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JAK/STAT autocontrol of ligand-producing cell number through apoptosis

Antoine Borensztejn[1,2], Elisabeth Boissoneau[1], Guillaume Fernandez[1], François Agnès*[,1,3] and Anne-Marie Pret[1,4,*]

SUMMARY

During development, specific cells are eliminated by apoptosis to ensure that the correct number of cells is integrated in a given tissue or structure. How the apoptosis machinery is activated selectively *in vivo* in the context of a developing tissue is still poorly understood. In the *Drosophila* ovary, specialised follicle cells [polar cells (PCs)] are produced in excess during early oogenesis and reduced by apoptosis to exactly two cells per follicle extremity. PCs act as an organising centre during follicle maturation as they are the only source of the JAK/STAT pathway ligand Unpaired (Upd), the morphogen activity of which instructs distinct follicle cell fates. Here we show that reduction of Upd levels leads to prolonged survival of supernumerary PCs, downregulation of the pro-apoptotic factor Hid, upregulation of the anti-apoptotic factor Diap1 and inhibition of caspase activity. Upd-mediated activation of the JAK/STAT pathway occurs in PCs themselves, as well as in adjacent terminal follicle and interfollicular stalk cells, and inhibition of JAK/STAT signalling in any one of these cell populations protects PCs from apoptosis. Thus, a Stat-dependent unidentified relay signal is necessary for inducing supernumerary PC death. Finally, blocking apoptosis of PCs leads to specification of excess adjacent border cells via excessive Upd signalling. Our results therefore show that Upd and JAK/STAT signalling induce apoptosis of supernumerary PCs to control the size of the PC organising centre and thereby produce appropriate levels of Upd. This is the first example linking this highly conserved signalling pathway with developmental apoptosis in *Drosophila*.

KEY WORDS: JAK/STAT, Apoptosis, Organising centre, Oogenesis, Polar cell, *Drosophila*, Unpaired (Outstretched), Diap1 (Thread), Hid (Wrinkled)

INTRODUCTION

Cell death by apoptosis is a normal part of metazoan development, during which it is necessary to attain a precise number of cells in certain cell populations (Bergmann et al., 2002; Monserrate and Brachmann, 2007; Fuchs and Steller, 2011). Although there have been great advances in understanding the molecular mechanisms that lead to caspase activation and consequent apoptosis, how the cell death machinery is activated *in vivo* during development is still poorly understood. *Drosophila* ovarian polar cells (PCs) have emerged as an exquisite and relatively simple model to unravel the mechanisms by which apoptosis is induced in a physiological context during development (Besse and Pret, 2003; Vachias et al., 2010; Khammari et al., 2011). PCs are specialised somatic cells located at each extremity, anterior and posterior, of maturing ovarian follicles, where they are embedded within the monolayered follicular epithelium (Fig. 1A). During early oogenesis, PCs are produced in excess (three to six cells) and supernumerary PCs are eliminated by apoptosis, such that only a pair of PCs survive at each extremity in 100% of follicles as of mid-oogenesis (stage 5 follicles) (Besse and Pret, 2003; Khammari et al., 2011). We have identified the components of the apoptosis machinery specifically responsible for PC apoptosis (Fig. 1B). In particular, the initiator caspase, Dronc (Nedd2-like caspase – FlyBase) and its specific adaptor Dark/Apaf1 (Apaf-1-related-killer – FlyBase), as well as the effector caspase, DrICE (Ice/Decay – FlyBase), are involved in PC apoptosis. Among members of the RHG family of IAP inhibitors, Hid (Wrinkled – FlyBase) is specifically required to induce PC apoptosis. We have also shown that in supernumerary PCs destined to die, *hid* transcription is specifically activated and *hid* function is necessary for downregulation of *Drosophila* Iap1 (Diap1; Thread – FlyBase). However, the signal inducing *hid* expression, and thereby activating the apoptotic cascade in supernumerary PCs, remains unknown.

PCs act as an important organising centre at different stages of follicle development through secretion of Unpaired (Upd; Outstretched – FlyBase), a demonstrated ligand of the JAK/STAT pathway (Grammont and Irvine, 2002; Xi et al., 2003). The *Drosophila* JAK/STAT pathway is more simple than its vertebrate counterpart, as there is only one each of the receptor (Domeless), the Janus kinase (Hopscotch) and the Signal-transducer and activator of transcription protein at 92E (Stat92E) (Zeidler et al., 2000; Arbouzova and Zeidler, 2006). During early oogenesis, Upd and JAK/STAT signal transduction are necessary for induction of interfollicular stalk cell differentiation and encapsulation of follicles as they emerge from the germarium (Fig. 1A) (Baksa et al., 2002; McGregor et al., 2002). During mid-oogenesis, Upd-mediated JAK/STAT signalling at anterior follicle poles displays morphogen activity in instructing distinct cell fates among neighbouring follicle cells (Beccari et al., 2002; Grammont and Irvine, 2002; Xi et al., 2003; Devergne et al., 2007; Starz-Gaiano et al., 2008). The five to seven follicle cells immediately adjacent to the PCs are specified as border cells (BCs); further away stretch cells are generated and even further centripetal cells are produced. The BC/PC group migrates to the oocyte (Fig. 1A), upon which it is responsible for generating the micropyle, the sperm entry point.

[1]Centre de Génétique Moléculaire (UPR3404), Centre National de la Recherche Scientifique, 1 avenue de la Terrasse, 91198 Gif-Sur-Yvette, France. [2]Université Pierre et Marie Curie Paris 6, Ecole Doctorale Complexité du Vivant, 4 Place Jussieu, 75005 Paris, France. [3]Université Paris-Sud 11, UFR des Sciences, 91405 Orsay, France. [4]Université de Versailles/St Quentin, UFR des Sciences, 78035 Versailles, France.

*Authors for correspondence (fagnes@cgm.cnrs-gif.fr; pret@cgm.cnrs-gif.fr)

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Restriction of PC number to two is physiologically necessary for PC organiser function, as the presence of excess PCs during late oogenesis, produced by blocking apoptosis, leads to defects in BC migration and stretch cell morphogenesis (Besse and Pret, 2003; Khammari et al., 2011).

As the STAT proteins have been implicated in apoptosis in mammals (Battle and Frank, 2002; Kim and Lee, 2007), and *Drosophila* JAK/STAT pathway mutants have been shown to affect PC number (Baksa et al., 2002; McGregor et al., 2002), we were interested in testing whether Upd could provide the signal for PC apoptosis. The interpretation of JAK/STAT pathway mutant phenotypes during early *Drosophila* oogenesis is hampered, however, by the multiple roles played by this pathway (McGregor et al., 2002; Xi et al., 2003; Starz-Gaiano et al., 2008). Using RNA interference (RNAi) and UAS/Gal4 for spatiotemporal gene inactivation, as well as clonal analysis of classical mutants, we provide evidence for a new role of *upd* and JAK/STAT pathway component genes in PC apoptosis.

MATERIALS AND METHODS

Drosophila stocks and crosses

The following fly stocks were used for RNAi experiments (IR corresponds to 'inverted repeat'): *UAS-updIR* (#3282), *UAS-domeIR* (#36355), *UAS-Stat92EIR* (#43866), *UAS-hopIR* (#102830) and *UAS-NotchIR* (#27228) from the Vienna Drosophila RNAi Center in Austria, and *UAS-UpdIR* (5993R-1, 5993R-2) from NIG-FLY in Japan. *FRT82B*, *Stat92E*^{397}/TM3,Sb and *FRT82B*, *Stat92E*^{1681}/TM3,Sb stocks [a gift from D. Montell (Starz-Gaiano et al., 2008)] were used to generate *Stat92E* mosaic mutant follicle. *2XStat92E*-GFP, an insertion (third chromosome) of a GFP construct fused downstream of two Stat92E-binding sites, was used to monitor Stat92E activity [a gift from E. Bach (Bach et al., 2007)]. The enhancer trap *upd-Gal4* was used to target PCs specifically (Khammari et al., 2011). *fruitless-Gal4* (Boquet et al., 2000) (whose ovarian expression pattern is first characterised in this study) was used to target expression in terminal follicle cells except PCs. *tubP-Gal80*^{ts} (7016) from the Bloomington Stock Center was used to avoid embryonic lethality associated with expression of *UAS-Stat92EIR*. *UAS-nls:*GFP allows expression of nuclear GFP. *UAS-mCD8:G*FP allows expression of GFP at the membrane and in the cytoplasm (Lee and Luo, 1999). UAS-p35 (Hay et al., 1994) allows expression of p35, a baculovirus caspase inhibitor. The *UAS-Upd* line was obtained from Martin Zeidler (Zeidler et al., 1999; Beccari et al., 2002). Two Notch transcriptional reporters were used, *GbeSu*(*H*)*m8-lacZ* and *E*(*spl*)*m*β*-lacZ* (Kramatschek and Campos-Ortega, 1994; Cooper et al., 2000; Furriols and Bray, 2001; Vachias et al., 2010). All crosses for Gal4-induced RNAi and reporter expression were performed at 25°C until eclosion and newly-eclosed adult females were placed at 29°C until dissection 5 or 9 days later. For crosses involving *Gal80*^{ts}, crosses were carried out at 20°C, shifted to 25°C at third instar larva and to 29°C at eclosion before dissecting 4- to 5-day-old females.

