A two-step Notch-dependant mechanism controls the selection of the polar cell pair in *Drosophila* oogenesis

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SUMMARY

Organisers control the patterning and growth of many tissues and organs. Correctly regulating the size of these organisers is crucial for proper differentiation to occur. Organiser activity in the epithelium of the *Drosophila* ovarian follicle resides in a pair of cells called polar cells. It is known that these two cells are selected from a cluster of equivalent cells. However, the mechanisms responsible for this selection are still unclear. Here, we present evidence that the selection of the two cells is not random but, by contrast, depends on an atypical two-step Notch-dependant mechanism. We show that this sequential process begins when one cell becomes refractory to Notch activation and is selected as the initial polar cell. This cell then produces a Delta signal that induces a high level of Notch activation in one other cell within the cluster. This Notch activity prevents elimination by apoptosis, allowing its selection as the second polar cell. Therefore, the mechanism used to select precisely two cells from among an equivalence group involves an inductive Delta signal that originates from one cell, itself unable to respond to Notch activation, and results in one other cell being selected to adopt the same fate. Given its properties, this two-step Notch-dependent mechanism represents a novel aspect of Notch action.

KEY WORDS: *Notch*, *neuralized,* Polar cells, Organizer, Apoptosis, *Drosophila*

INTRODUCTION

Organizers are essential to the development of epithelial sheets. They act on neighbouring cells by secreting factors that control their growth and differentiation. In the *Drosophila* ovary, each follicle is composed of a monolayer of epithelial cells that surrounds the germline cells (Wu et al., 2008) (Fig. 1A). Two pairs of epithelial cells, called polar cells (pc), located at either extremity of each follicle, are formally defined as the organizers of the follicular epithelium (Grammont and Irvine, 2002; Xi et al., 2003). Pc pair formation starts with the acquisition of a polar fate by four to six somatic cells (that we refer to as the pfc, for polar-fated cells) and ends when this number is reduced to two through an apoptotic process. The acquisition of a polar fate starts in region IIb of the germarium, when the cyst is surrounded by about eight to sixteen somatic cells, and spans two days until the follicle reaches stage 2 (Nystul and Spradling, 2010). No specific pc fate markers can be used to follow the acquisition of this fate during this period. Pfc can be detected only by lineage, because they exit the mitotic cycle soon after specification, in contrast to the other somatic cells that surround the germline (Besse and Pret, 2003; Margolis and Spradling, 1995; Nystul and Spradling, 2010; Tworoger et al., 1999). From stage 2 onwards, pfc start expressing the Fasciclin III (FasIII) protein at high levels. Three to four cells are usually seen at this stage, but a maximum of six can be observed by blocking the apoptotic process that occurs between stages 1 and 5 (Besse and Pret, 2003) (Fig. 1B). Previous data have shown that the induction of the pc fate requires the activation of the Notch (N) receptor via germline production of the Delta (Dl) ligand (Grammont and

Irvine, 2001; Lopez-Schier and St Johnston, 2001). In *Drosophila*, the N pathway is activated by the interaction of N with Dl or with a second ligand named Serrate (Ser), present at the surface of the adjacent signal-sending cell. This interaction releases the N intracellular domain, which translocates to the nucleus to regulate the transcription of target genes with the Su(H) transcription factor. In the absence of this signalling, no cell adopts a pc fate. However, the mechanisms involved in selecting a pair of pc from among the pfc (a process hereafter referred to as pc pair selection) have not yet been resolved.

By looking at the level of expression of N activity reporters and by manipulating the level of N activation within the cluster, we report that N activation is required for pc pair selection, and that it acts through an original two-step mechanism for this process: initially, an intrinsically N-refractory pfc produces a Dl signal that in turn activates N in another pfc of the cluster. These two cells will eventually form the pair of pc and are functionally indistinguishable from each other.

MATERIALS AND METHODS

Drosophila stocks and crosses

$neur^{if65}$, Dl^{rev10}, $Ser^{rev2-11}$ and Ser^{RX106} are null alleles (de Celis et al., 1993; de-la-Concha et al., 1988; Heitzler and Simpson, 1991; Sun and Artavanis-Tsakonas, 1996). $neur^{A101}$ is a hypomorph allele (Lai et al., 2005) and $neur^{il119}$ is a loss-of-function allele (Leviten and Posakony, 1996). The three different loss-of-function alleles, $neur^{IF65}$, $neur^{IL119}$ and $neur^{A101}$ display similar levels of phenotypic penetrance and expressivity (and thus, are hereafter collectively referred to as *neur*). CantonS is used as wild type. The Notch activity reporter lines used were $E(spl)m\beta\text{-}lacZ$ (Cooper et al., 2000), $E(spl)m\beta\text{-}CD2$ (de Celis et al., 1998) and $GbeSu(H)_{m8}\text{-}lacZ$ (Furriols and Bray, 2001). Most of the experiments have been done using the three reporters. $GbeSu(H)_{m8}$ was used for statistical analyses as it drives the strongest expression.

Mutant clones were generated by Flipase-mediated mitotic recombination (Xu and Rubin, 1993). Flipase expression was induced by heat shocking two-day old females at 38°C for 1 hour, then the females were placed at 25°C and were dissected 3 to 9 days after the heat shock. *neur* or *Dl* clones encompassing less than 25% of follicle cells, such as

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those affecting only the pfc, lead to the formation of normal-looking follicles. Clones encompassing more than 50% of follicle cells sometimes induced the formation of compound follicles and were excluded from our analyses.

P(UAS-Dl::N.ΔECN) (N^{act}) drives expression of a Dl:N fusion protein. Amino acids 1-27 of Dl (start and membrane transport signal sequence) are fused to amino acids 1742-2703 of N (transmembrane and intracellular regions) (Baker and Schubiger, 1996; Doherty et al., 1996). P(UAS-N.ICN) (N^{ICN}) is a form of N in which the extracellular domain including the signal peptide and transmembrane domain is deleted (Fortini et al., 1993; Go et al., 1998).

Ectopic expression of these forms of Notch was performed by generating Flip-out Gal4 clones using the *AyGal4 UASGFP* (Ito et al., 1997) transgene. Heat shocks were performed at 32.5°C for 20 minutes, then females were placed at 18°C and were dissected 42 hours to five days after the heat shock.

