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RESEARCH ARTICLE

IMP regulates Kuzbanian to control the timing of Notch signalling in *Drosophila* follicle cells

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ABSTRACT

The timing of *Drosophila* egg chamber development is controlled by a germline Delta signal that activates Notch in the follicle cells to induce them to cease proliferation and differentiate. Here, we report that follicle cells lacking the RNA-binding protein IMP go through one extra division owing to a delay in the Delta-dependent S2 cleavage of Notch. The timing of Notch activation has previously been shown to be controlled by cis-inhibition by Delta in the follicle cells, which is relieved when the miRNA pathway represses Delta expression. *imp* mutants are epistatic to *Delta* mutants and give an additive phenotype with *belle* and *Dicer-1* mutants, indicating that IMP functions independently of both cis-inhibition and the miRNA pathway. We find that the *imp* phenotype is rescued by overexpression of Kuzbanian, the metalloprotease that mediates the Notch S2 cleavage. Furthermore, Kuzbanian is not enriched at the apical membrane in *imp* mutants, accumulating instead in late endosomes. Thus, IMP regulates Notch signalling by controlling the localisation of Kuzbanian to the apical domain, where Notch cleavage occurs, revealing a novel regulatory step in the Notch pathway.

KEY WORDS: RNA-binding protein, Delta, ADAM10 protease, *Drosophila* oogenesis

INTRODUCTION

RNA-binding proteins (RBPs) play diverse roles in the post-transcriptional regulation of gene expression by controlling the splicing, stability, translation or subcellular localisation of specific mRNAs. One of the best studied classes of RBPs is the conserved family of IGF2 mRNA-binding proteins (IMPs, also known as the VICKZ family), which are characterised by four conserved KH domains, with KH3 and KH4 being most important for RNA binding, and two N-terminal RRM domains (Degrauwe et al., 2016). Initial studies on IMPs pointed to an important role in mRNA localisation. The *Xenopus* IMP3 orthologue, Vg1RBP/Vera (Igf2bp3), binds to the localisation signal in *vg1* (*gdf1*) mRNA and colocalises with it to the vegetal cortex of the *Xenopus* oocyte (Deshler et al., 1997; Havin et al., 1998). Similarly, the chicken IMP1, ZBP1 (IGF2BP1), binds to the 54-nucleotide localisation signal in β-actin mRNA to mediate its localisation to the periphery of fibroblasts and the dendrites of neurons (Farina et al., 2003; Tiruchinapalli et al., 2003). However, IMPs also regulate mRNA translation and mRNA stability. Mammalian IMP1-3 were initially identified as translational regulators of insulin-like growth factor II (*Igf2*) mRNA and ZBP1 represses the translation of β-actin mRNA until it reaches its destination (Hüttelmaier et al., 2005; Nielsen et al., 1999; Yao et al., 2006). One mechanism by which IMPs regulate mRNA translation and stability is by preventing the binding of siRNAs and miRNAs to their targets, either by masking the binding sites or by sequestering the mRNA away from the Argonaute/RISC complex (Degrauwe et al., 2016). In many cases, IMPs have been found to play an important regulatory role, although the relevant RNA targets have not been identified. For example, IMP1 and 3 are upregulated in a number of tumours, with their expression levels correlating with increased metastasis and poor prognosis (Degrauwe et al., 2016; Ioannidis et al., 2001; Nielsen et al., 2000).

Vertebrates contain three closely related IMP paralogues, which has hampered functional analysis, whereas *Drosophila* contains a single IMP orthologue with four well-conserved KH domains, allowing the genetic analysis of IMP function (Nielsen et al., 2000). IMP was found to bind directly to *oskar* and *gurken* mRNAs and localise with them to the posterior and dorsal sides of the oocyte, respectively (Geng and Macdonald, 2006; Munro et al., 2006). Although the IMP-binding sites are required for *oskar* mRNA translation and anchoring, loss of IMP has no obvious phenotype, suggesting that it functions redundantly with other proteins in the germ line. IMP is strongly expressed in the developing nervous system and RNAi knockdown causes neuronal loss and axon-pathfinding defects and a reduced number of boutons at the neuromuscular junctions (Boylan et al., 2008; Koizumi et al., 2007). *imp* mutant clones in the developing adult brain cause similar defects in axon elongation in mushroom body neurons, at least in part through IMP's role in regulating the localisation of *chic* mRNA (Medioni et al., 2014). These neural phenotypes may be related to IMP's function as temporal identify factor that acts in opposition to Syncrip to specify early-born neuronal fates and to promote neuroblast proliferative capacity (Liu et al., 2015; Narbonne-Reveau et al., 2016). IMP also acts as part of a temporal programme that controls the aging of the testis hub cells. IMP protects *unpaired* mRNA from repression by miRNAs in these cells and, as IMP levels fall with age, Unpaired signalling, which maintains the male germline stem cells, declines, leading to stem cell loss (Toledano et al., 2012).

Here, we analyse the function of IMP during the development of the somatic follicle cells of the *Drosophila* ovary and show that it also controls the temporal programme of development in this tissue. Unlike other well-characterised roles of IMP, we find that IMP functions independently of the microRNA pathway to regulate the timing of Delta/Notch signalling.

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RESULTS

IMP is required for proper timing of Notch signalling in follicle cells

To investigate the role of IMP in the follicle cell layer, we generated clones that were homozygous for the null allele *imp*^{7}, marked by the loss of RFP (Munro et al., 2006). The mutant cells showed no phenotypes during early oogenesis up until the end of stage 6. However, phalloidin staining of actin revealed that mutant cells at later stages were smaller in size and were more densely packed than the surrounding wild-type cells (Fig. 1A,A′). We observed the same phenotype with a second null allele, *imp*^{8} (Munro et al., 2006). *imp* mutant cells also have smaller nuclei (Fig. 1A,C,C′). The size and number of follicle cells is determined by the timing of the mitotic-to-endocycle transition, which takes place at stage 6, when the germ cells in the egg chamber produce the DSL ligand Delta to activate the Notch pathway in the follicle cells (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). Analysis of 56 *imp* mutant clones revealed that there are twice as many mutant cells in each clone than there are wild-type cells in the twin spot clone induced at the same time (Fig. 1B). Thus, *imp* mutant cells go through one extra round of mitosis, suggesting that Delta/Notch signalling is delayed.

Notch activation controls both the mitosis-to-endocycle switch and follicle cell differentiation. The late differentiation in the absence of IMP leads to delays in several other aspects of follicle cell development. For example, the lateral follicle cells move posteriorly to form a columnar epithelium around the oocyte during stages 8-9, but *imp* mutant cells do this more slowly and later than normal (Fig. 1C,C′). During stage 9 of oogenesis, the anterior-most follicle cells adopt the border cell fate, delaminate from the epithelium and migrate between the nurse cells to the anterior of the oocyte (Fig. 1D,E). When the entire border cell cluster is mutant for *imp*, the cells frequently fail to delaminate and remain at the anterior of the egg chamber, whereas those that do delaminate often only migrate part of the way to the oocyte (Fig. 1F,H). When the cluster contains both mutant and wild-type cells, the wild-type cells lead the migration with the mutant cells trailing behind (Fig. 1G). Thus, loss of IMP affects the timing of all aspects of follicle cell behaviour, suggesting that it plays a general role in this process. The localisation of IMP does not give any clues as it its function, however, as IMP protein is uniformly distributed throughout the cytoplasm of the follicle cells (Fig. S1A-A″).