To generate *Stat92E* mosaic mutant follicle cells, *Stat92E*^{397}, FRT82B and *Stat92E*^{1681}, FRT82B flies were crossed to hs-FLP ;; ubiquitin-nuclear-GFP, FRT82B flies. Clones marked by absence of GFP were induced by three 1-hour heat shocks at 37°C at mid pupa, at eclosion and 2 days after eclosion. Adult females were dissected for ovary analysis 4-5 days after eclosion.

Egg chamber immunostaining

Females were anaesthetised in CO_2 and decapitated and the ovaries were dissected in PBS. Ovaries were fixed for 20 minutes at room temperature in 4% formaldehyde in PBS. After several rinses, pre-absorption was carried out in PBS supplemented with 2% BSA and 0.3% Tween-20 for 2 hours at room temperature. Incubations with primary antibodies were carried out on a shaker overnight at 4°C. The ovaries were then rinsed several times in PBS supplemented with 0.3% Tween-20 and incubated 2 hours with secondary antibodies and DAPI. After several rinses in PBS, ovaries were incubated with TO-PRO-3 (Invitrogen) at 4°C at least overnight. Ovaries were then further dissected to separate ovarioles and mounted in Dabco.

The following primary and secondary antibodies were used for conventional immunofluorescence: rabbit anti-Upd (gift from D. Harrison, University of Kentucky, Lexington, USA) at 1:500, rabbit anti-GFP (Interchim) at 1:500, mouse monoclonal anti-Fasciclin 3 (DSHB, 7G10) at 1:20, guinea pig and rabbit polyclonal anti-Hid (gift from D. Ryoo, New York University, USA) at 1:100, mouse monoclonal anti-Diap1 (B. Hay laboratory, California Institute of Technology, Pasadena, CA, USA) at 1:200, mouse monoclonal anti-Lamin C (DSHB, LC28.26) at 1:500, rabbit anti-Slbo at 1:2000 (gift from C. Ghiglione) (Van de Bor et al., 2011), rabbit anti-human cleaved caspase 3 (1:20, Ozyme), mouse anti-Notch^{intra} (DSHB, C17.9C6) and anti-mouse-Cy3 and anti-rabbit-Alexa Fluor 488 secondary antibodies at 1:200 (Invitrogen). DAPI (1 ng/ml final concentration) and TO-PRO-3 (10 μM final concentration) were used to stain nuclei. Immunodetection of extracellular Upd was carried out on ovaries cultured as in a study by Prasad et al. (Prasad et al., 2007) and treated as described (Strigini and Cohen, 2000; Van de Bor et al., 2011).

Microscopy, image processing and cell counting

Epifluorescence images were taken using a Leica DMRB microscope, a Q-imaging Retiga 2000R camera and the Image-Pro Express 6 software. Confocal images were taken using the Nikon eclipse TE 2000-U microscope and the EZ-C1 3.30 software. Confocal image stacks were exported in original format and processed with ImageJ software (1.36b). Captured images from *z*-stack projections were processed and annotated using Adobe Photoshop CS3. The confocal images represent *z*-stack projections of three to five consecutive slices (0.2-0.3 μm per slice).

RESULTS

Upd is implicated in PC number reduction during early oogenesis

In order to avoid perturbing early *upd* function in the germarium, we targeted inhibition of *upd* in PCs using upstream activator sequence (UAS) transgenic constructs allowing expression of *upd* interfering dsRNAs (Montgomery, 2004) and a GAL4 enhancer-trap within the *upd* gene, which is expressed in PCs from stage 2 (Fig. 1C) (Khammari et al., 2011). Expression of a *UAS-updRNAi* construct (VDRC3282) in PCs led to strong reduction of Upd protein accumulation from stage 2 (supplementary material Fig. S1A,B) and strong border migration defects (Fig. 1E,F; supplementary material Fig. S1C-E), like those reported for *upd* and JAK/STAT pathway component mutants (Silver and Montell, 2001; Ghiglione et al., 2002; Silver et al., 2005). Rare apposed and multicyst follicles (<5% of ovarioles, data not shown) reflecting encapsulation defects during follicle formation as expected for reduction of JAK/STAT pathway activity (McGregor et al., 2002) confirmed that the conditions used do not affect *upd* function in the germarium to a great extent.

We assayed PC number in nascent stage 2 follicles using a GFP reporter construct driven by *upd-Gal4* (Fig. 1C,D) and using Fas3 immunodetection for stages 3-10 (Fig. 1E,F) as this marker is not specific to PCs before stage 3 (Fig. 1C,D) (Adam and Montell, 2004; Khammari et al., 2011). In both control and *upd* RNAi-expressing stage 2 follicles, the vast majority of follicle poles contain more than two PCs (average of 74% and 80%, respectively; Fig. 1G). By stages 3-4, in the control, only 20-24% of poles exhibited more than two PCs, indicative of PC number reduction by apoptosis (Fig. 1G). PC apoptosis continued through stages 5-6 in the control and beyond these stages, there were almost no poles with more than two PCs (Fig. 1G). By contrast, upon expression of *upd* RNAi, groups of three or four PCs (and rarely five or six PCs) were observed through stage 10 and beyond (Fig. 1F,G; data not shown) in a statistically significant proportion of follicle poles (Fig. 1G). Two other

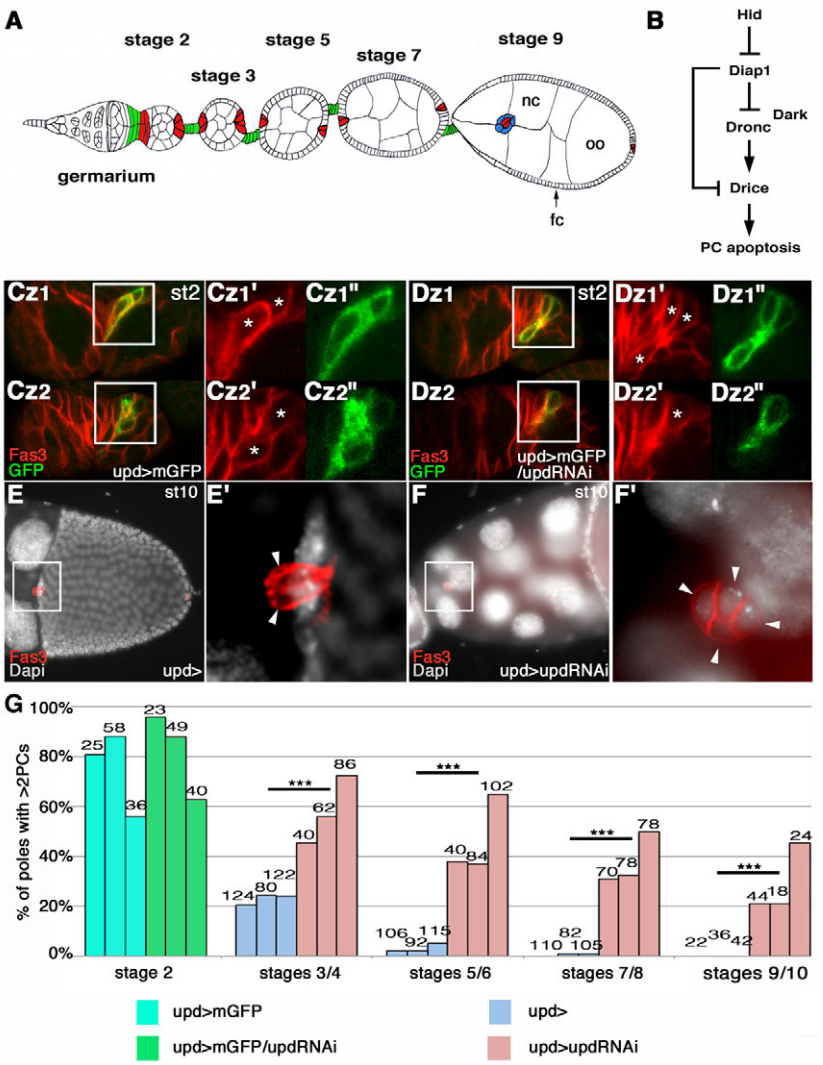

Fig. 1. *Upd* function is involved in PC number reduction to two. (**A**) Schematic drawing of an ovariole showing the anteriorly positioned germarium from which follicles (or egg chambers) are formed, subsequently maturing posteriorly (stages 2-9 of 14 stages are shown). PCs are indicated in red, interfollicular stalk cells in green and BCs in blue. Follicle cells surround the germline cyst composed of 15 nurse cells and one oocyte. fc, follicle cell; nc, nurse cell; oo, oocyte. (**B**) The apoptotic cascade involved in supernumerary PC apoptosis. (**C**,**D**) Control (C) and *upd* RNAi (D) stage 2 follicles with four anterior PCs (asterisks) immunostained for GFP and Fas3, the latter also marking adjacent follicular cells. Panels with primes correspond to magnified views of the boxed regions in the panels sharing the same letter. Two consecutive *z*-confocal sections (z1 and z2) are presented in order to visualise all the PCs in a given cluster. (**E-F′**) Epifluorescence images of control (E) and *upd* RNAi (F) stage 10 follicles displaying two and four anterior PCs, respectively, immunostained for Fas3 (arrowheads). Panels marked with primes are magnified views of boxed areas in panels sharing the same letter. DAPI staining reveals all nuclei for staging follicles. (**G**) Percentage of PC clusters containing more than two cells in control and *upd* RNAi-expressing follicles. Stage 2 PCs were counted using mCD8:GFP expressed specifically in PCs and stage 3-10 PCs using Fas3 immunostaining. The results of three independent experiments are presented in the same order for each stage and genotype. The numbers above each bar indicate the total number of follicle poles analysed for that point. Statistically significant differences according to a Chi-square test between control and experimental genotypes are indicated with a bar and asterisks according to same code that is used throughout the entire report (**P*<0.05, ***P*<0.01, ****P*<0.001). Genotypes: upd>mGFP: *upd-Gal4/+;UAS-mCD8:GFP/+*; upd>mGFP/updRNAi: *upd-Gal4/+;UAS-mCD8:GFP/UAS-updRNAi/+*; upd>: *upd-Gal4/+*; upd>updRNAi: *upd-Gal4/+;UAS-updRNAi*.