Follicle staining
The pfc/pc identity was assayed by the expression of the FasIII protein or of the PZ80 enhancer trap, an insertion in the *FasIII* gene (Karpen and Spradling, 1992), which are specifically expressed in the pfc/pc from stage 1 onwards. The A101 marker (an enhancer-trap within the *neur* gene) was not used in order to prevent feedback from affecting marker expression when the N pathway was manipulated. β-galactosidase activity detection and immunofluorescence staining were carried out as described previously (Grammont et al., 1997; Grammont, 2007). The following primary antibodies were used: rabbit anti-Neur [1:100 (Lai et al., 2005)], goat anti-β-galactosidase (1:1000, Biogenesis), rabbit anti-β-galactosidase (1:2000, Cappel), rabbit anti-DIAP1 [1/40 (Ryoo et al., 2002)], rabbit anti-Myc (1:100, Santa Cruz), mouse anti-CD2 (1:200, Serotec), mouse anti-GFP (1:500, Sigma-Aldrich), goat anti-GFP (1:1000, AbCam), mouse anti-FasIII 7G10 (1:200, DSHB), and rabbit anti-cleaved Caspase-3 (1:200, Cell Signaling Technology). Secondary antibodies coupled with Cy5, Cy3 (Jackson ImmunoResearch) and Alexa Fluor 488 (Molecular Probes) were used at a dilution of 1:1000.

Whole-mount in situ hybridization (*neur* cDNA provided by E. Lai, Sloan-Kettering Institute, New York, NY) to follicles was carried out as described previously (Sahut-Barnola et al., 1995).

Imaging
Confocal images were obtained using a Zeiss LSM510Meta microscope with 40×/1.3 Plan-NeoFluar or 63×/1.4 Plan-Apochromat objectives. Images were processed using LSM510Meta software (Zeiss), Photoshop 7 and Illustrator CS3 (Adobe Systems).

RESULTS
The level of N activity within the pfc induces a bias in the selection of one of the two pc
To determine whether the N pathway acts during pc pair selection, we analysed three reporters of N activation: the P(*E(spl)m*β*-lacZ*), P(*E(spl)m*β*-CD2*) and P(*GbeSu(H)$_{m8}$*) lines in young wild-type (WT) follicles. All yielded a similar expression pattern. The earliest significant expression was a transient and weak staining in the three or four cells located at the extremities of some stage 1 or stage 2 follicles (25%, *n*=32; see Movie 1 in the supplementary material). Soon after, 89.7% (*n*=39) of the clusters displayed N activation, but with an expression level that often varied within the pfc (Fig. 1C-E). Within these N-activated clusters, we analysed those containing only three pfc, as they were the most frequent, and found that 77% presented a differential expression, with the reporter being strongly expressed in one (or, more rarely, two) pfc (Fig. 1J). The remaining 23% presented a low and uniform expression (Fig. 1F). We wondered whether the levels of N activity are important for the selection of the two final pc or for the elimination of the others. Elimination of the supernumerary pfc can be followed through the

expression of cleaved Caspase-3 (cCasp3), a cell death marker. In 100% (*n*=23) of clusters with only one pfc displaying a high level of N reporter expression and with at least one apoptotic pfc, the cell with the highest level of N reporter expression was never the one marked by cCasp3 (Fig. 1G). This was particularly revealing in clusters composed of three cells, where we could be certain that the cell with the highest level of N activity would be retained as one of the two final pc. We ruled out the possibility that apoptosis might preclude the detection of N reporter expression as, in the few clusters that present two pfc with a high level of N activity, cCasp3 is also sometimes observed in one of them (20%, *n*=20; data not shown). Together, these observations show that: (1) N is most often activated within the cluster during pc pair selection; (2) the level of N activity reporter expression is frequently higher in one pfc; and (3) the cell with the highest level is never eliminated by apoptosis. This indicates that the pfc are not equivalent with respect to the level of Notch activity and strongly suggests that the dynamic level of N activation creates a bias in the selection of one of the final pc, by retaining the cell with the greatest activity. This in turn implies that at the end of pc pair selection, the expression level of the three N reporters should be higher in one of the two final pc. Indeed, 61% (*n*=18) of pc pairs detected in follicles between stage 2 and 5 displayed a differential expression (Fig. 1H-J). This percentage is probably an underestimation, as measurement of the Notch activity reporter in these pairs might have happened several hours after they had been formed. After stage 5, the expression became weaker and the difference between the two pc tended to be less noticeable, which suggests that the signalling that drives the differential expression is not active anymore.