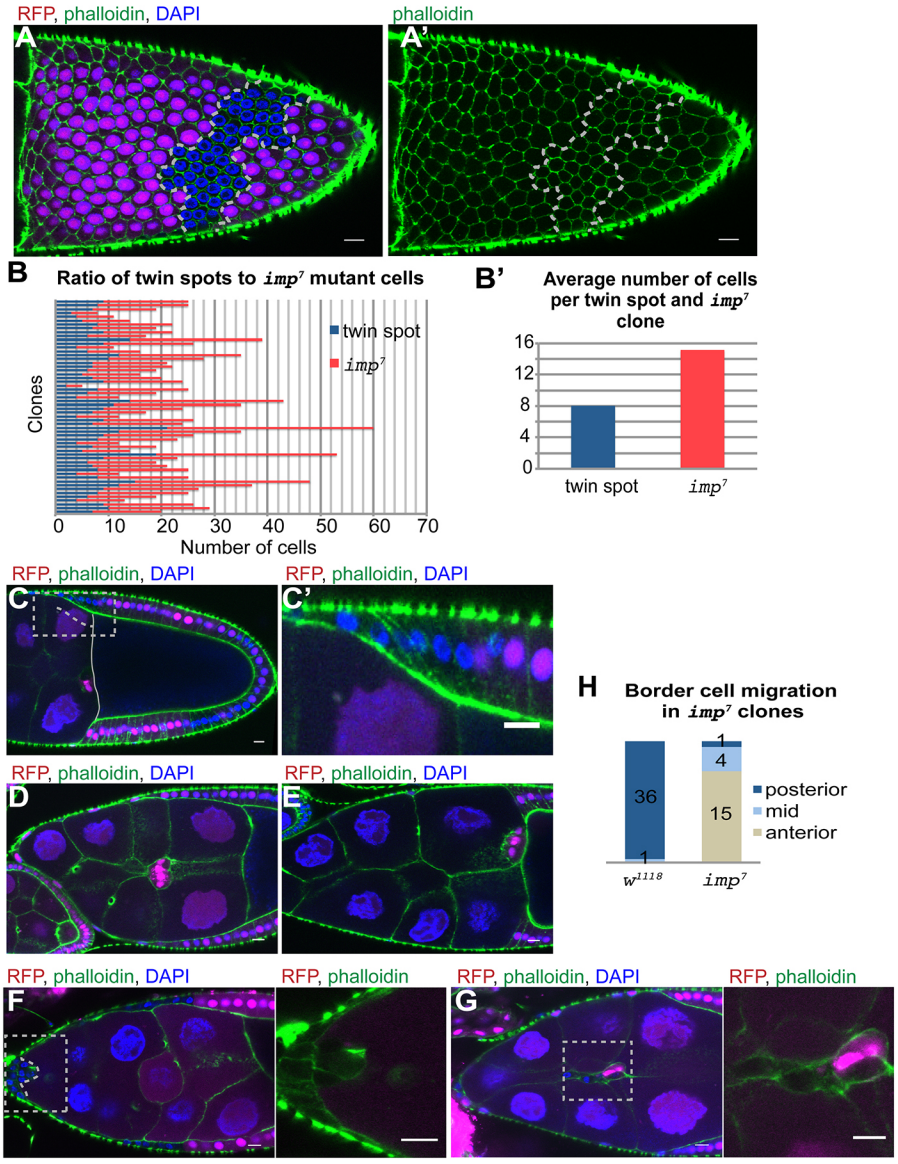

Fig. 1. *imp* mutant cells go through one extra division. (A) Surface view of a stage 10a egg chamber containing an *imp*^{7} mutant follicle cell clone (marked by the loss of RFP, magenta) stained with phalloidin (green) and DAPI (blue). The mutant cells are outlined in white. (B) Graph showing the number of wild-type and *imp*^{7} mutant cells in 56 independent twin spot clones. (B′) Histogram showing the average number of cells per wild-type clone and *imp*^{7} clone (*n*=56). (C,C′) Stage 10a chamber with an *imp*^{7} follicle cell clone that has not yet migrated posteriorly to envelop the oocyte (*n*=23). C′ shows magnification of the boxed area in C. Dashed line indicates mutant cells. (D,E) Wild-type stage 9 (*n*=12) (D) and stage 10b (*n*=21) (E) egg chambers showing the migration of the border cells between the nurse cells to reach the anterior of the oocyte at stage 10b. (F) Stage 9 egg chamber with an *imp*^{7} mutant clone that includes all of the border cells, which have failed to detach from the anterior (*n*=15). Image to the right is a magnification of the boxed area on the left. Dashed line indicates the border cell cluster. (G) Stage 9 egg chamber containing a mosaic of *imp*^{7} mutant and wild-type border cells. The mutant cells are found at the back of the cluster and trail behind the wild-type cells. The migration of these clusters is severely delayed and they often move only half way to the oocyte. Image to the right is a magnification of the boxed area on the left. (H) Quantification of the region to which wild-type border cell clusters and entirely mutant clusters have moved by stage 10b. Scale bars: 10 µm.

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To test whether IMP is required for the proper timing of Notch pathway activation in the follicle cells, we stained *imp* mutant clones for Cut and Hindsight (Hnt; also known as Pebbled). Cut is expressed from stages 1 to 6 of oogenesis and is downregulated at stage 7 in response to Notch activation (Sun and Deng, 2005). By contrast, Hnt is expressed only in post-mitotic cells that already received the Delta signal from the germ line (Sun and Deng, 2007). *imp* clones continue to express Cut at stage 7 in contrast to wild-type cells in the same egg chamber (Fig. 2A,A′). However, Cut expression is lost in the majority of mutant cells at stage 8 (Fig. 2B,B′). On the other hand, Hnt is not expressed in *imp* mutant cells at stage 7 as in wild type, but appears one stage later (Fig. 2C-D′). Both the continued expression of Cut and the late activation of Hnt in imp^7 MARCM (mosaic analysis with a repressible cell marker) clones are rescued by expressing UAS-IMP, indicating that these phenotypes are caused the loss of IMP (Fig. S2A-D′). Cut and Hnt both give a binary readout of whether Notch signalling has reached a threshold and we therefore examined the level of Notch signalling more directly by monitoring the expression

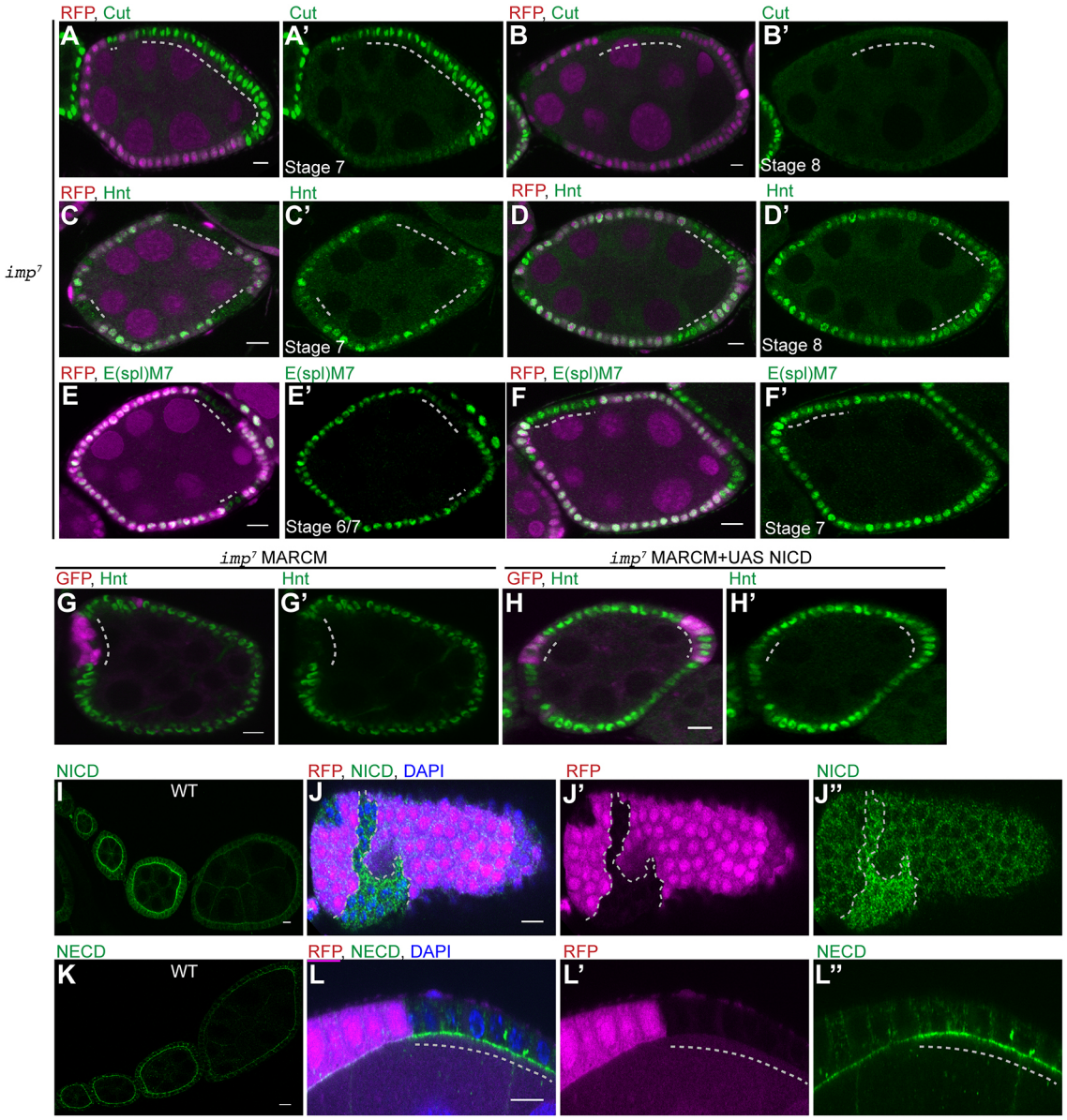

Fig. 2. IMP is required for the first cleavage of Notch. (A,A′) Cut expression in a stage 7 egg chamber containing an imp^7 follicle cell clone marked by the loss of RFP (magenta). Cut expression persists in the mutant cells (*n*=32). (B,B′) A stage 8 egg chamber containing an imp^7 follicle cell clone stained for Cut (*n*=41). Cut is lost from the majority of mutant cells. (C,C′) Hnt expression in a stage 7 egg chamber containing several imp^7 follicle cell clones marked by the loss of RFP. Hnt is expressed in the wild-type cells, but not in the mutant cells at stage 7 (*n*=28). (D,D′) A stage 8 egg chamber containing imp^7 follicle cell clones stained for Hnt. The mutant cells have turned on Hnt at this stage (*n*=47). (E-F′) E(spl)m7lacZ expression in stage 6/7 and stage 7 egg chambers containing imp^7 follicle cell clones marked by the loss of RFP. E(spl)m7lacZ is expressed at much lower levels in *imp* mutant cells than in wild-type cells at stage 6 (*n*=12), but at similar levels at stage 7 (*n*=21). (G,G′) A stage 7 egg chamber containing an imp^7 MARCM clone marked by the expression of GFP (magenta) and stained for Hnt (green). The mutant cells do not express Hnt at stage 7 (*n*=27). (H,H′) A stage 7 egg chamber containing an imp^7 MARCM clone expressing UAS-NICD stained for Hnt (green). Hnt expression is rescued by the expression of the NICD (*n*=21). (I-L″) Localisation of the NICD (I-J″) and the NECD (K-L″) in wild-type early-stage egg chambers and egg chambers containing *imp* mutant clones. Both NICD and NECD are enriched at the apical side of wild-type follicle cells until stage 6 when they are downregulated as a result of Delta signalling. NICD (J-J″; *n*=38) and NECD (L-L″; *n*=27) are not downregulated in imp^7 mutant cells (marked by the loss of RFP). Dashed lines indicate *imp* mutant clones. Scale bars: 10 µm.

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of the E(spl)m7lacZ reporter (Assa-Kunik et al., 2007). Staining for β-galactosidase revealed that *imp* mutant follicle cells show much weaker expression of the reporter than wild-type cells at stage 6, but express nearly normal levels by stage 7 (Fig. 2E-F′). As β-galactosidase is very stable, this suggests that Notch signalling is impaired but not abolished by the loss of IMP, so that it takes longer to reach the threshold for the follicle cell response to Delta signalling from the germline, resulting in the delay in follicle cell differentiation.