transgenic UAS-RNAi lines targeting *upd* mRNA also induced supernumerary PCs at late stages (data not shown). Importantly, reduction of *upd* never led to the production of PC clusters with more cells than that observed normally in the control at early stages before apoptosis (six PCs maximum), suggesting that there was no overproliferation of these cells. In addition, Phosphohistone-H3 immunostaining indicated that no cell division occurred in PC clusters from stage 2 onwards upon *upd* downregulation (data not shown). Finally, Upd depletion also led to a reduction in interfollicular stalk cell number as previously reported (McGregor et al., 2002), but this effect was independent of the increase in PC number (supplementary material Fig. S2). Taken together, these results implicate *upd* function specifically in PC number reduction.

upd is necessary for activation of the apoptotic machinery in supernumerary PCs

To determine if Upd-dependent reduction of PC number depends on apoptosis, we analysed the expression of three different apoptotic markers. Hid is a pro-apoptotic factor specifically transcribed in PCs destined to die and necessary for normal PC apoptosis (Khammari et al., 2011). Upregulation of Hid in PCs destined to die is readily detectable only if caspases are inhibited (e.g. upon expression of the baculovirus caspase inhibitor p35) (Ryoo et al., 2004; Khammari et al., 2011). In the control, 67% of clusters with more than two PCs showed Hid accumulation in supernumerary PCs between stages 2 and 6 (Fig. 2A,H), whereas 39% did so between stages 7 and 10 (Fig. 2H). By contrast, simultaneous expression of p35 and *upd* RNAi in PCs led to a minority of clusters with more than two PCs displaying Hid accumulation at early and late stages (6% and 7%, respectively) (Fig. 2B,H). These results show that *upd* is necessary for upregulation of Hid expression in PCs destined to die.

We also analysed the accumulation of Diap1, which has been shown to be downregulated in supernumerary PCs (Khammari et al., 2011). In the control, Diap1 downregulation in supernumerary PCs was observed at early stages in 47% of clusters with more than two PCs (Fig. 2C′, arrowhead, 2I). When *upd* RNAi was expressed, Diap1 downregulation was not significantly different from that in the control at early stages (Fig. 2I), possibly because apoptosis still occurs during these stages (Fig. 1G). However, at later stages (9-10), the majority of clusters (81%) with supernumerary PCs presented high levels of Diap1 in all PCs (Fig. 2D,I). These results implicate *upd* function in downregulation of Diap1 in supernumerary PCs and consequent apoptosis of these cells.

Using antibodies directed against activated human Caspase 3, we found that caspase activation in the control occurs in supernumerary PCs in 15% of early follicle poles (Fig. 2E,J). Upon RNAi-mediated downregulation of *upd*, 5% of early follicle poles

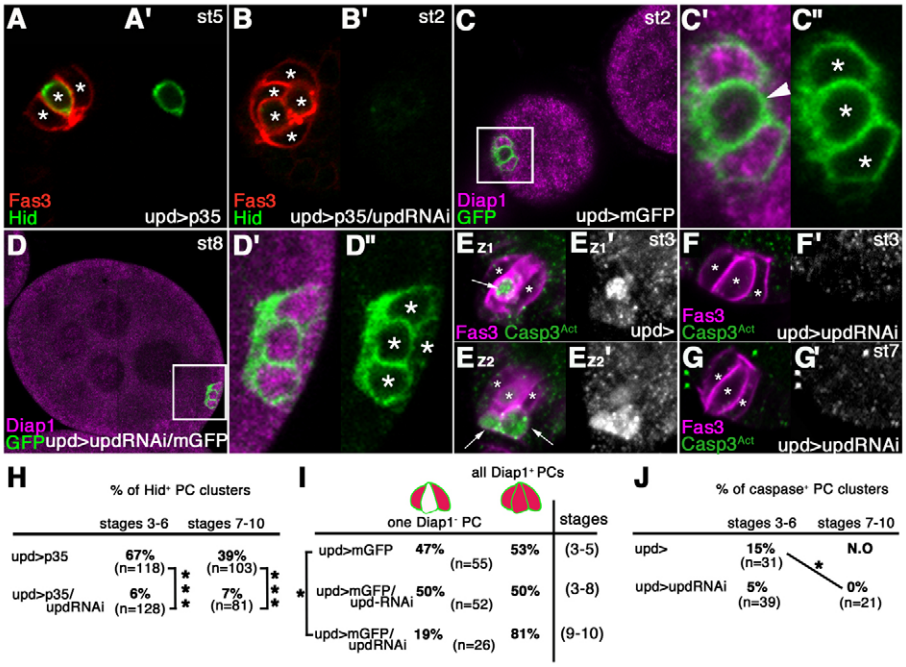

Fig. 2. *upd* regulates Hid, Diap1 and caspase accumulation in supernumerary PCs. (**A-B′**) Confocal images of PC clusters in control (A) and *upd* RNAi (B) follicles of the indicated stages immunostained for Fas3 to identify PCs (asterisks) and for Hid. Double- and single-channel (panels with primes) views are shown. Hid is detected in the supernumerary PCs in the control, but not in those present upon *upd* RNAi. (**C-D″**) Confocal images of control and *upd* RNAi follicles of indicated stages immunostained for GFP to identify PCs (asterisks) and for Diap1. Panels marked with primes are magnified views of boxed areas in panels sharing the same letter. In the control, a supernumerary PC (arrowhead in C′) displays a reduced level of Diap1 compared with its neighbours. Under *upd* RNAi conditions, all PCs, including two supernumerary cells in a cluster of four PCs (D′) display a high level of Diap1. (**E-G′**) Supernumerary PC clusters in control (E) and *upd* RNAi (F,G) follicles of the indicated stages immunostained for Fas3 to identify PCs and for activated caspases ($Casp^{Act}$). The three supernumerary PCs in the control (E, arrows) that accumulate activated caspases are visualised in two consecutive *z*-confocal sections (z1 and z2). No active caspase staining is detected in the supernumerary PCs under *upd* RNAi conditions (F,G). (**H**) Percentages of clusters with more than two PCs exhibiting Hid staining in supernumerary PCs at early (3-6) and late (7-10) stages for control and *upd* RNAi ovaries. (**I**) Percentages of clusters with more than two PCs exhibiting at least one supernumerary $Diap1^{-}$ PC or all $Diap1^{+}$ PCs for control and *upd* RNAi ovaries at the indicated stages. (**J**) Percentages of clusters with more than two PCs exhibiting caspase activity in supernumerary PCs at early (3-6) and late (7-10) stages for control and *upd* RNAi ovaries. For (H-J), n signifies the total number of clusters with more than two PCs analysed and asterisks, the statistically significant differences according to a Chi-square (H,I) or Fischer's exact (J) test (see Fig. 1 for *P*-value code). Genotypes: upd>p35: *upd-Gal4/+;UAS-p35/+*; upd>p35/updRNAi *upd-Gal4/+;UAS-p35/UAS-upd-RNAi*; upd>mGFP: *upd-Gal4/+;UAS-mcd8:GFP/+*; upd>updRNAi/mGFP: *upd-Gal4/+;UAS-mcd8/UAS-upd-RNAi*; upd>: *upd-Gal4/+*; upd>updRNAi: *upd-Gal4/+;UAS-upd-RNAi*. N.O, not observed.

displayed activated caspase staining (Fig. 2F,J), whereas after stage 6, no follicle pole with supernumerary PCs presented caspase activation (Fig. 1G,J). These results indicate that reducing *upd* impedes caspase activation. Therefore, the excess PCs observed at late stages of oogenesis upon *upd* RNAi-mediated inhibition exhibit prolonged survival associated with failure to induce Hid expression, which prevents downregulation of Diap1 and therefore caspase activation.