A high level of N activity autonomously prevents pfc from being eliminated by apoptosis
The correlation between the high expression of the N activity reporter in one pfc and the absence of apoptosis markers within the same cell suggests that high levels of N activation render cells resistant to apoptosis. To test this, we expressed a constitutively activated form of Notch (N^{act}) under the *act-gal4* driver (Doherty et al., 1996). Although it has already been shown that the expression of this form in the pfc cluster leads to the formation of extra pc (Grammont and Irvine, 2001), a detailed analysis of this phenotype has never been carried out. We found that 47% (*n*=57) of the pfc clusters in which some or all of the cells express N^{act} were composed of more than two cells after stage 5, compared with 4% (*n*=150) for the wild type (Fig. 2A,B). Three hypotheses can explain the presence of extra pc: an abnormal division of the pfc/pc, the differentiation of supernumerary pfc in the germarium and/or an abnormal elimination of pfc through apoptosis. We eliminated the first hypothesis because we never detected the phosphorylated form of Histone H3 in the N^{act}-expressing pfc/pc clusters from stage 1 onwards (data not shown). We then tested the second and third hypotheses by dissecting flies 42 hours or 5 days after induction of N^{act} expression under the *act-gal4* driver; the short or the long induction-to-dissection interval allowing the observation of clusters formed prior to induction and upon induction, respectively. After 5 days, clusters of stage 6 or older follicles were composed of 6-12 cells, indicating that a higher number of somatic cells adopt a pfc fate in the germarium, and thus validating the second hypothesis (Fig. 2B). After 42 hours, clusters of three to five cells were detected, revealing that the presence of extra pfc/pc is also due to the reduced elimination of the pfc (Fig. 2A). To confirm this, we examined cCasp3 expression in pre-stage 5 N^{act}-expressing clusters and found that only 2.5% (*n*=79) of them
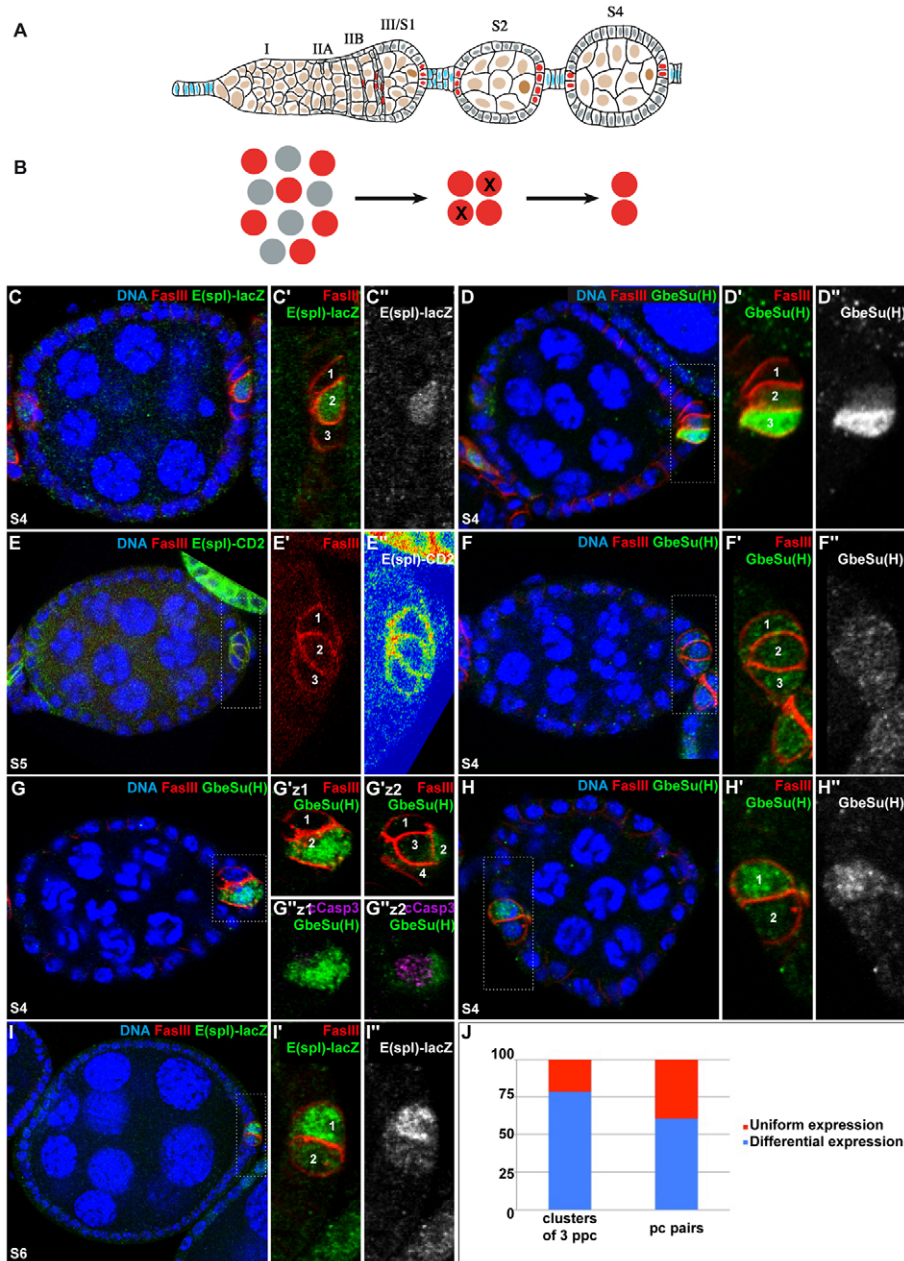
Fig. 1. One pfc/pc displays high Notch (N) activity and becomes one of the final pc. In all figures, anterior is to the left. The stages (S) of the follicles and the genotype are indicated on the first panel of each series. Panels marked with primes are magnified views of the boxed area in the corresponding letter (for example, A', A'' or A''' are magnifications of the boxed region in A). In some panels, the use of a colour gradient allows the visualization of differences in intensities of accumulation from none (blue) to strongest (red). (**A**) Schematic of the anterior part of a wild-type ovariole. The arrangement and identities of the different cell types – oocyte (brown), nurse cells (tan), follicle cells (grey), pfc/pc (red), stalk cells (light blue) – are shown in the germarium and in stage 2 and stage 4. Roman numerals indicate the different regions of the germarium. (**B**) Schematic of pc pair formation. Among the somatic cells (grey), few cells adopt a polar fate from region IIB until stage 2. At stage 2, three to four pfc are usually visible at each extremity. A pair is selected, while an apoptotic process eliminates the others from stages 1 to 5. (C-I) Expression of N reporters [*E(spl)mβ-lacZ*, *E(spl)mβ-CD2* and *GbeSu(H)$_{m8}$-lacZ*] in polar cells marked by Fas III. (**C-E**) Follicles with three posterior pfc. One of the pfc displays a high level of N activity. The others are faintly labelled (D,E) or not labelled (C). (**F**) Follicle with three posterior pfc displaying a uniform and low level of N activity. (**G**) Follicle with four posterior pfc at two different *z*-sections (z1 and z2). The cCasp3-expressing cell (visible in the z2 section) is not the one displaying the greatest level of N activity (mainly visible in the z1 section). This latter is selected. (**H,I**) Follicles with two anterior (H) or posterior (I) pc. One pc displays a higher level of N activity than the other one. (**J**) Percentage of wild-type clusters of three pfc and of pc pairs displaying a differential or a uniform expression of the N reporter before stage 6. Most of the clusters contain one pfc with a high N activity.

express this apoptotic marker compared with 21.7% (*n*=106) for the wild type (see Fig. S1A in the supplementary material). cCasp3 expression was also detected in some stage 7 or older follicles (see Fig. S1B in the supplementary material). We next checked whether the reduced apoptosis observed between stages 2 and 5 is due to an increase of N activity, driven by N^{act} expression in the pfc by looking at the expression of the P(*GbeSu(H)$_{m8}$*) or P(*E(spl)mβ-lacZ*) reporters. Indeed, most of the pfc per cluster showed high reporter expression (*n*=29; Fig. 2A). This level was usually even higher than those detected in wild-type pfc clusters. Thus, increasing N activity is sufficient to autonomously protect the pfc from apoptosis, even if N^{act} expression does not provide absolute protection.

One possible way for the cells to be protected from apoptosis is if they continuously express DIAP1 (also known as Thread), a known inhibitor of the caspases and of cell death. We thus examined DIAP1 expression levels in wild type and in N^{act}-expressing pfc clusters during pc pair selection. In the wild type, one pfc usually presented a level of DIAP1 expression that was lower than that of the other pfc. This pfc is most likely the one that will be eliminated next as it was not the one with a high level of N activity (85.7%; *n*=14; see Fig. S1C,D in the supplementary material). In N^{act}-expressing pfc clusters presenting extra pfc/pc, a uniform level of DIAP1 was observed between the pfc (69%, *n*=13; see Fig. S1E in the supplementary material). These data suggest that the level of N activity in the wild type regulates the level of DIAP1 expression in, at least, some pfc.