To test directly whether impaired Notch signalling is responsible for the *imp* phenotype, we used the MARCM system to express a constitutively active form of Notch, the Notch intracellular domain (NICD), in *imp* mutant cells (Go et al., 1998; Lee and Luo, 2001). Control *imp* mutant MARCM clones do not turn on Hnt at stage 7, but expressing NICD in the mutant cells restores timely Hnt expression (Fig. 2G-H′). This indicates that IMP controls Notch activity upstream of NICD production.

IMP acts at or before the first cleavage of Notch

Upon binding to its ligand, Delta, the extracellular domain of Notch is cleaved at the S2 site by the ADAM10 protease, Kuzbanian, to produce a transient form of the receptor, NEXT, which contains the transmembrane and intracellular domains of Notch (Lieber et al., 2002; Pan and Rubin, 1997). NEXT then undergoes a second cleavage at the S3 site mediated by the Presenilin/γ secretase complex (De Strooper et al., 1999; Struhl and Greenwald, 1999; Vaccari et al., 2008; Ye et al., 1999). This releases the NICD, which translocates to the nucleus to regulate transcription in association with Suppressor of Hairless protein [Su(H)] (Bray, 2016).

Staining with antibodies that detect the Notch intracellular and extracellular domains reveal that full-length Notch accumulates on the apical side of the follicle cells during early oogenesis and is then cleared from the membrane at stage 6, when signalling occurs (Fig. 2I,K). However, both antibodies detect high levels of Notch at stage 7-8 in *imp* mutant cells (Fig. 2J-J″,L-L″). The Notch extracellular domain is removed by the first cleavage and then disappears, perhaps because it is endocytosed with Delta into the signalling cell, as in other tissues (Langridge and Struhl, 2017; Nichols et al., 2007; Parks et al., 2000). The persistence of the extracellular domain signal in the mutant cells therefore indicates that loss of IMP inhibits Notch signalling before or at the first Kuzbanian-dependent cleavage of the Notch extracellular domain (NECD).

To test whether IMP is more generally involved in Notch signalling, we generated mutant clones in the wing imaginal disc. Notch activity is required for Cut expression in two rows of cells along the dorsal-ventral midline of the wing disc (Fig. 3A) (Micchelli et al., 1997). Large *imp* mutant clones that include the dorsal-ventral boundary have no effect on Cut expression (Fig. 3B,B′). Furthermore, the Notch signalling reporter, NRE-GFP (Housden et al., 2012) (Fig. 3C), is expressed at the same level in *imp* mutant and wild-type cells, indicating that the loss of IMP does not impair or delay Notch signalling in this tissue (Fig. 3D,D′). Consistent with this, adult wings containing *imp* clones have a normal bristle pattern and never show any of the wing-notching characteristic of *Notch* mutants, although they have some wing venation defects. *imp* null mutant clones in the eye imaginal disc also showed no phenotype, with the cone cells being specified normally as shown by Cut expression (Tomlinson et al., 2011) (Fig. 3E-E″). Thus, IMP seems to be specifically required for Notch activation in the ovary and is not a general component of the Notch signalling pathway.

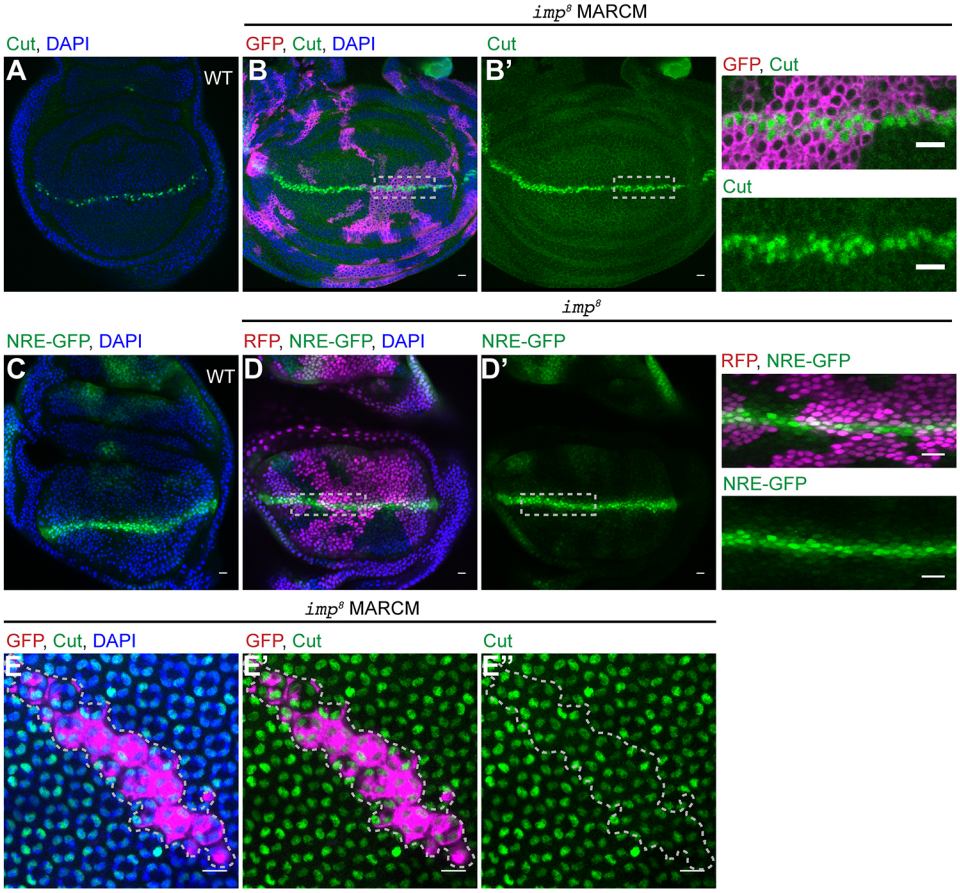

Fig. 3. IMP is not a general component of the Notch signalling pathway. (A-B′) Third larval instar wing imaginal discs stained for Cut (green). B shows a disc containing *imp*^{8} MARCM clones marked by GFP expression (magenta). Cut is expressed normally in mutant cells along the dorsal-ventral compartment boundary (*n*=49). Images to the right are magnifications of the boxed areas in B and B′. (C) Third larval instar wing imaginal disc expressing the NRE-GFP reporter. (D,D′) A wing disc containing *imp*^{8} clones at the dorsal ventral boundary. NRE-GFP is expressed at similar levels in *imp*^{8} mutant and wild-type cells (*n*=16). Images to the right are magnifications of the boxed areas in D and D′. (E-E″) 50 h pupal eye disc containing *imp*^{8} MARCM clones marked by GFP expression (magenta). Clone area is outlined by dashed lines. Mutant cone cells are indistinguishable from wild type and express the cone cell marker Cut (green; *n*=19). Scale bars: 10 µm.

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IMP does not act through the microRNA pathway

In addition to binding to Notch in trans to activate its cleavage and signalling, Delta expressed in the same cell can bind to Notch in cis to inhibit signalling (Cordle et al., 2008; de Celis and Bray, 1997; Micchelli et al., 1997; Miller et al., 2009; Sakamoto et al., 2002). Indeed cis-inhibition by Delta expressed in the follicle cells controls their competence to respond to Delta from the germ line, as *Delta* mutant follicle cell clones switch from mitosis to the endocycle too early and undergo precocious differentiation (Poulton et al., 2011). This inhibition is regulated by the microRNA pathway, which represses Delta expression in the follicle cells to relieve cis-inhibition at stage 6. Mutants in the conserved components of the microRNA pathway, *belle* and *Dicer-1*, therefore cause a delay in follicle cell development similar to that observed in *imp* mutants (Poulton et al., 2011). As IMP is an RNA-binding protein that modulates the miRNA pathway in other contexts (Toledano et al., 2012), this raises the possibility that it is required for the miRNA-dependent repression of Delta in the follicle cells.

We compared the phenotypes of *belle* and *Dicer-1* mutants with that of *imp* to confirm that they cause a similar inhibition of Notch activation. Like *imp* mutants, *belle* null mutant follicle cells go through one extra division and border cell migration is disrupted (Fig. 4A-C″). Furthermore, *belle* and *Dicer-1* mutant cells accumulate uncleaved Notch at their apical surfaces, as shown by the persistence of staining with antibodies against the NECD and NICD (Fig. 4D-E″; Fig. S3A-B″). If IMP functions in the same microRNA pathway as Belle and Dicer-1, cells carrying null mutations in two of these genes should show a delay in follicle cell differentiation identical to that observed in *imp*, *belle* and *Dicer-1* single-null mutants, as the removal of a second essential component of the pathway should have no further effect. On the other hand, one would expect an additive effect if IMP functions in a parallel pathway. We therefore generated *imp* null mutant clones marked by the loss of RFP and *belle* or *Dicer-1* null mutant clones marked by the loss of GFP in the same egg chambers. This allowed us to compare directly the phenotypes of cells mutant for either gene with the double mutant cells that express neither GFP nor RFP. Although the single-mutant clones produced the expected phenotypes, the double-mutant cells showed a more severe delay in Notch activation, as illustrated by their continued expression of Cut after the single mutant cells had lost expression (Fig. 4F-F‴; Fig. S3C-C‴). This suggests that IMP functions in a different pathway from Belle and Dicer-1 to control the timing of Notch activation in the follicle cells.

imp is epistatic to *Delta*

Although IMP does not appear to function in the microRNA pathway, it could still control Notch activation by repressing Delta