JAK/STAT activity in terminal follicle cells is necessary for PC apoptosis

Using a Stat92E transcriptional reporter, JAK/STAT activity was found continuously in terminal follicle cells (TFCs) and interfollicular stalks and sporadically in PCs themselves (Fig. 3A-C). Expression of *upd* RNAi in PCs reduced Stat92E activity in all three cell types (Fig. 3D-F). To investigate whether JAK/STAT signal transduction is required for PC apoptosis, and if so in which cells, we generated mosaic follicles via the FLP/FRT system (Golic, 1991) using two distinct amorphic alleles of the *Stat92E* gene ($Stat92E^{397}$ and $Stat92E^{1681}$) and assayed the number of PCs at late stages of oogenesis using the Fas3 marker. In these experiments, *Stat92E* homozygous mutant clones were identified by absence of GFP. Using this approach, induction of homozygous mutant follicle cell clones for either *Stat92E* allele was associated with the presence of poles with more than two PCs (three or four, rarely five PCs) after stage 5 of oogenesis (Fig. 4A′,B′,C′, clones encircled with dotted lines). In particular, we found a strong correlation between large *Stat92E* mutant TFC clones surrounding the entire PC cluster and the presence of supernumerary PCs in the cluster at later stages of oogenesis (90%; Fig. 4A,A′). The presence of supernumerary PCs at late stages was less frequently associated with *Stat92E* mutant TFC clones that were smaller and only partially enveloping the PC cluster (39%; Fig. 4B,B′) or at a distance from PCs (45%; Fig. 4C,C′). In addition, *Stat92E* mutant TFC clones not in contact with PCs and associated with supernumerary PCs after stage 6 of oogenesis were never more than three cell diameters away from the PC cluster (Fig. 4C,C′; data not shown). These results support the conclusion that *Stat92E* function is necessary specifically in TFCs in proximity to PCs for efficient apoptosis of these cells.

To further confirm that the JAK/STAT pathway is transduced in TFCs for supernumerary PC apoptosis induction, we expressed *dome*

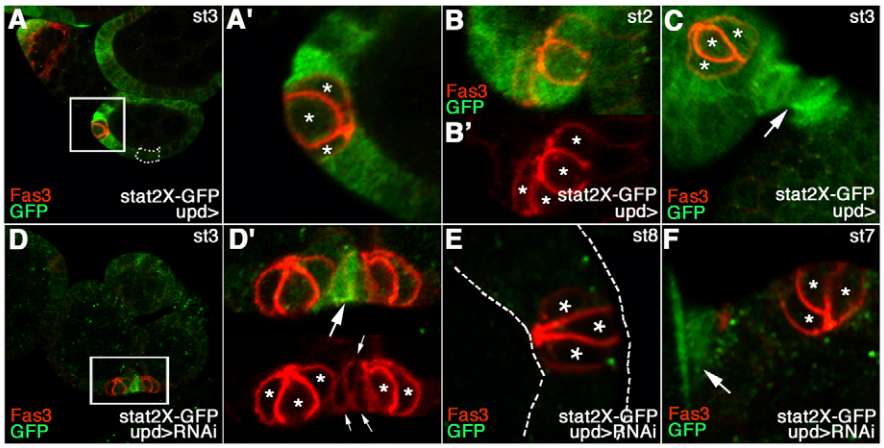

Fig. 3. *upd* controls Stat92E transcriptional activity in terminal follicle cells, interfollicular stalks and PCs. Confocal images acquired using identical parameters of control (**A-C**) and *upd* RNAi (**D-F**) follicles of the indicated stages immunostained for Fas3 to identify PCs (asterisks) and GFP to detect Stat92E reporter activity. B′ is a single-channel view of B. D′ is a magnification of the boxed area in D including both double- and single-channel views. In the majority of PC clusters with more than two PCs (71%, *n*=51), there is no Stat92E reporter activity (A,A′) and in the remaining cases, Stat92E activity is present in one or several PCs (B). High Stat92E reporter activity is present in terminal follicle cells surrounding PCs (A, boxed area and A′) and interfollicular stalks (C, arrow) and low in main body follicle cells (A, dashed lines). Upon induction of *upd* RNAi, Stat92E reporter activity is lost in terminal follicle cells (E, dashed lines) and PCs (E,F) and significantly diminished in interfollicular stalks (F, arrow). Genotypes: stat2X-GFP/upd>: *upd-Gal4/+;;2XStat92E-GFP/+*; stat2X-GFP/upd>updRNAi: *upd-Gal4/+;UAS-updRNAi/+;2XStat92E-GFP/+*.

and *hop* UAS-RNAi constructs in these cells using a *fruitless* (*fru*)-*Gal4* enhancer trap. This driver is not expressed in the germarium (Fig. 5A, dotted line), its expression appearing in TFCs, excluding PCs, from stage 2 (Fig. 5C). *fru-Gal4* driven reporter expression is higher in TFCs immediately adjacent to PCs (Fig. 5B,C) compared to main body follicle cells (Fig. 5C, dotted lines). Expression increases as oogenesis progresses, becoming particularly strong in BCs (Fig. 5A, arrow). *fru-Gal4* driver expression remains very low or absent in PCs (Fig. 5B,C, arrowheads). In addition, strong *fru-Gal4* driven GFP expression is also detected in terminal cells of each interfollicular stalk (Fig. 5D, arrows).

Whereas in the control, PC number was almost always two from stage 7, targeted expression of *dome* and *hop* RNAi with *fru-Gal4* led to the production of late-stage follicle poles with three to five PCs (Fig. 5I,J). At later stages of oogenesis, *fru-Gal4*-driven RNAi for both *dome* and *hop* induced strong BC migration defects like those previously reported for JAK/STAT mutants (Beccari et al., 2002) (supplementary material Fig. S1F-H), probably because

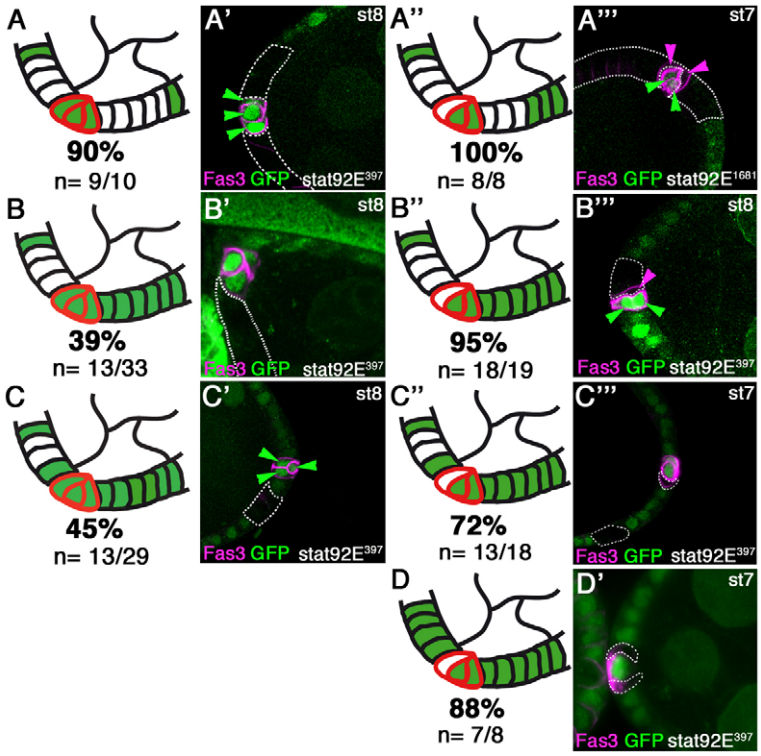

Fig. 4. *Stat92E* function is required in terminal follicle cells and PCs for PC number reduction to two. Schematic drawings of the different categories (**A-D**,**A″-C″**) of *Stat92E* homozygous mutant clones observed at follicle poles containing more than two PCs with corresponding confocal images (adjacent, right) (**A′-D′**,**A‴-C‴**) showing immunodetection of GFP to counterstain mutant cells (within dotted lines) and of Fas3 to count PCs. Mutant (white) and wild-type (green) epithelial follicle cells (black membranes) and PCs (red membranes) and the large adjacent germline cells are depicted. n represents the number of *Stat92E* mutant clones associated with more than two PCs after stage 6 over the total number of clones recovered (containing two or more than two PCs) after stage 6 for each category. The corresponding percentages are given just above each n. (A-C,A″-C″) Each line presents the same category of terminal follicle cell clone with (A-C) or without (A″-C″) associated mutant PCs. (A,A′) Large *Stat92E* mutant terminal follicle cell clones completely surrounding the PC cluster. (B,B′) Small *Stat92E* mutant terminal follicle cells in contact with only one side of a PC cluster. (C,C′) Small *Stat92E* mutant terminal follicle cell clones that are not in direct contact with PCs. (D) *Stat92E* mutant clones including only PCs. The size and position of each clone relative to the PC cluster was determined by analysing the stack of confocal images covering the entire depth of the follicle. Although germline *Stat92E* mutant clones were sometimes present, there was not a strict correlation between the presence of germline clones (not shown) and that of supernumerary PCs at late stages consistent with the absence of Stat92E reporter activity in germline cells (Fig. 3A).