One of the two pc is refractive to N activation

We expected that all the pfc that expressed N^{act} would display N reporter expression. Instead, 93% (*n*=48) of the clusters with three to five N^{act}-expressing cells had one cell that did not express the reporters or that expressed the reporters at a low level (Fig. 2A). Similarly, in the clusters of six to twelve N^{act}-expressing cells
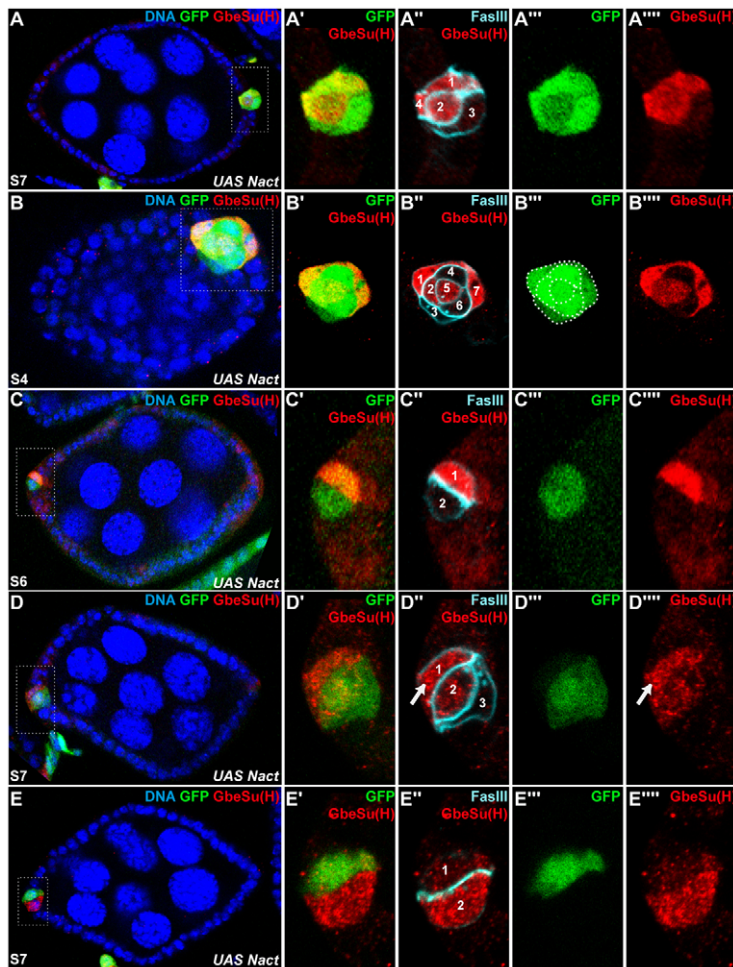
Fig. 2. One pfc/pc is refractory to N activation and becomes one of the final pc. Follicles from females carrying $GbeSu(H)_{m8}$ with N^{act}-expressing clones (marked with GFP), 42 hours (A,C-E) or 5 days (B) after induction. (**A**) Follicle with four posterior pfc expressing N^{act}. Three of them express the reporter. One cell is refractory to the N activation. (**B**) Follicle with seven posterior pfc expressing N^{act}. Three are not expressing the reporter. (**C**) Follicle with two anterior pc expressing N^{act}. Only one of them expresses the reporter. (**D**) Follicle with three anterior pfc. Two express N^{act} but only one expresses the reporter. The third pfc does not express N^{act} but expresses the reporter (arrow). (**E**) Follicle with two anterior pc. One pc expresses N^{act} but does not express the reporter. The other pc does not express N^{act} but expresses the reporter.

(formed by the commitment of too many somatic cells to pfc fate in the germarium), two to three cells per cluster did not display noticeable N activity (Fig. 2B). This suggests that in the wild type, one cell per cluster becomes refractory to N activation, after acquisition of the pfc fate. We then analysed pc pairs in which both cells express N^{act} and observed that, in 92% (n=43) of them, one cell expressed the N reporters at a high level and the other did not (Fig. 2C). The cell that was refractive to N target gene transcription was thus selected to be one of the two pc. By analysing mosaic clusters, we confirmed that N activation within the cluster depends on the cellular context as, in 35% (n=57) of them, one non-N^{act}-expressing cell presented a high level of N reporter expression (Fig. 2D,E). This reflects the wild type situation, where N signalling leads to increased N activity in one pfc, whether this cell is expressing N^{act} or not. Interpreted within the context of our previous conclusions, these results indicate that the two selected pfc differ from each other, as well as from the other pfc, with respect to their levels of N activity and to their intrinsic abilities to activate N. This implies that these two cells are selected by at least two different mechanisms, one of them being dependent on a high level of N activity.

To further elucidate the mechanism(s) by which one cell becomes refractory to N activation, we next tested a different constitutively active form of N, N^{ICN}. Unlike N^{act}, which is a membrane-targeted form of N that requires endocytosis to be active (Nagaraj and Banerjee, 2009), N^{ICN} is composed of only the intracellular part of N, and should consequently be less dependent

on intracellular trafficking. Expression of N^{ICN} in the pfc led to supernumerary pfc/pc in similar proportions to those observed with N^{act}. In 30% (n=26) of N^{ICN}-expressing clusters, a single N-refractory cell was detected (see Fig. S2A in the supplementary material) and in a further 40%, one pfc presented a noticeably lower level of N activity than do the others (see Fig. S2B in the supplementary material). Thus, the characteristic of one pfc being refractory to N activation is not specific to one form of activated N. However, 30% of the N^{ICN}-expressing pfc clusters displayed a high level of reporter expression in all the pfc, compared with the 7% observed in N^{act}-expressing pfc clusters (see Fig. S2C in the supplementary material). This indicates that, in the N-refractory cell, the cytoplasmic form of N is more active than the membrane-targeted form, which in turn suggests that the control of endocytic trafficking could play a role in blocking N activation in one pfc per cluster. To confirm this, we tried to detect the extracellular or the intracellular parts of the Notch protein in wild-type pfc clusters and in pfc clusters expressing N^{act} or N^{ICN}. In both cases, no significant signal could be revealed in any cells, precluding a detailed analysis of the intrinsic properties of the N-refractory cell.