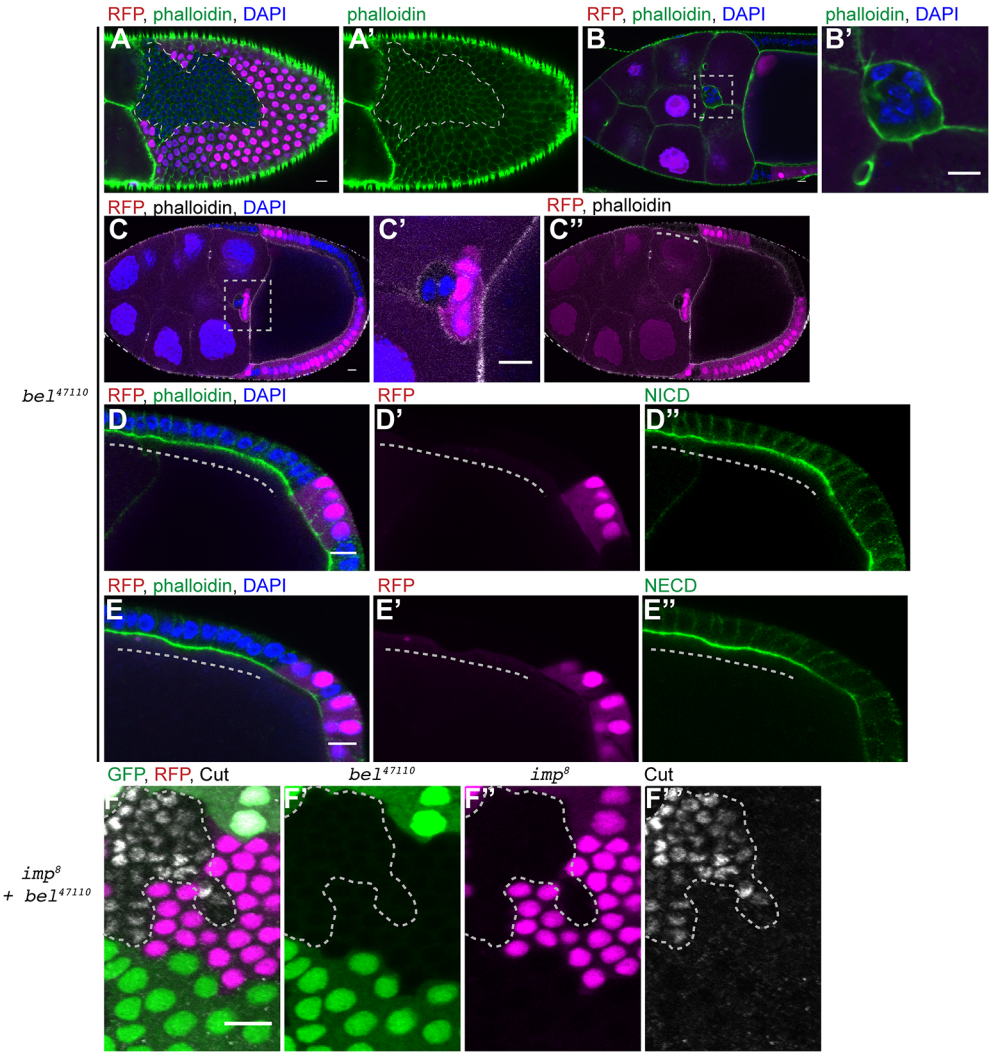

Fig. 4. IMP does not act through the microRNA pathway. (A-C″) Stage 10a egg chambers containing *belle*^{47110} mutant clones marked by the loss of RFP. The mutant cells show a similar phenotype to that observed in *imp* mutants. They go through one extra round of division and are therefore smaller than the wild-type cells (A,A′; *n*=19). When all of the border cells are mutant for *belle*, there is a delay in border cell migration (B,B′; *n*=11). In mosaic border cell clusters, the mutant cells lag behind the wild-type border cells (C-C″). The migration of mutant follicle cells to envelop the oocyte is also delayed (white dashed line in C″; *n*=17). B′ and C′ are magnifications of the boxed areas in B and C, respectively. (D-E″) Stage 9 egg chambers containing *belle*^{47110} mutant clones marked by the loss of RFP stained for NICD (D-D″) and NECD (E-E″). The mutant cells retain high levels of NICD (D-D″; *n*=15) and NECD (E-E″; *n*=13) at their apical membranes. Dashed lines indicate mutant cells. (F-F‴) A stage 9 egg chamber containing both *imp*^{7} mutant clones marked by the loss of RFP (magenta) and *belle*^{47110} mutant clones marked by the loss of GFP (green), stained for Cut (white). Cut is still expressed in the double-mutant cells (marked by the dashed line), but not in the single-mutant cells (*n*=24). Scale bars: 10 µm.

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expression in the follicle cells to relieve cis-inhibition. If this is the case, *Delta* should be epistatic to *imp*, with double-mutant clones showing the *Delta* phenotype of early differentiation and exit from the cell cycle. We first confirmed that *Delta* clones cause premature differentiation of the follicle cells and observed that mutant cells have larger nuclei, indicating premature entry into endocycle (Fig. 5A,A′). Furthermore, *Delta* mutant cells turn off Cut expression before stage 6, unlike wild-type cells, indicating that the cells lacking Delta differentiate precociously (Fig. 5B,B′).

To examine the epistatic relationship between *Delta* and *imp*, we generated clones of null mutants in each gene in the same egg chambers, marked by the loss of GFP and RFP, respectively. This revealed that *Delta imp* double-mutant cells show the same delay in Notch signalling as *imp* single-mutant cells (Fig. 5C-E‴). Double-mutant clones express Cut protein at stage 6/7, as do neighbouring wild-type cells, whereas *Delta* single-mutant clones have already turned Cut off at this stage (Fig. 5D-D‴). Moreover, Hnt expression is not switched on prematurely in *Delta imp* clones, as it is in *Delta* clones (Fig. 5C-C‴). More importantly, like single *imp* mutant clones, the *Delta imp* double-mutant clones express Cut longer than wild-type cells (Fig. 5E-E‴). To confirm this result, we also examined whether IMP regulates *Delta* mRNA stability or translation, using a sensor line that contains the 3′UTR of *Delta* downstream of the GFP coding sequence (Poulton et al., 2011). However, *imp* clones expressed the same level of GFP from the *Delta* sensor as wild-type cells (Fig. S4). The observation that the *imp* mutants are epistatic to *Delta* mutants in the follicle cells indicates that IMP regulates the timing of Notch activation independently of and in parallel with Delta cis-inhibition.

Kuzbanian is indispensable for Notch pathway activation in follicle cells

Because IMP is only required in the follicle cells, it cannot affect the expression of Delta in the germ line and must therefore affect either the ability of Notch to bind to Delta in trans or the first Delta-dependent cleavage of Notch at the S2 site, which is mediated by the ADAM family metalloprotease Kuzbanian (Lieber et al., 2002; Pan and Rubin, 1997). To confirm that Kuzbanian is required for Notch signalling in the follicle cells, we generated clones of an amorphic allele, *kuz*$^{e29\text{-}4}$ (Rooke et al., 1996). *kuz* mutant follicle cells show an identical phenotype to null mutations in Notch: mutant cells continue dividing after stage 6, leading to an increase in cell number and a decrease in cell size, and fail to differentiate, as they continue to express Cut and never turn on Hnt (Fig. 6A-B′). Staining for the extracellular and intracellular domains of Notch showed that *kuz* mutant cells maintain high levels of NICD and NECD at the follicle cell apical membrane, consistent with its role in the first cleavage of Notch, a phenotype that resembles that of *imp* (Fig. 6C-D′).

As *kuz* and *imp* mutants impair the same step in Notch activation, we investigated whether the *imp* phenotype results from a deficit in Kuzbanian activity by overexpressing Kuzbanian in *imp* mutant cells using the MARCM system (Lee and Luo, 2001). Control *imp* MARCM clones continue to express Cut and not Hnt at stage 7, as expected (Fig. 6E,E′,G,G′). By contrast, expression of Kuz

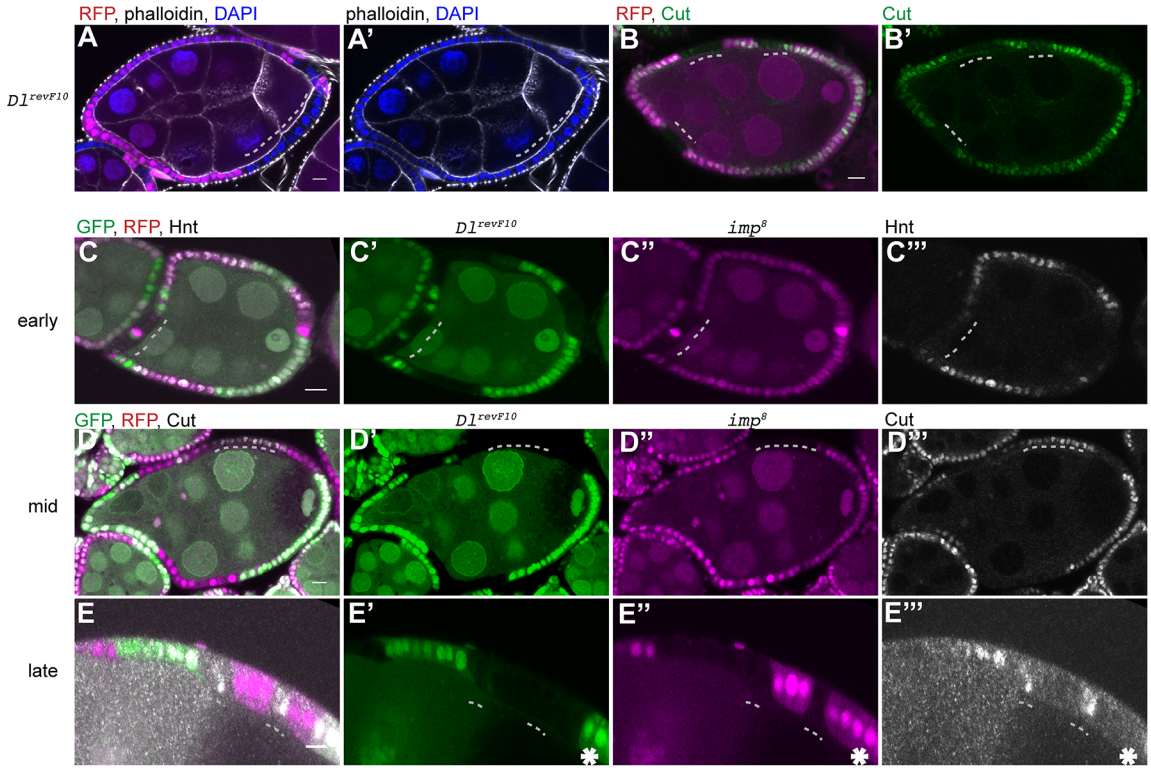

Fig. 5. *imp* is epistatic to *Delta* in follicle cells. (A-B′) Stage 6 egg chambers containing *Dl*^{revF10} mutant cells marked by the loss of RFP. The mutant cells are larger than wild type and have bigger nuclei, indicating that they have undergone the switch from mitosis to endoreplication prematurely (A,A′; *n*=18). The *Dl* mutant cells switch off Cut expression (green) before the wild-type cells (B,B′; *n*=17). Dashed lines indicate mutant cells. (C-E‴) Egg chambers containing both *Dl*^{revF10} clones marked by the loss of GFP (green) and *imp*^{8} mutant clones marked by the loss of RFP (magenta). (C-C‴) *imp Dl* double-mutant cells do not express Hnt (white) at stage 6/7, whereas *Dl* mutant cells do (*n*=34). (D-D‴) *imp Dl* double mutant cells still express Cut (white) at stage 6, unlike *Dl* mutant cells (*n*=56). (E-E‴) *imp Dl* double-mutant cells (marked by the dashed lines) still express Cut at stage 7, in contrast to wild-type cells (marked with an asterisk; *n*=23). Scale bars: 10 µm.