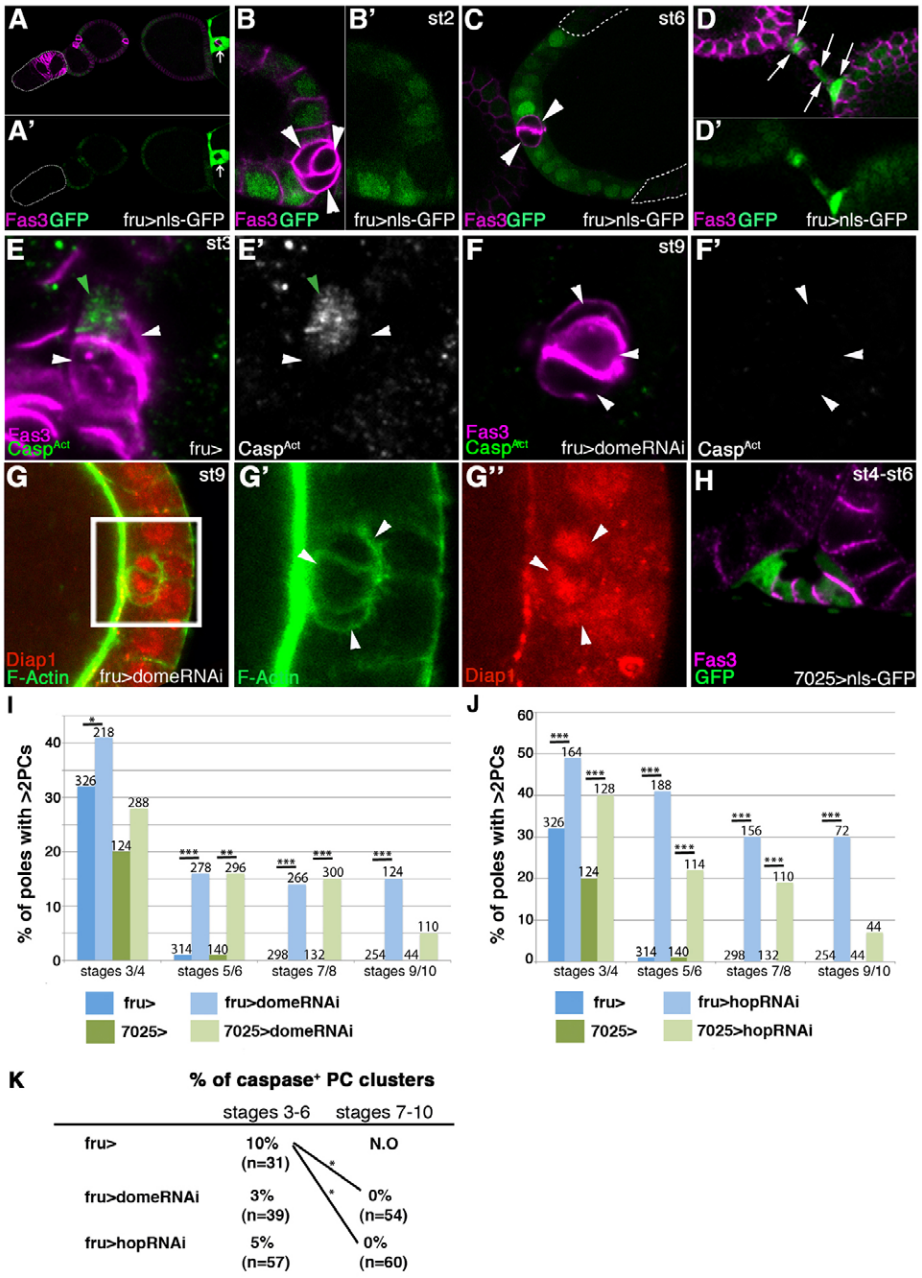

Fig. 5. *domeless* and *hopscotch* functions in TFCs and stalk cells are implicated in PC apoptosis. (**A-D'**) Confocal images of ovarioles that express nuclear GFP driven by the *fruitless* (*fru*)-*Gal4* driver. PCs immunostained for Fas3 are indicated by arrowheads (B,C), and GFP-expressing BCs and stalk cells by arrows (A,D, respectively). Panels with primes are single-channel views of the panel sharing the same letter. The germarium and main body follicle cells are delimited by dashed lines in A, A′ and C, respectively. Stages are indicated in B′ and C. (**E-F'**) Confocal images of PC clusters in control and *dome* RNAi follicles immunostained for Fas3 and activated caspases ($Casp^{Act}$). Panels with primes are single-channel views of the panel sharing the same letter. Stages are indicated. Caspase activity is detected in a supernumerary PC in the control (E,E′, green arrow), but not in the PC cluster of a later-stage *dome* RNAi follicle (F,F′). (**G-G″**) Confocal image of the posterior pole of a stage 9 *dome* RNAi-expressing follicle immunostained for Diap1 and stained for F-actin to reveal PCs by their position and shape (G and higher magnification of boxed area in G′, arrowheads). All three PCs accumulate Diap1 (G″, arrowheads). (**H**) Confocal image of two consecutive follicles and the intervening interfollicular stalk expressing GFP under the control of the *7025*-Gal4 driver. Immunostaining for Fas3 reveals the membranes of both interfollicular stalk cells and adjacent follicle cells. (**I**,**J**) Percentages of follicle poles exhibiting more than two PCs as a function of the stage of oogenesis, for control and *dome* or *hop* RNAi-expressing ovaries (I and J, respectively). The total number of follicle poles analysed is indicated above each bar. Statistically significant differences between control and experimental genotypes according to a Chi-square test are indicated with a bar and asterisks according to the code in Fig. 1. (**K**) Percentages of clusters of more than two PCs presenting caspase activity at early (3-6) and late (7-10) stages for control and *dome* and *hop* RNAi ovaries. In the control, no clusters of more than two PCs are observed at late stages. Statistically significant differences between control and experimental genotypes according to a Fisher's exact test are indicated with a bar and asterisks according to the code in Fig. 1. Genotypes: fru>nls-GFP: *UAS-nls:GFP/+;fru-Gal4/+*; fru>: *fru-gal4/+*; fru>domeRNAi: *fru-Gal4/UAS-domeRNAi*; fru>hopRNAi: *fru-Gal4/UAS-hopRNAi*; 7025>nls-GFP: *UAS-nls:GFP/+;7025-Gal4/+*; 7025>: *7025-Gal4/+*; 7025>domeRNAi: *7025-Gal4/UAS-dome-RNAi*; 7025>hopRNAi: *7025-Gal4/UAS-hopRNAi*. N.O, not observed.

expression of the *fru-Gal4* driver is much stronger in BCs than any other ovarian cells (Fig. 5A, arrow).

We next tested whether *dome* or *hop* RNAi-mediated reduction using *fru-Gal4* had an effect on the expression of apoptosis markers in supernumerary PCs normally destined to die. Caspase activation in the control, as evidenced by immunostaining for activated human Caspase 3, occurred in supernumerary PCs in 10% of early stage follicle poles (Fig. 5E,K). By contrast, reduction of *dome* and *hop* function led, respectively, to 3% and 5% of follicle poles exhibiting caspase activation in supernumerary PCs at early stages and no caspase activation in these cells at late stages (Fig. 5F,K). Therefore, *dome* and *hop* functions are implicated in caspase activation in supernumerary PCs normally destined to die.

In order to test Diap1 accumulation, it was necessary to use a different PC marker from Fas3, as available antibodies for these two proteins originate from the same species. Phalloidin coupled to a fluorochrome was used to detect subcortical F-actin allowing recognition of PCs due to their shape (Fig. 5G,G′). Upon *fru-Gal4* driven expression of *dome* and *hop* RNAi constructs, supernumerary PCs exhibited prolonged survival after stage 5 of oogenesis and always accumulated high levels of the anti-apoptotic factor Diap1 like their neighbouring PCs and TFCs ($n \geq 15$) (Fig. 5G,G″), indicating that JAK/STAT signal transduction is necessary for Diap1 downregulation normally observed in supernumerary PCs destined to die (Fig. 2C,C′). Taken together with the results for the *Stat92E* mutant TFC clone analysis, JAK/STAT pathway function is implicated specifically in TFCs, and possibly in stalk cells, for induction of PC apoptosis.

JAK/STAT activity in interfollicular stalks participates to PC apoptosis

As the *fru-Gal4* driver is expressed in both TFCs and interfollicular stalks, we wanted to test whether JAK/STAT signal transduction specifically in stalk cells is also implicated in PC apoptosis. It was not possible to recover *Stat92E* homozygous mutant stalk cell clones, probably because JAK/STAT signal transduction is necessary for stalk cell specification (McGregor et al., 2002). We therefore used *7025-Gal4*, which is expressed only in stalk cells throughout oogenesis (Fig. 5H), to induce *dome* and *hop* RNAi. At stages 3-4, before PC apoptosis is completed in the control, supernumerary PCs were observed more frequently upon expression of either *dome* or *hop* RNAi than in the control (Fig. 5I,J, green bars). Between stages 5 and 8, almost all follicle poles in the control exhibited PC number reduction to two, whereas expression of *dome* or *hop* RNAi in stalk cells led to 16% and 22% of follicle poles with supernumerary PCs, respectively. However, at stages 9-10, *dome* and *hop* RNAi expression in stalk cells was associated with only ~5% of follicle poles with extra PCs. These results suggest that at early stages, JAK/STAT signal transduction in stalk cells participates to PC apoptosis, but as oogenesis progresses, inhibition of JAK/STAT signal transduction in these cells can be compensated for and thus only causes a delay in PC number reduction. Indeed, this transient participation could be explained by the fact that interfollicular stalks and PCs are in proximity during early oogenesis, after which these two cell types are displaced away from each other (supplementary material Fig. S2A-C) (F.A. and A.-M.P., unpublished results).

