but failed to maintain one of them. This led to an open primary cell phenotype where the CCs enter in direct contact with the IPCs, before failing to establish a stable junction and maintain their niches, allowing a third cell to intercalate and adopt the typical primary cell shape (Fig. 6B; supplementary material Movie 4).

**vav interacts genetically with shg and cindr**

To further support the role of Vav in junction dynamics and adhesion, we tested whether decreasing adherens junction function in vav retinas amplified the supernumerary primary cell phenotype. Reducing adhesion by using a hypomorphic allele of shotgun (shgP34-1), the Drosophila E-cadherin, did not affect retina patterning (Fig. 7A). Strikingly, using the same shgP34-1 allele strongly enhanced the vav phenotype – from 25% of ommatidia with extra primary cells to the final number of primary cells (supplementary material Fig. S5B, Movie 2). These abnormal movements sometimes occurred for hours and in several cells around a single ommatidium (supplementary material Fig. S5C, Movie 3). Importantly, this resulted in a patterning delay (supplementary material Fig. S5A) without causing recruitment of extra primary cells. Similar abnormal movements have been observed in retina defective for cindr activity (Johnson et al., 2008).
61.5% in \textit{vav}; \textit{shg}^{P34-1} (Fig. 7C,G, \(n=1289\) ommatidia from six retinas). Interestingly, and in agreement with our proposal that extra CCs are mainly due to defects in EFGR signalling, the percentage of ommatidia with extra CCs was similar in \textit{vav} (32.2%) and \textit{vav}; \textit{shg}^{P34-1} (32.9%, \(n=1342\) ommatidia from six retinas, Fig. 7G).

Reducing the activity of Cindr, an adaptor protein that interacts with Cadherin and multiple components of the actin cytoskeleton, also affects cell movements during retina patterning (Johnson et al., 2008). Interestingly, \textit{cindr} phenotypes are also enhanced in a heterozygous \textit{shg} background, and live imaging movies of pupal retina. Live imaging from wild-type (wt) pupal retina (A) and \textit{vav} clones (B,C). The time at which the different pictures were taken is indicated. (A) Wild-type ommatidium from a DE-cadherin:GFP pupa, showing that once two primary cells (yellow) enter in contact (arrowheads), a stable junction is established. (B,C) Pupae of the following genotype were selected: \textit{vav}^{FRT19A/ubiGFPeyFlp122 FRT19A; DE-cadherin:GFP}+. \textit{vav} ommatidia from clonal regions are presented. (B) Over several hours, two primary cells (yellow) enter in contact and are separated successively. As they fail to establish a stable junction, a third cell intercalates, leading to the recruitment of three primary cells. Note that extra primary cells are unlikely to be due to IPC crowding, as extra primary cells in \textit{vav} ommatidia followed by live imaging were not associated with an increase of IPC number at the time of primary cell recruitment, as compared with \textit{vav} ommatidia with two primary cells (mean of 14.3 and 14.4 cells, respectively; \(n=14\) ommatidia with two and 14 with three final primary cells). (C) A junction between two primary cells that had been maintained for several hours is finally disrupted. Hence, the CC cluster (pink) enters in contact with the interommatidial cells (blue), leading to the open primary cell phenotype. Tertiary pigment cells are in green and bristles in orange.

Fig. 6. Unstable primary-primary cell junctions are associated with various phenotypes in \textit{vav} retina. Apical views of 50 h APF retinas stained with anti-Dlg antibody. (A) Retina patterning and cell number are not affected by \textit{shg}^{P34-1}, a hypomorphic allele for \textit{DE-cadherin}. (B) \textit{vav} retina showing extra CCs (white asterisks) and extra primary cells (pink asterisks). (C) In \textit{vav}; \textit{shg}^{P34-1} mutants, the extra primary cells phenotype is increased whereas CC number is not. (D) Retina patterning and cell number are not affected in \textit{vav}/+ retina. (E) GMR>\textit{cindr-IR2} retina display a series of patterning defects, including disorganisation of the retina, misplaced pigment cells and bristles, excess of IPCs, ommatidia misrotation, CC cluster arrangement, and a mild excess of primary cells. (F) In \textit{vav}/+: GMR>\textit{cindr-IR2} retinas, the extra primary cells phenotype is increased. (G) Quantification illustrates that decreasing adhesion in the \textit{vav} mutant or decreasing \textit{vav} levels in the presence of \textit{cindr} RNAi increases the primary cell (1°) but not CC excess (0.8% of ommatidia in GMR>\textit{cindr-IR2} (\(n=1154\) ommatidia from eight retinas) and 1.6% in \textit{vav}/+: GMR>\textit{cindr-IR2} (\(n=1437\) ommatidia from seven retinas)).
eyes with reduced cindr activity showed that there was junction instability and primary cell replacement in these retinas. We therefore tested whether vav and cindr interacted genetically. To address this question, we expressed a cindr RNA interference (RNAi) line in wild-type eyes (GMR>cindr-IR2) and in a vav heterozygous background (vav+/; GMR>cindr-IR2). Decreasing cindr function leads to a series of patterning defects, including a mild excess of primary cells (Fig. 7E) (Johnson et al., 2008). Whereas in vav heterozygous retinas, cell numbers were close to those in wild-type (98.9% and 99.9% of ommatidia with normal primary and CC numbers, respectively; n=1099 ommatidia from four retinas), halving the vav dose in cindr-IR2 retinas enhanced the cindr-IR2 phenotypes (Fig. 7F). As shown in Fig. 7G, this was particularly striking for the number of ommatidia with three primary cells (4.4% of ommatidia in GMR>cindr-IR2 (n=1154 ommatidia from eight retinas) versus 25.1% in vav+/; GMR>cindr-IR2 (n=1198 ommatidia from seven retinas)). These results indicate that vav and cindr interact genetically, in particular for the recruitment of primary cells, and are in agreement with a model where Vav, like Cindr, participates in a signalling pathway linking the cell-surface junctions with components of the actin cytoskeleton.