Dl activity within the cluster drives differential levels of N activity

The source of the signal capable of driving different levels of N activation within the cluster is unknown. We hypothesized that this signal might come from one or more of the pfc. This hypothesis is supported by the expression pattern of the *neuralized* (*neur*) gene,
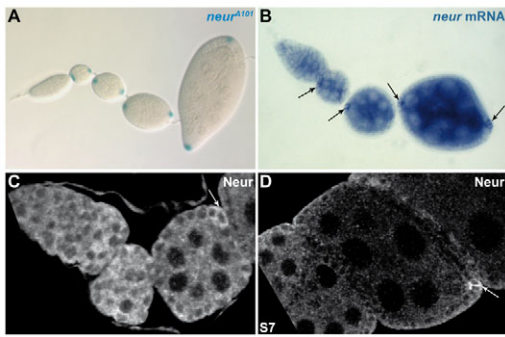
Fig. 3. *neuralized* is specifically expressed in the pfc/pc.
(**A**) Ovariole from the *neur^A101^*enhancer trap line. *lacZ* is detected first in the pfc once the follicle is formed and its expression is maintained at a high level in the pc until the end of oogenesis. (**B**) Wild-type ovariole displaying *neur* mRNA expression. From stage 2 to 7, *neur* transcript is detected in the germline cells and in the pfc/pc (arrows). (**C,D**) Anterior part of a wild-type ovariole (C) or a stage 7 follicle (D) displaying Neur protein expression, weakly in the pre-follicle cells in the germarium and at a high level in the pfc and pc until stage 7 (arrows).

which encodes an E3 ubiquitin ligase that acts in certain signal-sending cells to enhance Dl endocytosis and to upregulate its signalling activity (Lai et al., 2001; Pavlopoulos et al., 2001; Yeh et al., 2001). In the germarium, both the transcript and the protein product of the *neur* gene were observed at low levels in the pre-follicle cells and in the germline cells. From stages 2 to 7, high levels of mRNA and protein were detected in the pfc and pc,

reproducing the enhancer-trap pattern of the *neur^A101^* line (Fig. 3). These data suggest that Neur might upregulate Dl activity in the pfc/pc during pc pair selection.

We tested the possibility that *Dl* and *neur* might be required within the pfc to increase N activity in one pfc by examining P(*GbeSu(H)_{m8}*) reporter expression in clusters containing three pfc, all mutant for *neur* or *Dl*. Before stage 6, more *neur* mutant clusters (*n*=47) displayed either no expression or a low and uniform expression than was observed in wild type, whereas most *Dl* clusters (*n*=69) displayed no expression at all (Fig. 4A,C-D). Some *neur* or *Dl* clusters still present a differential reporter expression, but the differences in expression levels within the cluster were less noticeable than in wild type (compare Fig. 4B with 4E). This was observed regardless of the genotype of the surrounding cells, implying that Dl production from within the cluster is primarily responsible for the strong N activation in one pfc. After stage 6, 100% (*n*=66) of the clusters entirely mutant for *neur* or *Dl*, and 65% (*n*=55) of mosaic clusters, displayed either no expression or a low and uniform expression of the N reporter (Fig. 4F,G). These results show that the Dl signal produced by the pfc plays a crucial role in driving differential N activity within the cluster.

The production of Dl from within the pfc is required for efficient apoptosis

We next reasoned that as a high level of N activity in the pfc confers resistance to apoptosis, the absence of Dl production from within the cluster should lead to follicles with a single pc per extremity, with all pfc except the N refractory cell being eliminated. We thus counted the number of pfc/pc from stages 3 to 8 in clusters that were mosaic or entirely mutant for *neur* or *Dl*. At
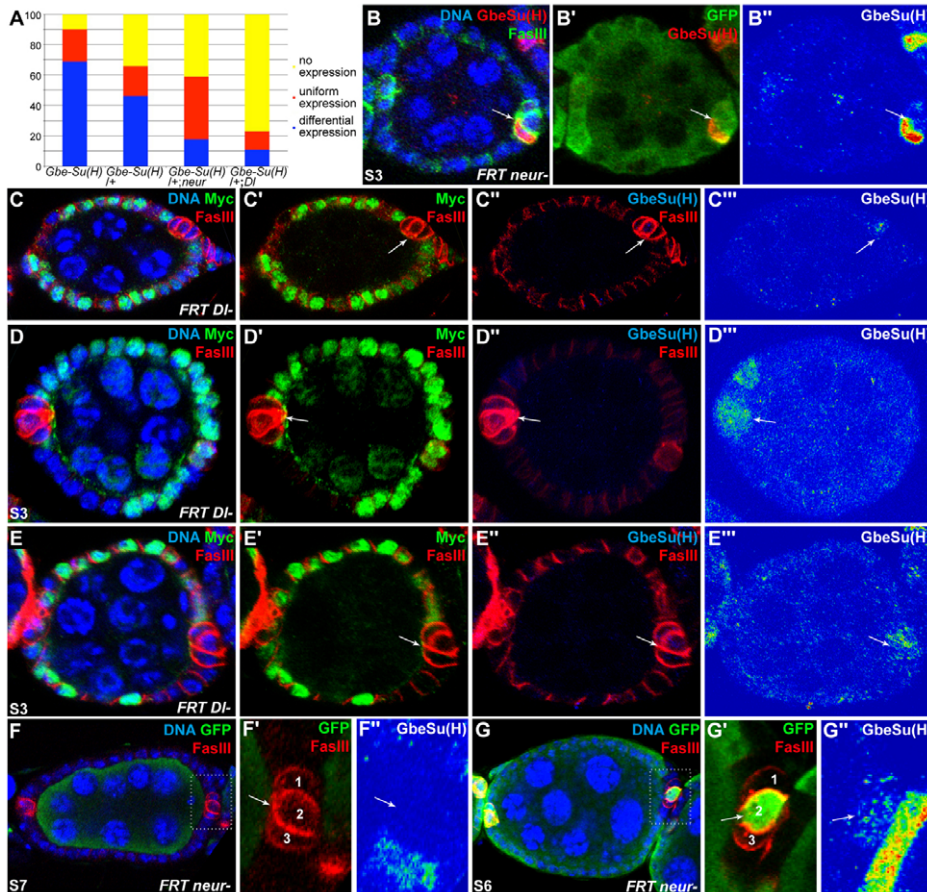