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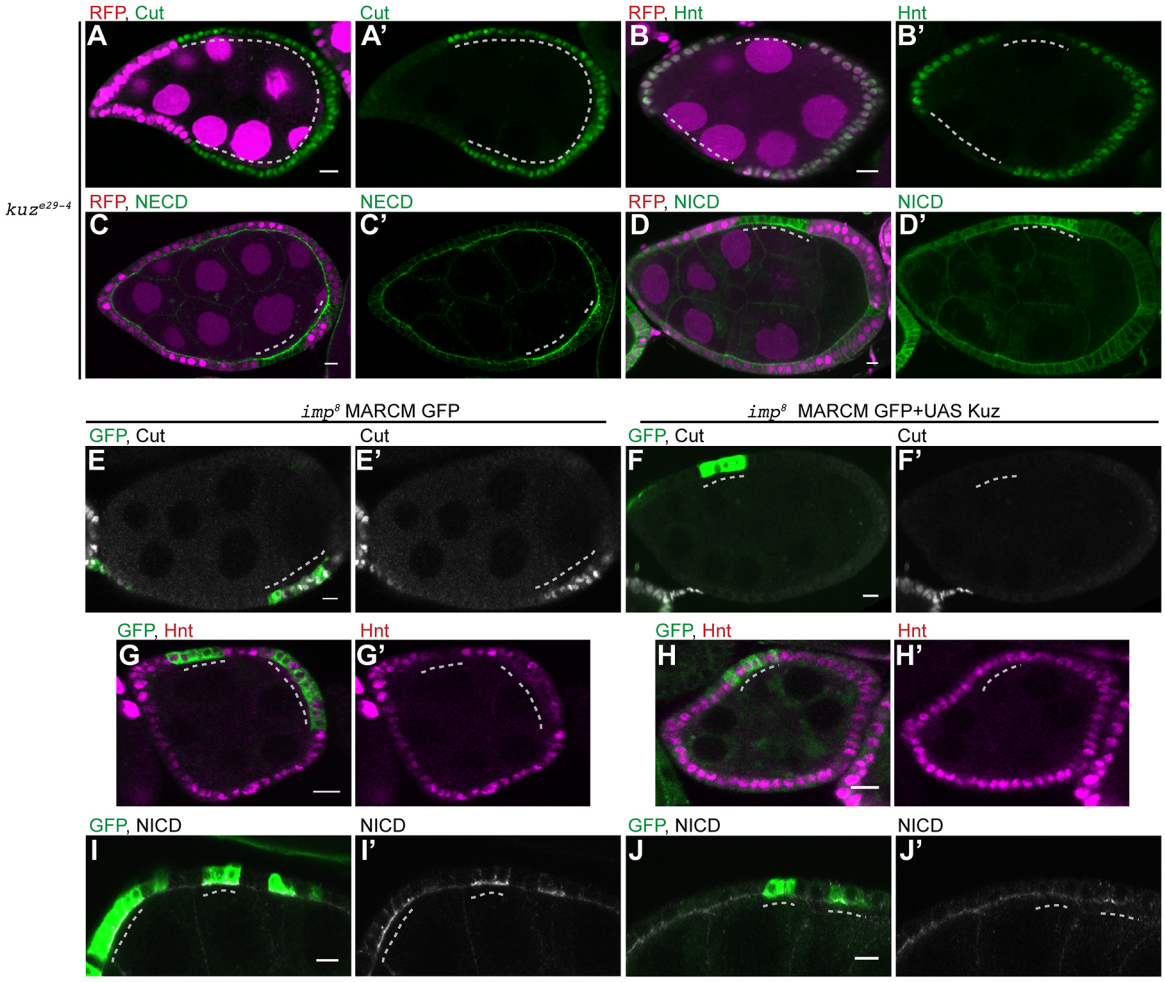

Fig. 6. Kuzbanian is required for Notch activation in the follicle cells. (A-D′) Egg chambers containing *kuz*$^{e29-4}$ mutant cells marked by the loss of RFP. The *kuz* mutant cells continue to express Cut at stage 7 (A,A′; *n*=17) and do not express Hnt (B,B′; *n*=16). Loss of Kuz leads to the persistence of high levels of NECD (C,C′; *n*=12) and NICD (D,D′; *n*=15) at the apical membrane of the follicle cells after stage 6. (E-J′) *imp*^{8} MARCM clones marked by the expression of GFP (green) without any additional transgenes (E,G,I) or with UAS- Kuz (F,H,J). Kuzbanian expression in *imp* mutant cells restores the timely repression of Cut (F,F′; *n*=37) and activation of Hnt (H,H′; *n*=43), in contrast to control *imp* mutant cells at stage 7 (E,E′,G,G′). Control *imp* mutant clones retain high levels of NICD at the apical membrane of the follicle cells after stage 6 (I,I′), whereas NICD is downregulated in *imp* mutant cells expressing Kuzbanian, as in wild-type cells (J,J′; *n*=46). Dashed lines indicate mutant cells. Scale bars: 10 µm.

eliminates the delay in Cut repression and restores timely Hnt expression (Fig. 6F,F′,H,H′). Furthermore, although Notch remains at high levels in the apical plasma membrane of *imp* mutant cells at stage 7, it disappears on schedule in mutant cells overexpressing Kuzbanian, as it does in wild type (Fig. 6I-J′). Thus, increasing the levels of Kuzbanian rescues the Notch signalling defect in *imp* mutant follicle cells, suggesting that IMP is required for normal Kuzbanian activity. As a control, we also overexpressed Fringe, which modifies Notch to make it more responsive to Delta (Brückner et al., 2000; Goto et al., 2001; Moloney et al., 2000). However, Fringe overexpression has no effect on the *imp* mutant phenotype (Fig. S6). The observation that the delay in Notch signalling in *imp* mutants is not rescued by the overexpression of another component that acts upstream of Notch cleavage reveals that rescue is specific to Kuzbanian overexpression.

IMP is required for the apical accumulation of Kuzbanian

Because no anti-Kuz antibodies are available, we took advantage of a GFP-tagged *kuzbanian* BAC transgene that rescues the lethality of *kuz* mutants (Dornier et al., 2012). Kuz-GFP is expressed at very low levels in the follicle cells, with the highest expression at stages 5-6. Live imaging of egg chambers with two copies of this transgene revealed that Kuz-GFP is enriched at the apical side of the follicle cells and in intracellular speckles (Fig. 7A-A″). This weak apical enrichment is reduced or lost in the majority of *imp* mutant cells (63% of 38 clones; Fig. 7B,B′). Instead, most mutant cells show a marked increase in the size and brightness of the speckles, with 56% of clones containing large intracellular foci (Fig. 7C-D′). Unlike the signal at the apical membrane, the intracellular foci of Kuzbanian remain intact after fixation, which allowed us to stain for markers for different vesicular compartments. The Kuzbanian-positive punctae showed the strongest colocalisation with Rab7 (Fig. 7E-F″) (34/35 punctae), a marker for late endosomes, whereas they showed minimal colocalisation with the Golgi marker GM130 (2/20) (Fig. 7G-G″). The formation of these endosomal foci of Kuz-GFP is not a consequence of impaired Notch signalling, because they did not form in *bel* mutant clones (Fig. S5A-A″), which show a very similar phenotype to *imp* mutants, or in follicle cells adjacent to *Delta*^{revF10} germline clones, in which Notch signalling does not occur (Fig. S5B-C′). These results suggest that loss of IMP disrupts the intracellular trafficking of Kuzabanian, leading to a reduction in its levels at the apical plasma membrane, where Notch cleavage occurs.