**DISCUSSION**

In this study, we provide evidence for distinct roles of the vav gene in regulating cell shaping and patterning of the Drosophila eye. On the one hand, Vav acts reiteratively to limit cell recruitment by counteracting EGFR signalling, and, on the other hand, it regulates adhesion and junction dynamics to limit excessive cell recruitment.

**The EGFR signalling pathway is antagonised by Vav**

The EGFR is a well-characterised signal transducer that has been highly conserved during evolution (Shilo, 2003). EGFR activity is subject to modulation by multiple regulators to ensure that the appropriate level of signalling is provided to specific cells (Bogdan and Klämbt, 2001). The increasing number of positive and negative regulators identified highlights the fundamental role of this well-conserved pathway in the regulation of adherens junction dynamics in the development of epithelial morphogenesis and reshaping, and various members of the Rho GTPases and their GEF regulators have been implicated in these processes during Drosophila embryonic (reviewed in Takeichi, 2014) and eye (Warner and Longmore, 2009a,b; Yashiro et al., 2014) development. Recently, with the development of live imaging tools, the use of the Drosophila eye has proven to be an excellent system to study the role of adhesion and junction dynamics in vivo (Larson et al., 2008). In particular, the recruitment of primary cells depends on both the integration of combinatorial signalling pathways (Nagaraj and Banerjee, 2007) and the regulation of cell-cell adhesion (Bao, 2014). Once the first two primary cell candidates are selected, they have to form stable junctions that enwrap the CCs and isolate them to prevent additional primary cell recruitment from the IPC pool. Accordingly, Cindr acts on E-Cadherin and the actin cytoskeleton to maintain adherens junctions, thus limiting excessive movements leading to extra primary cell recruitment (Johnson et al., 2008). Our live imaging experiments showed that junctions between primary cells were unstable in the absence of Vav, causing various patterning defects. Because vav genetically interacts with E-Cadherin and cindr, and given that the ortholog of cindr, known as CIN85 or CD2AP, directly associates with Vav in human B cells (Niño et al., 2012), we can envision that Vav might act with Cindr to modulate cell junction stability in the retina (both proteins being part of a complex and providing a link between junction proteins such as Cadherins and the actin cytoskeleton). Taken together, our data reveals a new Vav function in the regulation of adherens junction dynamics in the Drosophila eye.

In summary, Vav, which is located at the crossroads of distinct signalling pathways, is playing different roles during eye development, from recruitment of photoreceptors and accessory cells to photoreceptor axon targeting, but is also involved in cell dynamics and junction remodelling to ensure the well-ordered generation of appropriate numbers of the different cell types forming the perfectly structured Drosophila eye.

**MATERIALS AND METHODS**

**Drosophila genetics**

All crosses were conducted at 25°C. The following fly strains were provided by Bloomington Drosophila Stock Center: the Canton S strain as the wild-type reference, salm-lacZ, UAS-ergos, spi1, GMR-Gah4, shootgun34+. Other fly strains used were: vav1, vav2, vav3FRT19A (Malarte et al., 2010), vav153FRT19A (Kyoto Stock Center), yw, ubiGFPeyFlp122FRT19A (Kevin Legent, IJM, Paris), FRT19Aaarm-lacZ, eyFLP/TM6b (a gift from Fernando Casares, CABD, Seville, Spain), UAS-Rhombo1 and UAS-Rhombo3 (a gift from Matthew Freeman, University of Oxford, UK), kekkon-lacZ (Christian Ghiglione, IBV, Nice, France), trioFRT80B (a gift from Barry Dickson, IMP, Vienna, Austria), yweyFLP gl-lacZ, 3LevuFRT80M0 and rac1FRT80M0 (a gift from Takashi Suzuki, TIT, Yokohama, Japan), E-cadherin-GFP (a gift from Yang Hong, University of Pittsburgh, Pittsburgh, PA, USA) and cindr-IR2 line (a gift from Ruth Johnson, Wesleyan University, Middletown, CT, USA). Unless otherwise specified, vav1 and vav2 were both used in experiments conducted on whole mutant retinas and vav2FRT19A was used in clonal analyses.
Generation of vav alleles using Crispr/Cas9