Fig. 4. Dl production from among the pfc is required to generate differential expression of the N reporter.
(**A**) Percentage of clusters of three pfc displaying a differential (blue), a uniform (red) or no (yellow) expression of the N reporter before stage 6 in *GbeSu(H)_{m8}*/ *GbeSu(H)_{m8}* follicles, in *GbeSu(H)_{m8}*/+ follicles and in follicles with *neur* or *Dl* mutant clones encompassing all the pfc. Over 35 clusters were counted per genotype. Most *Dl* clusters display no expression. (**B-G**) Follicles with *neur* or *Dl* mutant clones (marked by the absence of Myc or GFP) generated in females carrying *GbeSu(H)_{m8}*. (B) Follicle without mutant clones bearing three posterior pfc (arrow). One pfc expresses the reporter at a high level. (C) Follicle with three mutant pfc (arrow). No expression of the reporter is observed. (D) Follicle with three mutant pfc displaying a uniform and low expression of the reporter (arrow). (E) Follicle with three mutant pfc (arrow). A weak differential expression of the reporter is observed. (F) Follicle with three mutant posterior pfc (arrow). No expression of the reporter is observed. (G) Follicle with three posterior pfc. One pfc is wild type (arrow). An extremely weak expression of the reporter is detected. In F and G, these post-stage 5 follicles present extra pc. Their presence is not due to high N activity.
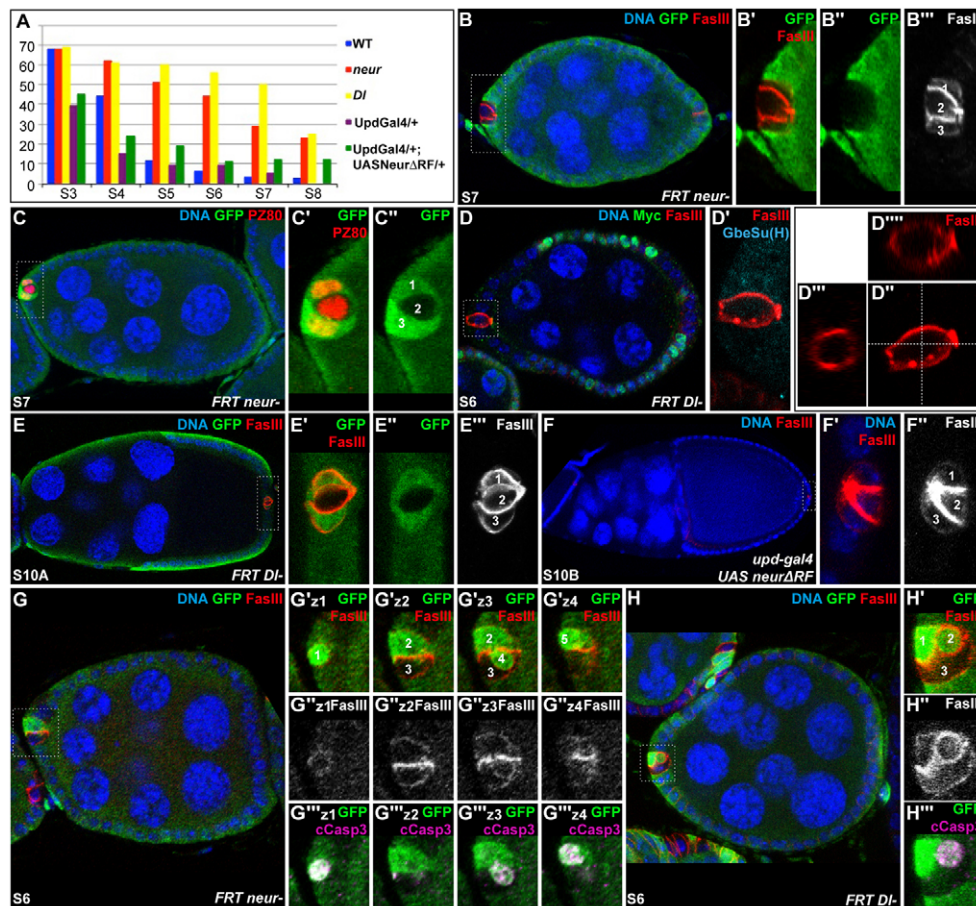
Fig. 5. *neur* and *Delta* are required in the N-refractory cell for pc pair selection. In B-E,G,H, follicles with *neur* (C,D,G) or *Dl* (B,E,H) mutant clones are marked by the absence of GFP. (**A**) Percentages of clusters containing more than two pfc in stages 3 to 8 wild-type follicles (blue), in follicles with *neur* (red) or *Dl* (yellow) mutant clones encompassing some or all of the pfc/pc, in follicles from UpdGal4/+ (purple) and from UpdGal4/+; UASNeurΔRF/+ (green) females. Over 20 clusters were counted per stage of each genotype. The pfc number reduction is delayed and incomplete in *neur* or *Dl* mutant clusters. (**B**) Follicle with three anterior pfc. The clone is encompassing only the pc. All the follicle cells are wild type. The cluster is essential to produce Dl. (**C**) Follicle with three anterior pfc. Only one pc is mutant for *neur*. All the follicle cells are wild type. Thus, the presence of wild-type pfc is not sufficient to prevent the phenotype from being displayed. (**D**) Follicle with only one anterior pc. The *Dl* mutant clone encompasses the anterior part of the follicle and the pc. The orthogonal sections represented by the dotted lines on the XY focal plan (**D'**) were used to generate the *yz* (**D''**) and the *xz* (**D'''**) focal plans. In absence of Dl, one cell always survives to apoptosis. This cell is mutant in most cases (80%, *n*=88), indicating that the production of Dl by this cell is essential, but not always sufficient, to protect another cell from apoptosis. (**E**) Follicle with three posterior pfc. Only one pc is mutant for *Dl*. All the follicle cells are wild type. Thus, the presence of wild-type pfc is not sufficient to prevent the phenotype from being displayed. (**F**) Follicle expressing *neurΔRF* under the driver *upd-gal4* with three posterior pfc. (**G**) Follicle with five anterior pfc through four consecutive *z*-confocal sections (z1 to z4). Four pfc are wild type (visible in z1 to z4) and one is mutant (visible in z2). Three wild-type pfc are labelled by cCasp3 (visible in z1, z3 and z4). Only one cell is mutant in this cluster composed of five pfc, indicating that a single mutant cell is sufficient to produce the phenotype. This mutant pfc is selected. (**H**) Follicle with three anterior pfc. Two pfc are wild type and one is mutant. One wild-type cell is labelled by cCasp3. The mutant pfc is selected.

stage 3, the number of mutant clusters with more than two pfc was similar to the number obtained for the wild-type clusters, indicating that the process of pfc specification was not delayed by the absence of Neur or Dl from within the cluster. However, in contrast to in the wild type, the number of pfc in mutant clusters did not decrease dramatically between stages 3 and 5, which led to the presence of supernumerary pfc/pc after stage 5 (Fig. 5A). From stages 6 to 8, 32% (*n*=93) and 44% (*n*=139) of the pfc clusters that were mosaic or entirely mutant for *neur* and *Dl*, respectively, were composed of more than two pfc/pc, as compared with 4% (*n*=150) for the wild type (Fig. 5B,C). Sixty-eight percent of the *neur* clusters and 28% of *Dl* clusters were composed of exactly two pc, as compared with 96% for the wild type. The final 28% of the *Dl* clusters had only

one pc (Fig 5D; see also Fig. S3A in the supplementary material). Both phenotypes (extra pc or a single pc) were observed regardless of the genotype of the surrounding cells and could also be detected when the mutant clones encompassed only the pfc or only some of the pfc, indicating that Dl production from within the cluster is crucial for pc pair selection. These phenotypes were never observed in follicles with *Ser* mutant clones.