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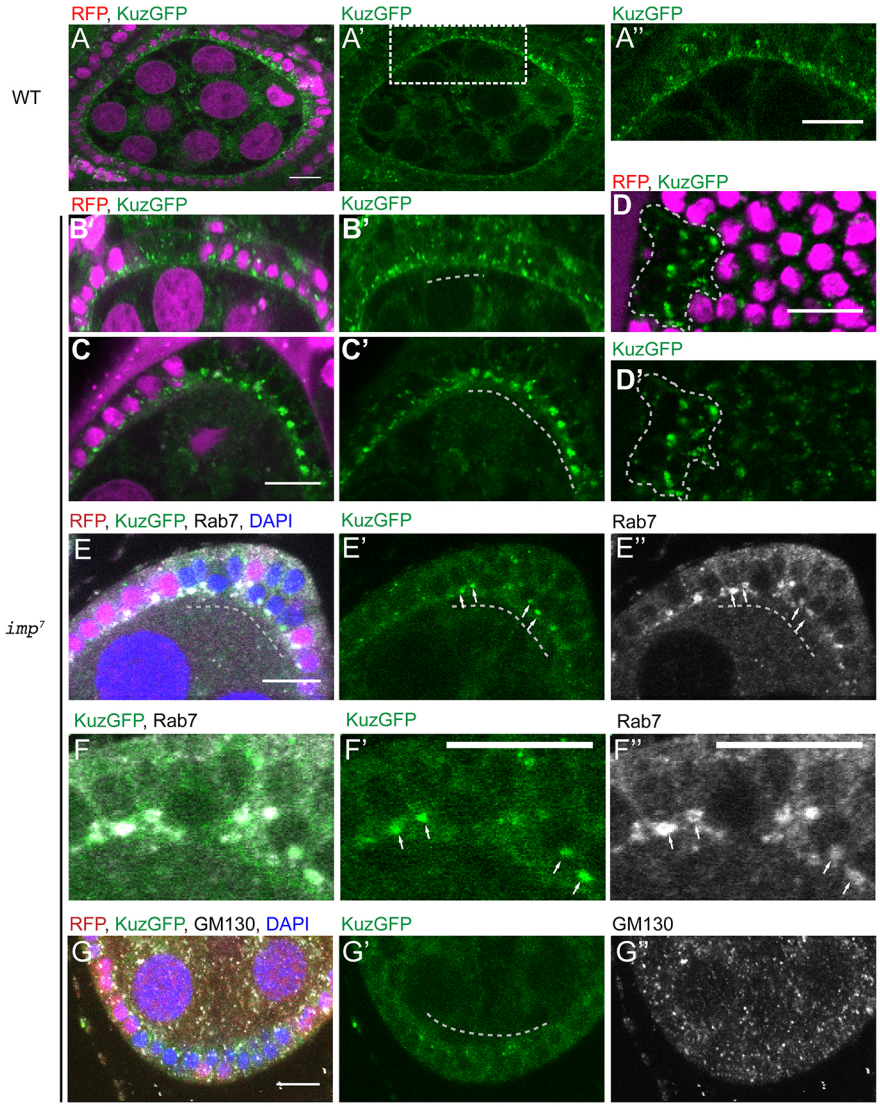

Fig. 7. Loss of IMP disrupts Kuzbanian localisation. (A-D′) Live egg chambers from females carrying two copies of a Kuz-GFP BAC transgene. (A,A′) Kuzbanian-GFP localises to the apical membrane of the follicle cells and to intracellular punctae (n=68). (B-D′) *imp*^{7} mutant cells marked by the loss of RFP (magenta) show a decrease in the amount of Kuz-GFP at the apical membrane (B,B′; n=38) and an increase in the Kuz-GFP found in bright intracellular foci (C-D′; n=27). (E-F″) The large intracellular Kuz-GFP foci colocalise with Rab7 (white in E,E″,F,F″; n=35), a marker for late endosomes, but do not colocalise with the Golgi marker, GM130 (white in G,G″; n=20). Dashed lines indicate *imp* mutant cells. Scale bars: 10 µm.

DISCUSSION

Although Delta/Notch signalling uses a very simple signal transduction pathway, its activity can be regulated at several levels to control the timing and direction of signalling in a context-dependent manner. Factors controlling the endocytosis of Notch, such as Numb, can determine the direction of signalling, whereas the ubiquitin ligases that induce Delta endocytosis, Neuralized and Mind bomb, determine where and when signalling occurs (Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001, 2005; Le Borgne et al., 2005; Pavlopoulos et al., 2001). Whether cells respond to a particular DSL ligand can also be regulated by the glycosylation of the NECD by the Fringe family of proteins, making *Drosophila* Notch more responsive to Delta and less responsive to Serrate (Brückner et al., 2000; Goto et al., 2001; Moloney et al., 2000). Indeed, the modification of Notch by Fringe in the polar/stalk follicle cell precursors renders these cells more responsive to Delta than the other follicle cells, thereby restricting polar cell fate to the ends of the early egg chamber (Grammont and Irvine, 2001). Finally, signalling can be modified by cis-inhibition by DSL ligands, which impede Notch interactions with activating ligands in trans. This mechanism plays a role in controlling the timing of follicle cell differentiation until Delta is downregulated in the follicle cells by the microRNA pathway (Poulton et al., 2011). Here, we present evidence for a new mechanism that regulates the activity of the Notch pathway through the localisation of the ADAM metalloprotease Kuzbanian.

Our results show that mutants in the RNA-binding protein IMP have impaired Delta/Notch signalling in mid-oogenesis, which results in one extra round of follicle cell division. This leads to a delay in follicle cell differentiation that is likely to be the cause of some of the later *imp* mutant phenotypes, which include impaired border cell delamination, a delay in the formation of the columnar epithelium around the oocyte and delayed migration of the centripetal follicle cells. Continuing Delta-Notch signalling has also been found to be required for border cell migration after the cells have been specified, and this correlates with higher levels of Kuzbanian expression (Wang et al., 2007). Thus IMP may also play a role in the later Notch signalling that is needed for normal border cell migration.

Mutants in the microRNA pathway give a very similar phenotype to *imp* because of a failure to repress Delta translation, but our results show that IMP functions in parallel to this pathway. Firstly, double mutants between *imp* and *belle* or *Dicer-1* show an additive delay in follicle cell differentiation. Secondly, *imp* mutants are epistatic to *Delta* mutants, indicating that IMP does not function by relieving cis-inhibition. Instead, we find that the delay in Notch

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signalling can be efficiently rescued by overexpression of Kuzbanian. Furthermore, loss of IMP disrupts the enrichment of Kuzbanian at the apical plasma membrane, which is where Notch must be cleaved for signalling to occur. Thus, IMP is required in some way for the localisation of Kuzbanian to the site where germline Delta binds to Notch in trans to trigger the first cleavage, identifying a new regulatory step in the Notch pathway.

The loss of IMP does not disrupt other Delta/Notch signalling processes, such as the formation of the dorsal ventral boundary in the wing, indicating that it is not a general component of the pathway. This raises the question of why IMP is specifically required in the follicle cells. One possibility is that this relates to the different geometry of the Delta/Notch interaction in the follicle cells compared with other signalling events. Most examples of Delta/Notch signalling occur between adjacent cells in epithelial tissues, where Delta and Notch can only interact at the lateral membrane, usually at the level of the adherens junctions (Andersson et al., 2011; Bray, 2016; de Celis et al., 1996; Kidd et al., 1989; Micchelli and Blair, 1999; Woods and Bryant, 1993). By contrast, the germ cells of the egg chamber, which produce the activating Delta signal, contact the apical side of the follicle cells, and both Notch and Kuzbanian therefore need to be localised apically for signalling to occur. Notch localisation is not affected in *imp* mutants, however, as it accumulates at high levels at the apical side of mutant cells. Thus, IMP appears to disrupt Kuzbanian localisation specifically rather affecting apical trafficking more generally.

As IMP is a cytoplasmic RNA-binding protein, it presumably acts by regulating the stability, translation or localisation of specific mRNAs. One possibility is that IMP acts on *kuz* mRNA directly. Indeed, we have observed by qPCR that *kuz* RNA is enriched in immunoprecipitations of IMP (data not shown), although we cannot detect the mRNA by fluorescent *in situ* hybridisation, presumably because it is present at very low levels. It seems unlikely that IMP regulates the translation of *kuz* mRNA, as Kuzbanian protein levels do not appear to change in *imp* mutants. There is also no evidence for a role of IMP in the localisation of *kuz* mRNA, as IMP itself is not localised and Kuzbanian is a secreted transmembrane protein that must be translated at the endoplasmic reticulum and trafficked to the cell surface through the Golgi complex. One possibility is that IMP controls Kuzbanian localisation through 3′UTR-dependent protein localisation in a similar way to that in which human HuR (ELAVL1) binds to the long 3′UTR of CD47 mRNA to recruit SET protein, which then facilitates CD47 protein trafficking to the cell surface (Berkovits and Mayr, 2015). In this scenario, IMP binding to the 3′UTR of *kuz* mRNA would recruit a co-factor, which then associates with the cytoplasmic domain of Kuzbanian protein to direct its trafficking to the apical plasma membrane.

It seems more likely that IMP enhances the translation or stability of another mRNA that encodes a factor that either directs the apical trafficking of Kuzbanian or anchors it in the apical plasma membrane. Possible candidates include the TspC8 Tetraspanins, Tsp86D and Tsp3A, which have been shown to enhance the trafficking of Kuzbanian to the cell surface in S2 cells and in the migrating border cells (Dornier et al., 2012). However, the accumulation of Kuzbanian protein in Rab7-positive late endosomes in *imp* mutant cells suggests that Kuzbanian is being endocytosed from the apical membrane in the absence of IMP, arguing that the phenotype results from the loss of a factor that stabilises Kuzbanian at the membrane and prevents its endocytosis, rather than a factor that facilitates its delivery there. Various cross-linking approaches, such as RIPseq, i-CLIP, PAR-iCLIP and eCLIP have shown that IMPs bind to hundreds to thousands of mRNAs, predominantly in their 3′ UTRs (Conway et al., 2016; Hansen et al., 2015). IMP-binding sites are particularly enriched in mRNAs encoding cytoskeletal components and trafficking factors, many of which are plausible candidates for the relevant target of IMP in regulating Kuzbanian localisation and Notch signalling in the follicle cells.

MATERIALS AND METHODS

Drosophila mutant stocks and transgenic lines

We used the following mutant alleles and transgenic constructs: imp^{7} and imp^{8} (Munro et al., 2006), GFP-IMP trap line 126.1 (a gift from Alain Debec, University Paris Diderot, Paris, France; Morin et al., 2001), UAS-NICD (Go et al., 1998; a gift from S. Bray, University of Cambridge, Cambridge, UK), bel^{47110} and Dl-3′UTR sensor (Poulton et al., 2011), $Delta^{revF10}$ and $Delta^{M1}$ (Sun and Deng, 2005; a gift from S. Bray), $Dicer\text{-}1^{Q1147X}$ (Lee et al., 2004; a gift from A. Brand, The Gurdon Institute, Cambridge, UK), Kuz-GFP and $kuz^{e29\text{-}4}$ (Dornier et al., 2012; Rooke et al., 1996), UAS-kuz (Sotillos et al., 1997; Kyoto Stock Center 108440), E(spl)M7-lac (a gift from Sarah Bray; Assa-Kunik et al., 2007) and NRE-GFP (a gift from Sarah Bray; Housden et al., 2012). The following stocks were used to generate mitotic clones: ubiRFP-nls, hsflp, FRT19A (BDSC 31418), FRT40A ubiRFP-nls (BDSC 34500), FRT82B, ubiRFP-nls (BDSC 30555) and FRT82B ubiGFP (BDSC 5188). MARCM, following the method of Lee and Luo (2001), was carried out using UAS-GFP-mCD8 (Lee and Luo, 1999) as the marker.