JAK/STAT activity is necessary in PCs for efficient PC apoptosis

Upd-dependent Stat92E reporter activity was present sporadically in PCs themselves (Fig. 3B), indicating that JAK/STAT signal transduction occurs in these cells, as in TFCs and stalks. We therefore tested whether the activity of JAK/STAT pathway components is necessary in PCs for apoptosis. We recovered a small number of $Stat92E^{397}$ and $Stat92E^{1681}$ homozygous clones composed only of PCs in follicles after stage 5 and almost all of these (7/8) exhibited supernumerary (one to three) PCs (Fig. 4D,D′). In addition, we recovered numerous *Stat92E* mutant PC clones associated with nearby TFC clones after stage 5 and the vast majority of these exhibited supernumerary (one to three) PCs (Fig. 4A″,A‴,B″,B‴,C″,C‴). Importantly, while 39% and 45% of small TFC *Stat92E* mutant clones were associated with the presence of supernumerary PCs depending on the position of the clone (Fig. 4B,B′,C,C′), when at least one PC was also mutant for *Stat92E*, the frequency of supernumerary PCs after stage 5 was much higher (95% and 72%, respectively; Fig. 4B″,B‴,C″,C‴). Taken together, these results implicate *Stat92E* function within PCs for PC apoptosis.

RNAi-mediated knockdown of *dome*, *hop* and *Stat92E* specifically in PCs was also performed using the *upd-Gal4* driver. At stages 3-4, 30% of follicle poles in the control had not completed PC number reduction, whereas 50% and 55% of follicles expressing *dome* and *hop* RNAi in PCs, respectively, had not done so (Fig. 6A). As of stage 5, in the control almost 100% of follicle poles had undergone PC number reduction to two, whereas expression of *dome* and *hop* RNAi in PCs led to the presence of supernumerary PCs (one to three) at least through stage 10 (Fig. 6A). RNAi targeting of *Stat92E* using the *upd-Gal4* driver led to embryonic lethality and expression of a tubulin promoter-Gal80^{ts} construct was used to circumvent this problem. Under these conditions, 46% (n=234) of follicle poles between stages 7 and 10 of oogenesis presented more than two PCs (three to five), whereas 100% of follicle poles in the control contained only two PCs (n=106) ($P<10^{-15}$ according to a Chi-square test). Therefore, reducing *dome*, *hop* or *Stat92E* function in PCs impedes efficient PC number reduction to two.

We next tested whether RNAi-mediated reduction of *dome* in PCs would affect Hid expression in supernumerary PCs normally destined to die. When p35 was expressed alone, 48% of early-stage follicles and 31% of late-stage follicles exhibited Hid accumulation in supernumerary PCs (Fig. 6B,D). By contrast, co-expression of *p35* and *dome* RNAi in these cells led to only 10% and 7.2% of early- and late-stage follicles, respectively, accumulating detectable levels of Hid (Fig. 6C,D). These results implicate *dome* function in PCs for Hid expression and consequent apoptosis.

As expression of one of the Stat92E transcriptional reporters is variable within PC clusters in early-stage wild-type follicles (Fig. 3B; data not shown), we tested whether any correlation exists between activation of this reporter and that of Hid in supernumerary PCs normally destined to die. To do this, p35 was expressed in PCs and Stat92E activity was detected using the Stat2X-GFP reporter. Interestingly, when Hid accumulation was detected in supernumerary PCs, in the majority of cases (80%, n=40), Stat92E reporter activity was highest in the Hid$^+$ PC compared with that in the other PCs of the same cluster (Fig. 6E). This result suggests that JAK/STAT signal transduction is also implicated cell autonomously within supernumerary PCs for apoptosis.

Finally, when the *upd-Gal4* PC driver was combined with the *fru-Gal4* TFC/stalk driver to reduce *dome* expression by RNAi in all three cell types, 62% of follicle poles between stages 7 and 10 (n=55) displayed supernumerary PCs (1-4), whereas only 15% and 14%, respectively, of follicle poles had supernumerary PCs at these stages when *dome* was targeted with each driver alone (Fig. 5I; Fig. 6A). Taken together, our results strongly suggest that JAK/STAT

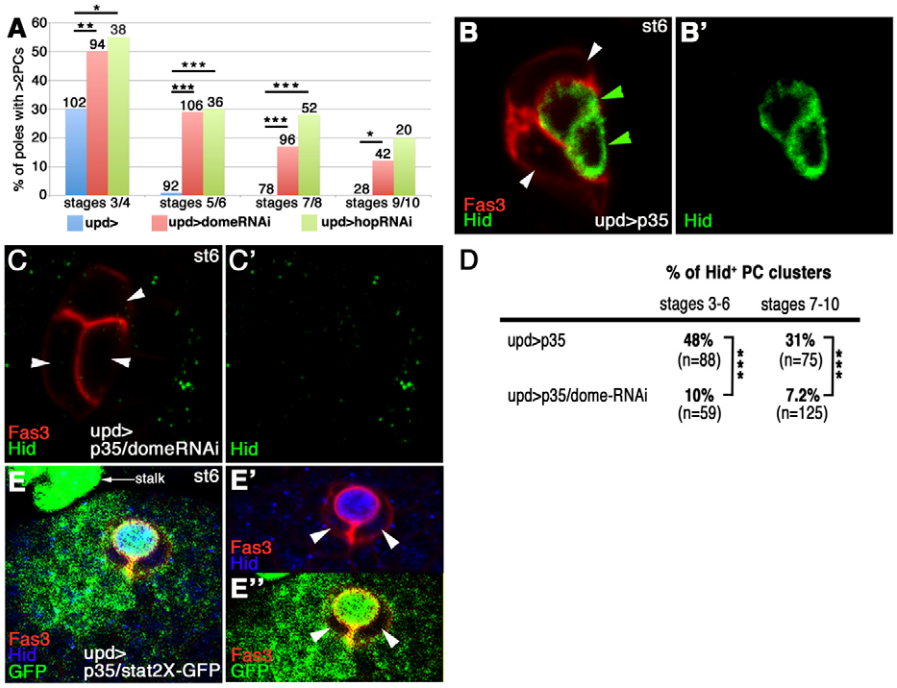

Fig. 6. JAK/STAT signal transduction in PCs is involved in Hid activation and PC number reduction to two. (**A**) Percentage of PC clusters containing more than two cells in control and *dome* and *hop* RNAi follicles of the stages indicated. The total number of follicle poles analysed is indicated above each bar. Statistically significant differences between control and experimental genotypes according to a Chi-square test are indicated with a bar and asterisks according to the code in Fig. 1. (**B-C′**) Confocal images of PC clusters of control (B) and *dome* RNAi (C) follicles immunostained for Hid and Fas3 to identify PCs (arrowheads). Panels with primes are single-channel views of the panels sharing the same letter. Hid is detectable in supernumerary PCs in the control (B, green arrows, B′), but not in a *dome* RNAi-expressing follicle (C,C′). (**D**) Percentages of clusters of more than two PCs exhibiting Hid staining in supernumerary PCs at early (3-6) and late (7-10) stages for control and *dome* RNAi follicles. n indicates the total number of clusters with more than two PCs analysed. Statistically significant differences between control and experimental genotypes according to a Chi-square test are indicated with a bar and asterisks according to the code in Fig.1. (**E-E″**) Confocal image of Stat92E GFP reporter expression (E,E″, green) in follicles expressing p35 immunostained for Fas3 to identify PCs (E-E″) and Hid (E,E′). The Hid-positive supernumerary PC presents a high level of Stat92E reporter activity compared with the two other PCs (arrowheads). Genotypes: upd>: upd-*Gal4*/+; upd>domeRNAi>: *upd-Gal4/+;;UAS-dome-RNAi/+*; upd>hopRNAi: *upd-Gal4/+;;UAS-hop-RNAi/+*; upd>p35: *upd-Gal4/+;UAS-p35/+*; upd>p35/domeRNAi: *upd-Gal4+;UAS-p35+;/UAS-domeRNAi/+*; upd>p35/stat2X-GFP: *upd-Gal4/+;UAS-p35/+;2XStat92E-GFP/+*.

signal transduction in all three cell types, TFCs, PCs and stalk cells, is necessary for fully efficient reduction of PC number to two.

DISCUSSION

A role for STAT in cell death and survival has been clearly documented in mammals, and depending on which of the seven mammalian Stat genes is considered and on the cellular context, both pro- and anti-apoptotic functions have been characterised (Battle and Frank, 2002; Stephanou and Latchman, 2005; Kim and Lee, 2007; Brumatti et al., 2010). In the *Drosophila* developing wing, phosphorylated Stat92E has been shown to be necessary for protection against stress-induced apoptosis, but not for wing developmental apoptosis (Betz et al., 2008). Here we provide evidence that Upd and the JAK/STAT pathway control developmental apoptosis during *Drosophila* oogenesis.

JAK/STAT signalling controls PC apoptosis

We demonstrate that the JAK/STAT pathway ligand, Upd, and all components of the JAK/STAT transduction cascade (the receptor Dome, JAK/Hop and Stat92E) are involved in promoting apoptosis of supernumerary PCs produced during early oogenesis. We argue that this pathway is essential for this event for several reasons. Indeed, in the strongest mutant context we tested, follicle poles containing large TFC and PC clones homozygous for *Stat92E* amorphic alleles, almost all of these (95%) maintained more than two PCs through oogenesis. Also, RNAi-mediated reduction of *upd*, *dome* and *hop* blocked PC number reduction and deregulated several apoptosis markers, inhibiting Hid accumulation, Diap1 downregulation and caspase activation in supernumerary PCs. Altogether, our data, along with what has already been shown for JAK/STAT signalling in this system, fit the following model (Fig. 7). Upd is secreted from PCs and diffuses in the local environment (supplementary material Figs S1, S3) (Harrison et al., 1998; Xi et al., 2003). Signal transduction via Dome/Hop/Stat92E occurs in nearby TFCs, interfollicular stalks and PCs themselves, leading to specific target gene transcription in these cells, as revealed by a number of pathway reporters (Fig. 3; data not shown) (Harrison et al., 1998; Ghiglione et al., 2002; Silver et al., 2005). An as-yet-unidentified Stat92E-dependent pro-apoptotic relay signal (X) is produced in TFCs, interfollicular stalks and possibly PCs, which promotes supernumerary PC elimination via specific expression of *hid* in these cells, consequent downregulation of Diap1 and finally caspase activation. An additional cell-autonomous role for JAK/STAT signal transduction in supernumerary PC apoptosis of these cells is also consistent with, though not demonstrated by, our results.