We then examined the extra pfc/pc phenotype in greater detail. Extra pfc/pc could be seen up to stage 10B (Fig. 5E) and could be generated by expressing a dominant-negative form of Neur (*NeurΔRF*) (Lai and Rubin, 2001) under the *upd-gal4* driver, which drives expression in the pfc from stage 1 onwards (10.3%, *n*=76; Fig. 5A,F). As discussed above, three major hypotheses could

explain the production of extra pfc/pc. The first hypothesis, an abnormal proliferation of pfc/pc, is ruled out by the two following observations: the phosphorylated form of Histone H3 was not detected in the *neur* mutant pfc/pc clusters (data not shown), and the percentage of *Dl* or *neur* mutant follicles with extra pc decreased with the age of the follicles (Fig. 5A). The second hypothesis, differentiation of supernumerary pfc in the germarium, is also not supported by our data, as no more than 6 pfc per cluster were ever observed in *Dl* or *neur* mutant follicles. The third hypothesis, an abnormal elimination of pfc through apoptosis, is the most likely to explain the extra pfc/pc observed in *Dl* or *neur* mutant clusters, as cCasp3 expression was detected in only 5.4% (*n*=110) of pre-stage 5 *Dl* mutant clusters compared with in 21.7% (*n*=106) for wild type (see Fig. S3B in the supplementary material). In turn, this abnormal pfc elimination could be due to inefficient apoptosis, or to the blocking of apoptosis by a high level of N activity. The fact that the latter is ruled out by our data (Fig. 4F,G) indicates that the production of a Dl signal from within the pfc is required to drive an efficient apoptotic process to eliminate pfc between stages 1 and 5.

We then examined the single pc phenotype in particular. This phenotype was seen only in follicles older than stage 5, indicating that two to five pfc were present before this stage. As only a few cells were eliminated before stage 5 in *Dl* mutant clusters, pfc elimination must also occur after this stage. Indeed, cCasp3 expression was still detected after stage 5, indicating that an apoptotic process could occur beyond this stage when extra pfc/pc are present (a process hereafter referred to as 'prolonged apoptosis'; Fig. 5G,H). However, follicles without pc were never observed (*n*=316), indicating that at least one pc is always resistant to apoptosis. This resistance cannot be attributed to an autonomously high level of N activity, as N reporter expression was almost never detected in the surviving pc (77%, *n*=26; Fig. 5D). As we have demonstrated in wild type that the two final pc are the N-refractory pfc and the pfc with the highest level of N activity, the single pc retained in these *Dl* mutant clusters must be the N-refractory cell. These data show that: (1) a prolonged apoptosis, independent of the Dl signal from within the pfc, might occur after stage 5 to eliminate some or all the pfc, except the N-refractory cell; (2) the production of Dl from within the cluster is crucial in inducing N activity in one pfc in order to protect it from apoptosis; and (3) the selection of the N-refractory cell is independent of this Dl production.

The production of Dl by the N refractory pfc is crucial to select the pc pair

We show above that a Dl signal from within the cluster is required both to increase N activity in one pfc and to drive efficient apoptosis in the others. We wondered whether all of the pfc produce this signal. We first observed that *neur* or *Dl* mosaic clusters present extra pfc/pc (Fig. 5C,E) and no N reporter expression after stage 5 (Fig. 4G), indicating that the presence of wild-type pfc in these clusters was not sufficient to prevent both phenotypes from being displayed. Second, we were able to detect some rare stage 6 follicles (*n*=5) with clusters composed of one mutant pfc, one wild-type pfc and three wild-type apoptotic pfc (Fig. 5G). The fact that clusters almost never contain more than five pfc indicates that the loss of Dl production from a single pfc is sufficient to drive an inefficient apoptotic process between stages 1 and 5. This suggests two alternative scenarios. In one, all the pfc are required to send an efficient signal. In the other, not all the pfc are involved in sending this signal and, depending on which cells

are mutant, the phenotypes may or may not be present. If all the pfc were required to produce an efficient signal, then we would expect all *Dl* or *neur* mosaic clusters to display prolonged apoptosis and/or to lack N activity in one pfc. In fact, not all such clusters display these phenotypes (see Fig. S3B in the supplementary material). However, 100% (*n*=34) of the clusters displaying prolonged apoptosis had at least one mutant pfc that always remained unlabelled by cCasp3, whereas labelled pfc could be either mutant or wild type. Tellingly, we obtained identical results for mosaic clusters in which all the pfc, except for two, were undergoing apoptosis (*n*=8 for *Dl*; *n*=7 for *neur*), which indicates that, in such clusters, one mutant pfc, which is sometimes the only mutant pfc of the cluster, is always selected to become a final pc (Fig. 5G,H). This demonstrates that this mutant cell plays a key role in the appearance of the prolonged apoptosis phenotype. Furthermore, because in a third of *Dl* mutant clones encompassing the pfc, only the mutant N-refractory cell survived, we deduce that the mutant cell responsible for the prolonged apoptosis and the lack of N activity in one pfc is the N-refractory pfc. Thus, this cell is essential for the production of an efficient Dl signal.