Reagents

The following antibodies were used: mouse anti-Cut [Blochlinger et al., 1990; Developmental Studies Hybridoma Bank (DSHB), 2B10], mouse anti-Hnt (Yip et al., 1997; DSHB, 1G9), mouse anti-NICD (Fehon et al., 1990; DSHB, C17.9C6), mouse anti-NECD (Diederich et al., 1994; DSHB, C458.2H). All primary antibodies from DSHB were used at a dilution of 1:100. Anti-GM130 was purchased from Abcam (Sinka et al., 2008; ab30637) and used at 1:500. Anti-Rab7 (rabbit) was kindly provided by the Nakamura lab and used at 1:1000 (Tanaka and Nakamura, 2008). AlexaFluor 488- and AlexaFluor 647-conjugated secondary antibodies were purchased from Jackson ImmunoResearch and used at a dilution of 1:1000 (115-545-003, 711-545-152, 115-607-020 and 111-605-003). F-actin was stained with Alexa Fluor 568 or Alexa Fluor 647 phalloidin (Invitrogen) at 1:1000. Ovaries were mounted in Vectashield with DAPI (Vector Laboratories). The cell membranes were labelled with CellMask Orange Plasma Membrane Stain or CellMask Deep Red Plasma Membrane Stain (Thermo Fisher Scientific).

Immunostaining

Ovaries from adult flies or imaginal wing discs from third instar larvae were dissected in PBS and fixed for 20 min in 4% paraformaldehyde and 0.2% Tween 20 in PBS. The tissues were then incubated in 10% bovine serum albumin (BSA) in PBS to block for 1 h at room temperature. The incubation with primary antibody was performed at 4°C overnight in PBS, 0.2% Tween 20 and 1% BSA.

Immunostaining on pupal eye discs was performed as described by Richard et al. (2006).

Imaging

Fixed preparations were imaged using an Olympus IX81 (40×/1.3UPlan FLN Oil or 60×/1.35 UPlanSApo Oil). Live imaging was performed using a Leica SP8 (63×/1.4 HCX PL Apo CS Oil) or Olympus IX81 (40×/1.3 UPlan FLNOil or 60×/1.35 UPlanSApo Oil) inverted confocal microscope. For live observations, ovaries were dissected and imaged in 10S Voltalef oil (VWR Chemicals).

Drosophila genetics

Follicle cell clones of *imp*, *Dl*, *bel*, *Dcr-1* and *kuz* were induced by incubating larvae or pupae at 37°C for 2 h every 12 h over a period of at least 3 days. Adult females were dissected at least 2 days after the last heat shock. Wing imaginal disc clones and eye imaginal disc clones of *imp* were induced by heat shocking first and second instar larvae for 30 min per day over a period of 2 days. Larvae were dissected at least 1 day after the last heat shock.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: W.F., D.S.J.; Methodology: W.F.; Validation: W.F.; Formal analysis: W.F., C.F.; Investigation: W.F., C.F.; Data curation: W.F.; Writing - original draft: W.F.; Writing - review & editing: D.S.J.; Supervision: D.S.J.; Project administration: D.S.J.; Funding acquisition: D.S.J.

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Supplementary information
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References

Andersson, E. R., Sandberg, R. and Lendahl, U. (2011). Notch signaling: simplicity in design, versatility in function. *Development* **138**, 3593-3612.
Assa-Kunik, E., Torres, I. L., Schejter, E. D., St Johnston, D. and Shilo, B.-Z. (2007). Drosophila follicle cells are patterned by multiple levels of Notch signaling and antagonism between the Notch and JAK/STAT pathways. *Development* **134**, 1161-1169.
Berkovits, B. D. and Mayr, C. (2015). Alternative 3′ UTRs act as scaffolds to regulate membrane protein localization. *Nature* **522**, 363-367.
Blochlinger, K., Bodmer, R., Jan, L. Y. and Jan, Y. N. (1990). Patterns of expression of cut, a protein required for external sensory organ development in wild-type and cut mutant Drosophila embryos. *Genes Dev.* **4**, 1322-1331.
Boylan, K. L. M., Mische, S., Li, M., Marqués, G., Morin, X., Chia, W. and Hays, T. S. (2008). Motility screen identifies Drosophila IGF-II mRNA-binding protein–zipcode-binding protein acting in oogenesis and synaptogenesis. *PLoS Genet.* **4**, e36.
Bray, S. J. (2016). Notch signalling in context. *Nat. Rev. Mol. Cell Biol.* **17**, 722-735.
Brückner, K., Perez, L., Clausen, H. and Cohen, S. (2000). Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature* **406**, 411-415.
Conway, A. E., Van Nostrand, E. L., Pratt, G. A., Aigner, S., Wilbert, M. L., Sundararaman, B., Freese, P., Lambert, N. J., Sathe, S., Liang, T. Y. et al. (2016). Enhanced CLIP uncovers IMP protein-RNA targets in human pluripotent stem cells important for cell adhesion and survival. *Cell Rep.* **15**, 666-679.
Cordle, J., Johnson, S., Tay, J. Z. Y., Roversi, P., Wilkin, M. B., de Madrid, B. H., Shimizu, H., Jensen, S., Whiteman, P., Jin, B. et al. (2008). A conserved face of the Jagged/Serrate DSL domain is involved in Notch trans-activation and cis-inhibition. *Nat. Struct. Mol. Biol.* **15**, 849-857.
de Celis, J. F. and Bray, S. (1997). Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the Drosophila wing. *Development* **124**, 3241-3251.
de Celis, J. F., Garcia-Bellido, A. and Bray, S. J. (1996). Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc. *Development* **122**, 359-369.
De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J. et al. (1999). A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518-522.
Deblandre, G. A., Lai, E. C. and Kintner, C. (2001). Xenopus neuralized is a ubiquitin ligase that interacts with XDelta1 and regulates Notch signaling. *Dev. Cell* **1**, 795-806.
Degrauwe, N., Suvà, M.-L., Janiszewska, M., Riggi, N. and Stamenkovic, I. (2016). IMPs: an RNA-binding protein family that provides a link between stem cell maintenance in normal development and cancer. *Genes Dev.* **30**, 2459-2474.
Deng, W. M., Althauser, C. and Ruohola-Baker, H. (2001). Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in Drosophila follicle cells. *Development* **128**, 4737-4746.
Deshler, J. O., Highett, M. I. and Schnapp, B. J. (1997). Localization of Xenopus Vg1 mRNA by Vera protein and the endoplasmic reticulum. *Science* **276**, 1128-1131.
Diederich, R. J., Matsuno, K., Hing, H. and Artavanis-Tsakonas, S. (1994). Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. *Development* **120**, 473-481.
Dornier, E., Coumailleau, F., Ottavi, J.-F., Moretti, J., Boucheix, C., Mauduit, P., Schweisguth, F. and Rubinstein, E. (2012). TspanC8 tetraspanins regulate ADAM10/Kuzbanian trafficking and promote Notch activation in flies and mammals. *J. Cell Biol.* **199**, 481-496.
Farina, K. L., Hüttelmaier, S., Musunuru, K., Darnell, R. and Singer, R. H. (2003). Two ZBP1 KH domains facilitate beta-actin mRNA localization, granule formation, and cytoskeletal attachment. *J. Cell Biol.* **160**, 77-87.
Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M. A. T. and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. *Cell* **61**, 523-534.
Geng, C. and Macdonald, P. M. (2006). Imp associates with squid and Hrp48 and contributes to localized expression of gurken in the oocyte. *Mol. Cell. Biol.* **26**, 9508-9516.
Go, M. J., Eastman, D. S. and Artavanis-Tsakonas, S. (1998). Cell proliferation control by Notch signaling in Drosophila development. *Development* **125**, 2031-2040.
Goto, S., Taniguchi, M., Muraoka, M., Toyoda, H., Sado, Y., Kawakita, M. and Hayashi, S. (2001). UDP-sugar transporter implicated in glycosylation and processing of Notch. *Nat. Cell Biol.* **3**, 816-822.
Grammont, M. and Irvine, K. D. (2001). fringe and Notch specify polar cell fate during Drosophila oogenesis. *Development* **128**, 2243-2253.
Hansen, H. T., Rasmussen, S. H., Adolph, S. K., Plass, M., Krogh, A., Sanford, J., Nielsen, F. C. and Christiansen, J. (2015). Drosophila Imp iCLIP identifies an RNA assemblage coordinating F-actin formation. *Genome Biol.* **16**, 123.
Havin, L., Git, A., Elisha, Z., Oberman, F., Yaniv, K., Schwartz, S. P., Standart, N. and Yisraeli, J. K. (1998). RNA-binding protein conserved in both microtubule- and microfilament-based RNA localization. *Genes Dev.* **12**, 1593-1598.
Housden, B. E., Millen, K. and Bray, S. J. (2012). Drosophila reporter vectors compatible with ΦC31 integrase transgenesis techniques and their use to generate new notch reporter fly lines. *G3 (Bethesda)* **2**, 79-82.
Hüttelmaier, S., Zenklusen, D., Lederer, M., Dictenberg, J., Lorenz, M., Meng, X. H., Bassell, G. J., Condeelis, J. and Singer, R. H. (2005). Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1. *Nature* **438**, 512-515.
Ioannidis, P., Trangas, T., Dimitriadis, E., Samiotaki, M., Kyriazoglou, I., Tsiapalis, C. M., Kittas, C., Agnantis, N., Nielsen, F. C., Nielsen, J. et al. (2001). C-MYC and IGF-II mRNA-binding protein (CRD-BP/IMP-1) in benign and malignant mesenchymal tumors. *Int. J. Cancer* **94**, 480-484.
Itoh, M., Kim, C.-H., Palardy, G., Oda, T., Jiang, Y.-J., Maust, D., Yeo, S.-Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L. et al. (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell* **4**, 67-82.
Kidd, S., Baylies, M. K., Gasic, G. P. and Young, M. W. (1989). Structure and distribution of the Notch protein in developing Drosophila. *Genes Dev.* **3**, 1113-1129.
Koizumi, K., Higashida, H., Yoo, S., Islam, M. S., Ivanov, A. I., Guo, V., Pozzi, P., Yu, S.-H., Rovescalli, A. C., Tang, D. et al. (2007). RNA interference screen to identify genes required for Drosophila embryonic nervous system development. *Proc. Natl. Acad. Sci. USA* **104**, 5626-5631.
Lai, E. C., Deblandre, G. A., Kintner, C. and Rubin, G. M. (2001). Drosophila neuralized is a ubiquitin ligase that promotes the internalization and degradation of delta. *Dev. Cell* **1**, 783-794.
Lai, E. C., Roegiers, F., Qin, X., Jan, Y. N. and Rubin, G. M. (2005). The ubiquitin ligase Drosophila Mind bomb promotes Notch signaling by regulating the localization and activity of Serrate and Delta. *Development* **132**, 2319-2332.
Langridge, P. D. and Struhl, G. (2017). Epsin-dependent ligand endocytosis activates Notch by force. *Cell* **171**, 1383-1396.e12.
Le Borgne, R., Remaud, S., Hamel, S. and Schweisguth, F. (2005). Two distinct E3 ubiquitin ligases have complementary functions in the regulation of delta and serrate signaling in Drosophila. *PLoS Biol.* **3**, e96.
Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-461.
Lee, T. and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. *Trends Neurosci.* **24**, 251-254.
Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J. and Carthew, R. W. (2004). Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* **117**, 69-81.
Lieber, T., Kidd, S. and Young, M. W. (2002). kuzbanian-mediated cleavage of Drosophila Notch. *Genes Dev.* **16**, 209-221.
Liu, Z., Yang, C.-P., Sugino, K., Fu, C.-C., Liu, L.-Y., Yao, X., Lee, L. P. and Lee, T. (2015). Opposing intrinsic temporal gradients guide neural stem cell production of varied neuronal fates. *Science* **350**, 317-320.
Lopez-Schier, H. and St Johnston, D. (2001). Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during Drosophila oogenesis. *Genes Dev.* **15**, 1393-1405.