New vav mutant alleles were generated by CRISPR- and Cas9-mediated non-homologous end joining. Two pairs of guide RNA (gRNA) target sites were used: 5′-GTACTTGAGAATCGGCTGATGG-3′ and 5′-TCTCAAGTATCATCTGCTGCTG-3′. gRNAs were cloned in the pCFD3-diU6:3gRNA plasmid (Addgene). Pairs of gRNAs (250 ng/µl each) were injected (BestGene) in embryos (600 for each pair) laid by Vasa-Cas9 females (Gratz et al., 2014). 278 F0 and 735 F1 crosses were established and progenies were screened by a T7 endonuclease assay (Zhang et al., 2014), by PCR or by lethality to identify deletions in the DH or SH2 domains. Deletions were further mapped by sequencing. vanΔBio5 is a 41-nucleotide deletion leading to a frame shift in which 30 different amino acids are followed by a stop codon. vanΔSHD is a 10-nucleotide deletion leading to a frame shift that removes the six last amino acids of the SH2 domain and introduces one amino acid followed by a stop codon, thus removing the entire SH3 domain.

Mosaic analysis

vav mutant clones were obtained using the FLP-FRT technique (Xu and Rubin, 1993). Recombination was induced by eyeless FLP activity. Mutant clones were marked by the absence of anti-GFP or anti-β-galactosidase antibody staining. Genotypes used for clonal analysis in larvae were: vanFRT19A/arm-lacZ/FRT19A: eyFpl/+; vanFRT19A/arm-lacZ/FRT19A: eyFpl/+; vanFRT19A/arm-lacZ/FRT19A; kek-lacZ+/-; RacΔ25 RacΔ2 mutant null clones were generated from the following pupae: ywFPL gf-lacZ; 3Lcl+; FRT80BMit+/+ FRT19A arm-lacZ; RacΔ25 RacΔ2mit3 mit4. These clones were generated as described previously (Walther and Pichaud, 2007). For pupal dissection, staged at 25°C. The following primary antibodies were used: mouse anti-Dlg (1:50), mouse anti-Elav (1:500), mouse anti-Pros (1:10), mouse anti-Cut (1:10) and rat anti-Elav (1:500). Secondary antibodies labelled with Alexa Fluor 488, Alexa Fluor 568 and Alexa Fluor 647 were purchased from Molecular Probes and were used at a concentration of 1:500. Immunostaining was performed according to standard procedure and as described previously (Walther and Pichaud, 2007). For pupal dissection, white prepupae were collected (0 h after puparium formation, APF) and staged at 25°C. The following primary antibodies were used: mouse anti-Dlg (1:50), rat anti-Elav (1:500), mouse anti-Pros (1:10), mouse anti-Cut (1:100) (Developmental Studies Hybridoma Bank); rabbit anti-Salmb (1:500, kind gift from Fernando Casares), guinea pig anti-Sens (1:1000), a kind gift from Hugo Bellen, BCM, Houston, TX, USA), mouse anti-diPERK (1:100, Sigma), fixation into PBS containing 1 mM okadaic acid prior to fixation, rabbit anti-GFP (1:1000, Molecular Probes), and mouse anti-β-galactosidase (1:5000, Promega) antibodies. To label nuclei, retinas were incubated with the DNA dye TO-PRO-3 (1:1000, Molecular Probes). The secondary antibodies labelled with Alexa Fluor 488, Alexa Fluor 568 (Molecular Probes) or Cy5 (Jackson ImmunoResearch) were used at 1:200. Samples were mounted in Vectashield (Vector Laboratories), and images were captured with a Leica TCS-SP2 or a Nikon eclipse TE 2000-U confocal microscope and the EZ-C1 3.30 software. Confocal image stacks were processed with ImageJ software (1.46r).

Time-lapse experiments

Pupae were mounted as described in Bardet et al., 2013. Images were acquired with a Ropper spinning-disk confocal mounted on an Eclipse Ti microscope (Nikon) using the Metamorph software. We imaged eight vav mutant mosaic retinas between 15-35 h APF. In the clonal region, and after elimination of ommatidia for which focus was lost or that moved out of frame during the course of the movie, morphogenesis was reconstituted and analysed for 36 ommatidia.

Image treatments

Raw fluorescence images were processed to increase the signal to noise ratio (Kervrann and Boulanger, 2006). After a maximum projection of the image stacks, the different parts of the eye filmed separately were stitched and registered using the Fiji plug-in ‘Stitching’ (Preibisch et al., 2009) and ‘StackReg’, respectively.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.M. performed the experiments and analysed the data. M.M. and P.-L.B. designed and performed the live imaging. M.M. and Y.B. conceived the experiments and wrote the paper. P.-L.B. and M.-D.M.B. edited the manuscript.

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Supplementary material

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References