DISCUSSION
A model for pc pair selection: the first selects the second

Controlling the number of final pc is essential in limiting the activity of the organizer, which triggers the differentiation of the neighbouring cells. Indeed, increasing or decreasing the pc number affects the number of border cells (population of about eight cells in wild type immediately adjacent to the pc) (Ghiglione et al., 2008; Grammont and Irvine, 2002; Silver et al., 2005; Silver and Montell, 2001; Xi et al., 2003). As supernumerary pc are quite rare in wild type, a robust mechanism must exist to guarantee the selection of only two cells from among a group of cells with similar developmental histories and similar developmental potential. At least three models can be invoked to explain such a selection: (1) the two cells are chosen randomly; (2) two cells acquire similar selective properties; or (3) two cells acquire selective properties that are different from each other, as well as from the others. Our data rule out the first two, as we demonstrate in several ways that the pfc are no longer equivalent at the time of selection, and that the two selected cells display different properties. By contrast, our results fit and even improve upon, the third model by revealing that the selection of the two cells with the same eventual fate occurs sequentially, and that the selection of the second depends on a signal from the first (Fig. 6). We propose that among the cluster, one pfc becomes unable to respond to the N pathway and also resistant to apoptosis by a mechanism that remains to be determined. In turn, this pfc produces a Dl signal that activates the N pathway in a second pfc and prevents it from being eliminated by apoptosis. In parallel, this signalling also promotes an efficient apoptotic process that eliminates all of the other pfc. Cells other than the N-refractory pfc (follicle cells, stalk cells, germline cells and/or other pfc) could participate to some extent in the specific N activation in the second selected pfc. Nevertheless, our data show that the role of the N-refractory cell in producing Dl is preponderant. This is in agreement with multiple studies in which it has been shown that cells that do not respond to N activation are more efficient in producing the ligands (Greenwald, 1998). One important aspect of the mechanism we describe is that the selection of the second pfc is tightly linked to the elimination of the other pfc, as the same signal is required for both. This renders the mechanism of pc pair selection highly efficient. The
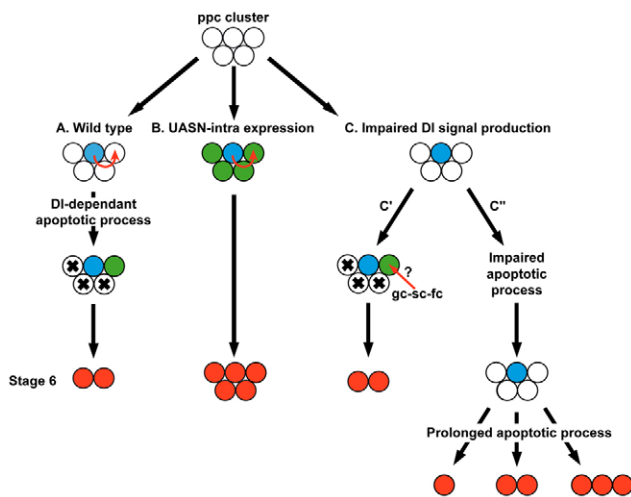
Fig. 6. A model for pc pair selection: the first selects the second. (**A**) In wild-type clusters composed of five pfc (white), one cell becomes refractory to N activation (blue) and produces a Dl signal that induces (red arrow) a high level of N activity in one other pfc (green), protecting it from apoptosis. Both become the pc (red). This signalling also participates in the elimination of the other pfc (black cross). The mechanisms by which (1) the N-refractory cell acquires this characteristic, (2) the N-refractory cell becomes more efficient in sending the signal, and (3) one pfc is more efficient at receiving the signal, remain unknown. (**B**) In N^{act}-expressing clusters, all pfc, except for the N-refractory cell, have a high level of N activity and are resistant to apoptosis driven by the Dl signal. (**C**) In clusters with the N-refractory cell impaired for producing an efficient Dl signal, two situations are observed. (**C′**) Some mutant clusters still display a differential pattern of N activity within the pfc, suggesting that a Dl signal from the germline, stalk and/or follicle cells, is occasionally sufficient. The apoptotic process thus occurs normally and two pc are selected before stage 6. (**C″**) No pfc have a high level of N activity and the apoptotic process is not efficient, resulting in the presence of more than two pfc at stage 5. An apoptotic signal, independent of the Dl signal produced by the N-refractory cell, applies after this stage and reduces the number of pfc. In addition to the N-refractory cell, the number of pc (none, one or two) that are retained might depend on the efficiency of this apoptotic process.

implication of the N pathway in the protection of cells from apoptosis or in the induction of apoptosis has already been described in several developmental contexts (Giraldez and Cohen, 2003; Lundell et al., 2003; Orgogozo et al., 2002; Singh et al., 2006). In the process of pc pair selection, the regulation of DIAP1 expression by Notch possibly provides a direct link that merits further exploration.

The mechanisms by which one cell becomes more efficient in sending the signal remain unknown. Recently, it has been shown that the first anterior and posterior pfc are specified in region IIb of the germarium and that additional pfc are formed later during stages 1 and 2 (Nystul and Spradling, 2010). One possibility is that this difference in timing is necessary for the first specified pfc to become refractory to N activation and thus more efficient in producing Dl. Moreover, the difficulty in detecting *neur* mRNA or protein prevents us from rejecting the possibility that *neur* is unequally distributed among the pfc. Similarly, it would also be interesting to determine why one pfc is more efficient at receiving the signal. The simplest explanation is that this depends upon the extent of contact between the sending and the receiving cells.

A novel mechanism for N to establish cell fate

Three modes of action – lateral inhibition, lineage decisions and boundary formation – have been proposed to describe how N activity regulates cell differentiation (Bray, 1998; Bray, 2006; Fiuza and Arias, 2007). Two of the main criteria used to define these modes are the state of the sending and receiving cells (equivalent or not), and the outcomes induced (positive or negative). In lateral inhibition, N signalling occurs between equivalent cells with roughly equivalent developmental properties (Simpson, 1997). The signal-sending activity increases in one cell, which in turn increases the signal-receiving activity in the others, preventing them from adopting the fate of the signal-sending cell (negative outcome). In lineage decisions, the signalling happens between daughter cells and depends on the asymmetrical inheritance of some N regulators, such as Numb or Partner of Numb (Guo et al., 1995). Their presence in one cell autonomously antagonises N activation, whereas the activation does occur in the sibling cell, preventing it from taking on the same identity (negative outcome). During boundary formation, N signalling takes place at the interface between two compartments of differentially fated cells (Irvine, 1999). The cells of the first row of each compartment are both the sending and the receiving cells and acquire identical new characteristics (positive outcome). In the mechanism we describe here, pc pair selection commences within a group of equivalent cells, with one cell acquiring specific properties and then signalling to another one to induce an identical fate (positive outcome). Based on the criteria mentioned above, this mechanism cannot be classed with either of the first two modes. First, it differs from the lineage decision mode, as the outcome of this mode is necessarily negative and because the two selected polar cells are not always sibling cells (Bray, 1998; Bray, 2006; Fiuza and Arias, 2007). Second, although the mechanisms used in pc pair selection and in the lateral inhibition mode both act upon a set of equivalent cells and generate Dl-expressing cells and N-expressing cells, the outcomes established are different. The mechanism we describe here is more closely related to the inductive signalling mode, as both generate positive outcomes and promote the acquisition of organizing activities by the Dl-sending and -receiving cells. But the fact that, in the signalling used to select the pc pair, the Dl-sending cell might be in part refractory to N activation because of differentially regulated endocytic trafficking possibly reveals an atypical N mechanism. Support for the existence of such a mechanism comes from recent data about the role of EGFR signalling and *phyllopod* during *Drosophila* eye formation (Nagaraj and Banerjee, 2009). The authors show that EGFR signalling suppresses some N^{act} phenotypes by activating *phyllopod*, which acts to regulate the residence time of the N components in early endocytic vesicles. In light of these observations, analysis of known trafficking regulators during pc pair formation could help to resolve the mechanisms that allow one pfc to become refractory to N activation. Thus, our data might define a novel aspect of the inductive signalling mode or, alternatively, might be representative of a fourth mode of N action. The discovery of other, similar examples would allow clarification of this point.

Competing interests statement

Supplementary material

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