Medioni, C., Ramialison, M., Ephrussi, A. and Besse, F. (2014). Imp promotes axonal remodeling by regulating profilin mRNA during brain development. *Curr. Biol.* **24**, 793-800.
Micchelli, C. A. and Blair, S. S. (1999). Dorsoventral lineage restriction in wing imaginal discs requires Notch. *Nature* **401**, 473-476.
Micchelli, C. A., Rulifson, E. J. and Blair, S. S. (1997). The function and regulation of cut expression on the wing margin of Drosophila: Notch, Wingless and a dominant negative role for Delta and Serrate. *Development* **124**, 1485-1495.
Miller, A. C., Lyons, E. L. and Herman, T. G. (2009). cis-Inhibition of Notch by endogenous Delta biases the outcome of lateral inhibition. *Curr. Biol.* **19**, 1378-1383.
Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S. et al. (2000). Fringe is a glycosyltransferase that modifies Notch. *Nature* **406**, 369-375.
Morin, X., Daneman, R., Zavortink, M. and Chia, W. (2001). A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in Drosophila. *Proc. Natl. Acad. Sci. USA* **98**, 15050-15055.
Munro, T. P., Kwon, S., Schnapp, B. J. and St Johnston, D. (2006). A repeated IMP-binding motif controls oskar mRNA translation and anchoring independently of Drosophila melanogaster IMP. *J. Cell Biol.* **172**, 577-588.
Narbonne-Reveau, K., Lanet, E., Dillard, C., Foppolo, S., Chen, C.-H., Parrinello, H., Rialle, S., Sokol, N. S. and Maurange, C. (2016). Neural stem cell-encoded temporal patterning delineates an early window of malignant susceptibility in Drosophila. *eLife* **5**, e13463.
Nichols, J. T., Miyamoto, A., Olsen, S. L., D'Souza, B., Yao, C. and Weinmaster, G. (2007). DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. *J. Cell Biol.* **176**, 445-458.
Nielsen, J., Christiansen, J., Lykke-Andersen, J., Johnsen, A. H., Wewer, U. M. and Nielsen, F. C. (1999). A family of insulin-like growth factor II mRNA-binding proteins represses translation in late development. *Mol. Cell. Biol.* **19**, 1262-1270.
Nielsen, J., Cilius Nielsen, F., Kragh Jakobsen, R. and Christiansen, J. (2000). The biphasic expression of IMP/Vg1-RBP is conserved between vertebrates and Drosophila. *Mech. Dev.* **96**, 129-132.
Pan, D. and Rubin, G. M. (1997). Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during Drosophila and vertebrate neurogenesis. *Cell* **90**, 271-280.
Parks, A. L., Klueg, K. M., Stout, J. R. and Muskavitch, M. A. (2000). Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development* **127**, 1373-1385.
Pavlopoulos, E., Pitsouli, C., Klueg, K. M., Muskavitch, M. A. T., Moschonas, N. K. and Delidakis, C. (2001). neuralized Encodes a peripheral membrane protein involved in delta signaling and endocytosis. *Dev. Cell* **1**, 807-816.
Poulton, J. S., Huang, Y.-C., Smith, L., Sun, J., Leake, N., Schleede, J., Stevens, L. M. and Deng, W.-M. (2011). The microRNA pathway regulates the temporal pattern of Notch signaling in Drosophila follicle cells. *Development* **138**, 1737-1745.
Richard, M., Grawe, F. and Knust, E. (2006). DPATJ plays a role in retinal morphogenesis and protects against light-dependent degeneration of photoreceptor cells in the Drosophila eye. *Dev. Dyn.* **235**, 895-907.
Rooke, J., Pan, D., Xu, T. and Rubin, G. M. (1996). KUZ, a conserved metalloprotease-disintegrin protein with two roles in Drosophila neurogenesis. *Science* **273**, 1227-1231.
Sakamoto, K., Ohara, O., Takagi, M., Takeda, S. and Katsube, K.-I. (2002). Intracellular cell-autonomous association of Notch and its ligands: a novel mechanism of Notch signal modification. *Dev. Biol.* **241**, 313-326.
Sinka, R., Gillingham, A. K., Kondylis, V. and Munro, S. (2008). Golgi coiled-coil proteins contain multiple binding sites for Rab family G proteins. *J. Cell Biol.* **183**, 607-615.
Sotillos, S., Roch, F. and Campuzano, S. (1997). The metalloprotease-disintegrin Kuzbanian participates in Notch activation during growth and patterning of Drosophila imaginal discs. *Development* **124**, 4769-4779.
Struhl, G. and Greenwald, I. (1999). Presenilin is required for activity and nuclear access of Notch in Drosophila. *Nature* **398**, 522-525.
Sun, J. and Deng, W.-M. (2005). Notch-dependent downregulation of the homeodomain gene cut is required for the mitotic cycle/endocycle switch and cell differentiation in Drosophila follicle cells. *Development* **132**, 4299-4308.
Sun, J. and Deng, W.-M. (2007). Hindsight mediates the role of notch in suppressing hedgehog signaling and cell proliferation. *Dev. Cell* **12**, 431-442.
Tanaka, T. and Nakamura, A. (2008). The endocytic pathway acts downstream of Oskar in Drosophila germ plasm assembly. *Development* **135**, 1107-1117.
Tiruchinapalli, D. M., Oleynikov, Y., Kelič, S., Shenoy, S. M., Hartley, A., Stanton, P. K., Singer, R. H. and Bassell, G. J. (2003). Activity-dependent trafficking and dynamic localization of zipcode binding protein 1 and beta-actin mRNA in dendrites and spines of hippocampal neurons. *J. Neurosci.* **23**, 3251-3261.
Toledano, H., D'Alterio, C., Czech, B., Levine, E. and Jones, D. L. (2012). The let-7-Imp axis regulates ageing of the Drosophila testis stem-cell niche. *Nature* **485**, 605-610.
Tomlinson, A., Mavromatakis, Y. E. and Struhl, G. (2011). Three distinct roles for notch in Drosophila R7 photoreceptor specification. *PLoS Biol.* **9**, e1001132.
Vaccari, T., Lu, H., Kanwar, R., Fortini, M. E. and Bilder, D. (2008). Endosomal entry regulates Notch receptor activation in Drosophila melanogaster. *J. Cell Biol.* **180**, 755-762.
Wang, X., Adam, J. C. and Montell, D. (2007). Spatially localized Kuzbanian required for specific activation of Notch during border cell migration. *Dev. Biol.* **301**, 532-540.
Woods, D. F. and Bryant, P. J. (1993). Apical junctions and cell signalling in epithelia. *J. Cell Sci.* **1993** Suppl. 17, 171-181.
Yao, J., Sasaki, Y., Wen, Z., Bassell, G. J. and Zheng, J. Q. (2006). An essential role for beta-actin mRNA localization and translation in Ca2+-dependent growth cone guidance. *Nat. Neurosci.* **9**, 1265-1273.
Ye, Y., Lukinova, N. and Fortini, M. E. (1999). Neurogenic phenotypes and altered Notch processing in Drosophila Presenilin mutants. *Nature* **398**, 525-529.
Yip, M. L., Lamka, M. L. and Lipshitz, H. D. (1997). Control of germ-band retraction in Drosophila by the zinc-finger protein HINDSIGHT. *Development* **124**, 2129-2141.