Relay signalling allows for spatial and temporal positioning of multiple signals in a tissue and thus exquisite control of

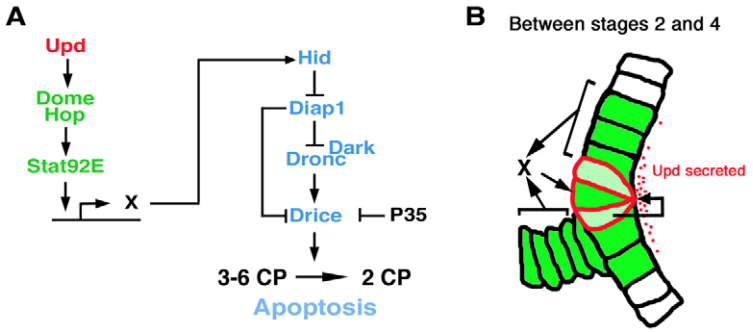

Fig. 7. Model for JAK/STAT pathway-dependent regulation of supernumerary PC apoptosis. (**A**) Schematic representation of the interaction between Upd (red) and the JAK/STAT signalling pathway (green) and the apoptotic cascade activated in supernumerary PCs (blue) via an as yet unidentified relay factor X. (**B**) Drawing of a transverse view of a follicle pole showing the follicular epithelium (vertically oriented with apical to the right), part of an interfollicular stalk to the left and three PCs (red membranes). Upd (red dots) is secreted apically from PCs and diffuses locally, inducing activation of the JAK/STAT signalling pathway in terminal follicle cells, stalks and PCs (green cells). An unknown JAK/STAT-dependent relay signal (X) is produced by TFCs, interfollicular stalks, and possibly PCs, eliciting the apoptotic cascade in supernumerary PCs. The JAK/STAT pathway may also be necessary in supernumerary PCs for direct induction of apoptosis without a relay signal (arrow in PCs).

differentiation and morphogenetic programmes (Papayannopoulos et al., 1998; Strigini and Cohen, 1999; Tickle, 2003). In the *Drosophila* developing eye, the role of Upd and the JAK/STAT pathway in instructing planar polarity has been shown to require an as-yet-uncharacterised secondary signal (Zeidler et al., 1999). In the ovary, the fact that JAK/STAT-mediated PC apoptosis depends on a relay signal may provide a mechanism by which PC apoptosis and earlier JAK/STAT-dependent stalk-cell specification can be separated temporally.

Although neither the identity, nor the nature, of the relay signal are known, it is possible to propose that the signal is not likely to be contact-dependent, and could be diffusible at only a short range. Indeed, *Stat92E* homozygous mutant TFC clones in contact with PCs, as well as those positioned up to three cell diameters away from PCs, are both associated with prolonged survival of supernumerary PCs, whereas clones further than three cell diameters away from PCs are not. In addition, fully efficient apoptosis of supernumerary PCs may require participation of all surrounding TFCs, stalk cells and possibly PCs, for production of a threshold level of relay signal. In support of this, large stat mutant TFC clones are more frequently associated with prolonged survival of supernumerary PCs, and the effects of removing JAK/STAT signal transduction in several cell populations at the same time are additive. Interestingly, the characterisation of two other *Drosophila* models of developmental apoptosis, interommatidial cells of the eye and glial cells at the midline of the embryonic central nervous system, also indicates that the level and relative position of signals (EGFR and Notch pathways) is determinant in selection of specific cells to be eliminated by apoptosis (Bergmann et al., 2002; Monserrate and Brachmann, 2007).

Our results indicate that only the supernumerary PCs respond to the JAK/STAT-mediated pro-apoptotic relay signal, whereas two PCs per pole are always protected. Indeed, we found that overexpression of Upd did not lead to apoptosis of the mature PC pairs and delayed rather than accelerated elimination of supernumerary PCs (supplementary material Fig. S1I). Recently, it was reported that selection of the two surviving PCs requires high Notch activation in one of the two cells and an as-yet-unknown Notch-independent mechanism for the second cell (Vachias et al., 2010). Intriguingly, expression of both Notch and Stat reporters is dynamic in PC clusters and PC survival and death fates are associated with respective activation of the Notch and JAK/STAT pathways (Vachias et al., 2010; Fig. 3B; Fig. 6E). However, we found that RNAi-mediated downregulation of *upd* did not affect either expression of Notch or that of two Notch activity reporters (supplementary material Fig. S4). Therefore, JAK/STAT does not promote supernumerary PC apoptosis by downregulating Notch activity in these cells. Identification of the relay signal and/or of Stat target genes should help further elucidate the mechanism underlying the induction of apoptosis in selected PCs.

JAK/STAT signalling controls the size of the PC organising centre via apoptosis

Interfollicular stalk formation during early oogenesis has been shown to depend on activation of the JAK/STAT pathway (Baksa et al., 2002; McGregor et al., 2002). The presence of more than two PCs during these stages may be important to produce the appropriate level of Upd ligand to induce specification of the correct number of stalk cells. Later, at stages 7-8 of oogenesis, correct specification of anterior follicle cell fates (border, stretch and centripetal cells) depends on a decreasing gradient of Upd signal emanating from two PCs positioned centrally in this field of cells (Liu and Montell, 1999; Bai and Montell, 2002; Grammont and Irvine, 2002; Besse and Pret, 2003). Attaining the correct number of PCs per follicle pole has been shown to be relevant to this process and BC specification seems to be particularly sensitive to the number of PCs present (Liu and Montell, 1999; Bai and Montell, 2002; Grammont and Irvine, 2002). We have previously shown that apoptosis of supernumerary PCs is physiological necessary for PC organiser function, as blocking caspase activity in PCs such that more than two PCs are present from stage 7 leads to defects in PC/BC migration and stretch cell morphogenesis (Besse and Pret, 2003; Khammari et al., 2011). We now show that the excess PCs produced by blocking apoptosis lead to increased levels of secreted Upd and induce specification of excess BCs compared with the control, and these exhibit inefficient migration (supplementary material Fig. S3). These results indicate that reduction of PC number to two is necessary to limit the amount of Upd signal such that the correct numbers of BCs are specified for efficient migration to occur. Taken together with the role we show for Upd and JAK/STAT signalling in promoting PC apoptosis, it is possible to propose a model whereby Upd itself controls the size of the Upd-producing organising centre composed of PCs by inducing

apoptosis of supernumerary PCs. Interestingly, in the polarising region in the vertebrate limb bud, which secretes the morphogen Sonic Hedgehog (Shh), Shh-induced apoptosis counteracts Fgf4-stimulated proliferation to maintain the size of the polarising region and thus stabilise levels of Shh (Sanz-Ezquerro and Tickle, 2000). It is likely that signal autocontrol via apoptosis of signal-producing cells will prove to be a more widespread mechanism as our knowledge of apoptosis control during development advances.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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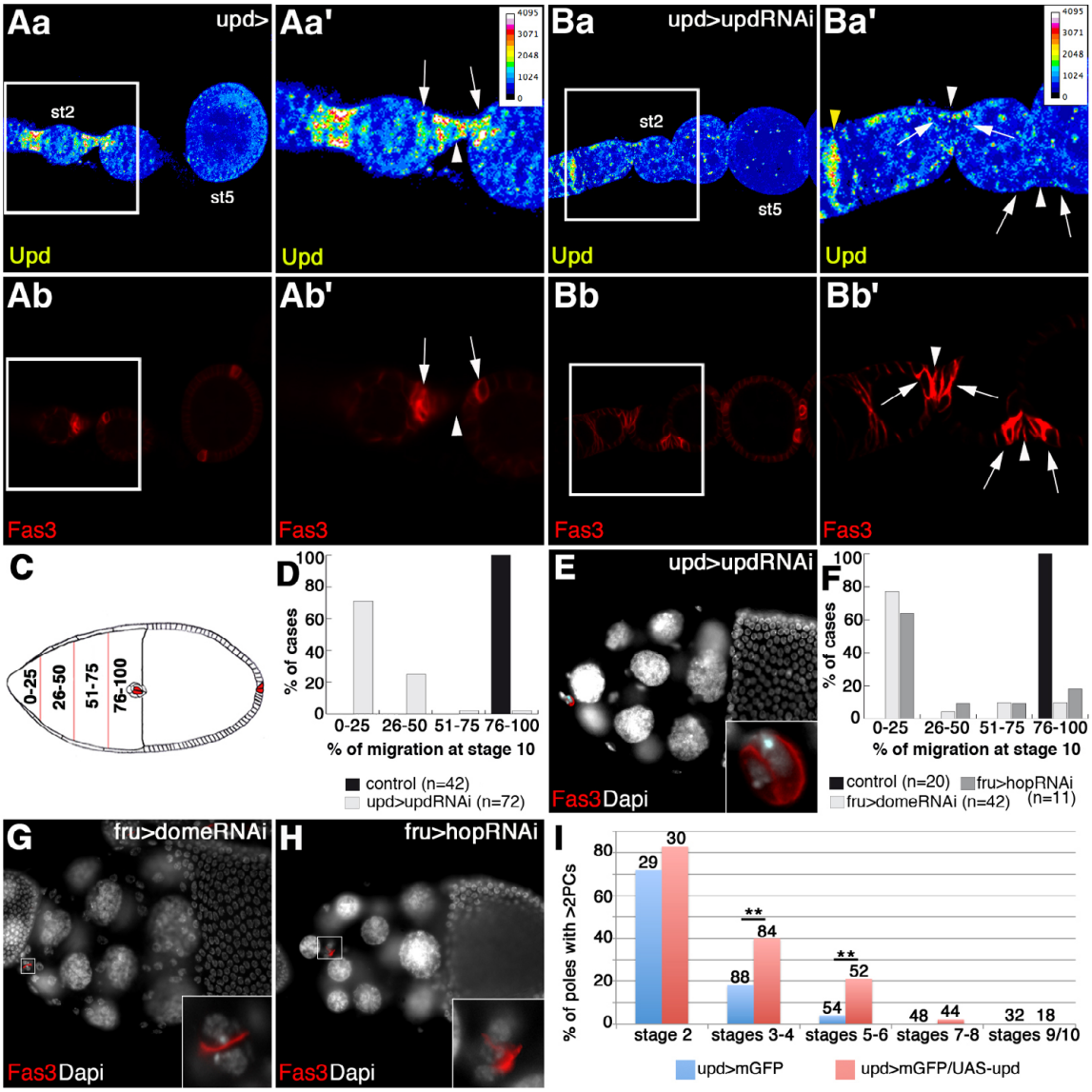

Fig. S1. Validation of JAK/STAT pathway RNAi lines using immunodetection and a border cell migration assay. (**A**,**B**) Confocal images of control and *Upd* RNAi ovarioles (anterior to the left) immunostained for Upd (Aa,Ba) and Fas3 (Ab,Bb). Panels marked with primes are magnified views of boxed areas in panels sharing the same letter. Arrows and arrowheads point to PCs and interfollicular stalks, respectively. A false colour code was used to compare Upd signal intensities (scale indicated). Yellow arrowhead indicates Upd signal in the germarium. (**C**) Schematic drawing of a stage 10 follicle (anterior to the left) in which the distance of PC/border cell migration toward the oocyte is expressed as the percentage of the nurse cell compartment length (0% at the anterior pole and 100% at the oocyte). PCs are shown in red. (**D**,**F**) The percentage of follicles as a function of the percentage of PC/border cell migration according to (C) for control follicles and follicles expressing RNAi targeting *Upd* in PCs (D) and *dome* (F) or *hop* (F) in terminal follicle cells and stalks. The number (n) of follicles analyzed for each condition is indicated. (**E**,**G**,**H**) Epifluorescence images of stage 10 follicles (anterior to left) immunostained for Fas3 to identify PCs and thereby to determine the extent of PC/border cell migration. DAPI staining reveals all nuclei and allows identification of stage 10 follicles. Upon expression of *Upd* RNAi in PCs (D,E) and *dome* (F,G) or *hop* (F,H) RNAi in TFCs and stalk cells, PC/BC migration is severely impaired. In the three examples shown (E,G,H), only two anterior PCs are present, indicating that the PC/BC migration defects occur even when PC number is not altered. (**I**) Percentage of PC clusters containing more than two cells in control and *Upd*-overexpressing follicles. PCs were counted using the mCD8:GFP reporter expressed specifically in PCs from stages 2-10. The numbers above each bar indicate the total number of follicle poles analysed for a given stage and statistically different results according to a Chi-square test are indicated with a bar and asterisks (see Fig. 1 for code). Genotypes: upd>: *upd-Gal4*/+; upd>updRNAi *upd-Gal4*/+;*UAS-updRNAi*/+; fru>: *fru-Gal4*/+; fru>domeRNAi: *fru-Gal4/UAS-domeRNAi* ; fru>hopRNAi: *UAS-hopRNAi/+; fru-Gal4*/+; upd>mGFP: *upd-Gal4*/+;*UAS-mCD8:GFP*/+; upd>mGFP/UAS-upd: *upd-Gal4*/+;*UAS-mCD8:GFP/UAS-upd*.

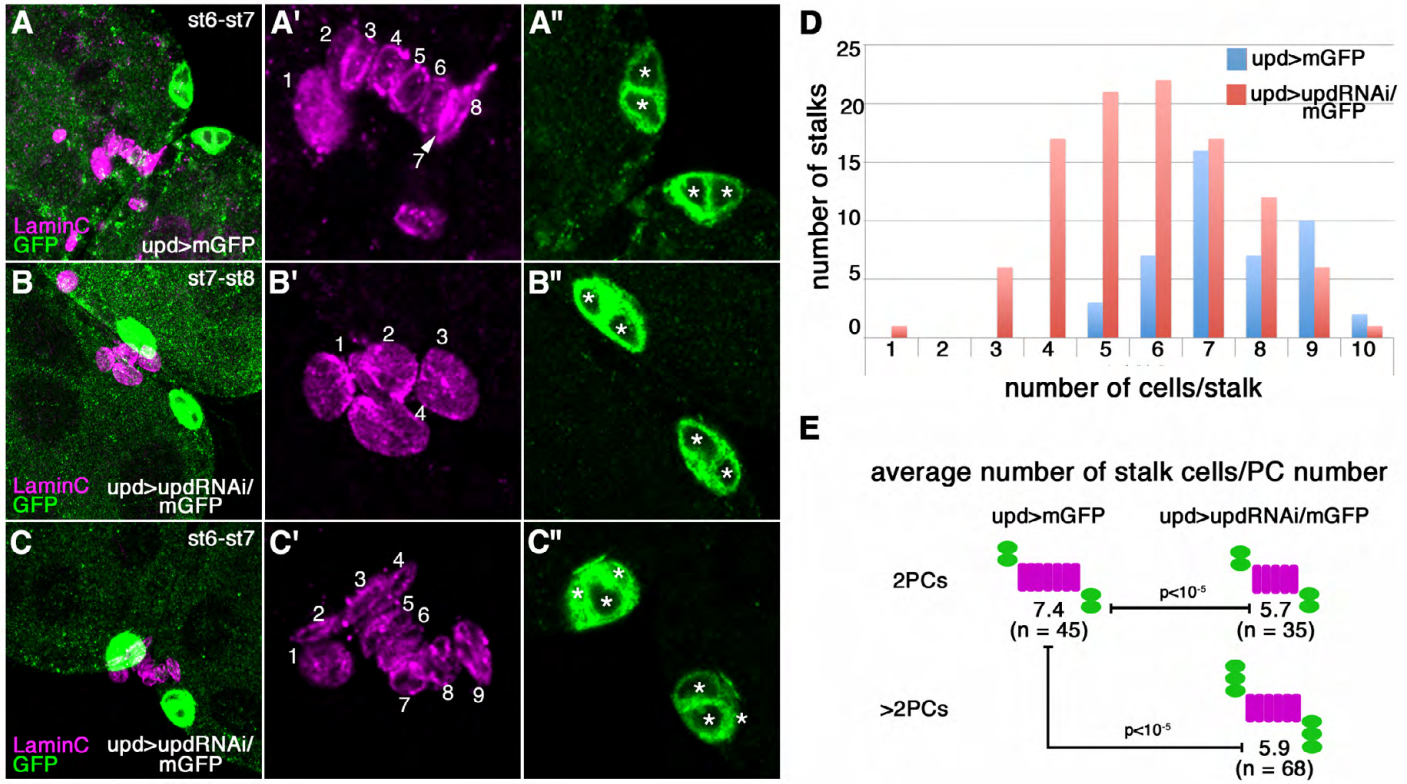

Fig. S2. *upd* controls the number of PCs and interfollicular stalk cells independently. (**A-C″**) Confocal images of stage 6-8 follicles of control and *Upd* RNAi ovaries immunostained for GFP and LaminC to identify PCs and interfollicular stalks, respectively. The panels labelled with single primes correspond to magnified views of stalk cells (each numbered) and with double primes to that of PCs (asterisks) in the panel of the same letter. (**D**) The number of stalks assayed as a function of the number of cells per stalk for control and *Upd* RNAi ovaries. (**E**) Schematic drawing presenting the average number of cells per stalk (indicated below the magenta stalk) flanked by two PCs on each side (green) or more than two PCs on at least one side (green) for control and *Upd* RNAi ovaries. n indicates the total number of stalks analysed. *P*-values indicate statistically significant differences between the average number of cells per stalk between the conditions compared using ANOVA. Only stalks after stage 6 were included in the analysis (D,E). Genotypes: upd>mGFP: *upd-Gal4*/+; *UAS-mCD8:GFP*/+ and upd>updRNAi/mGFP: *upd-Gal4*/+;*UAS-updRNAi*/*UAS-mCD8:GFP*.

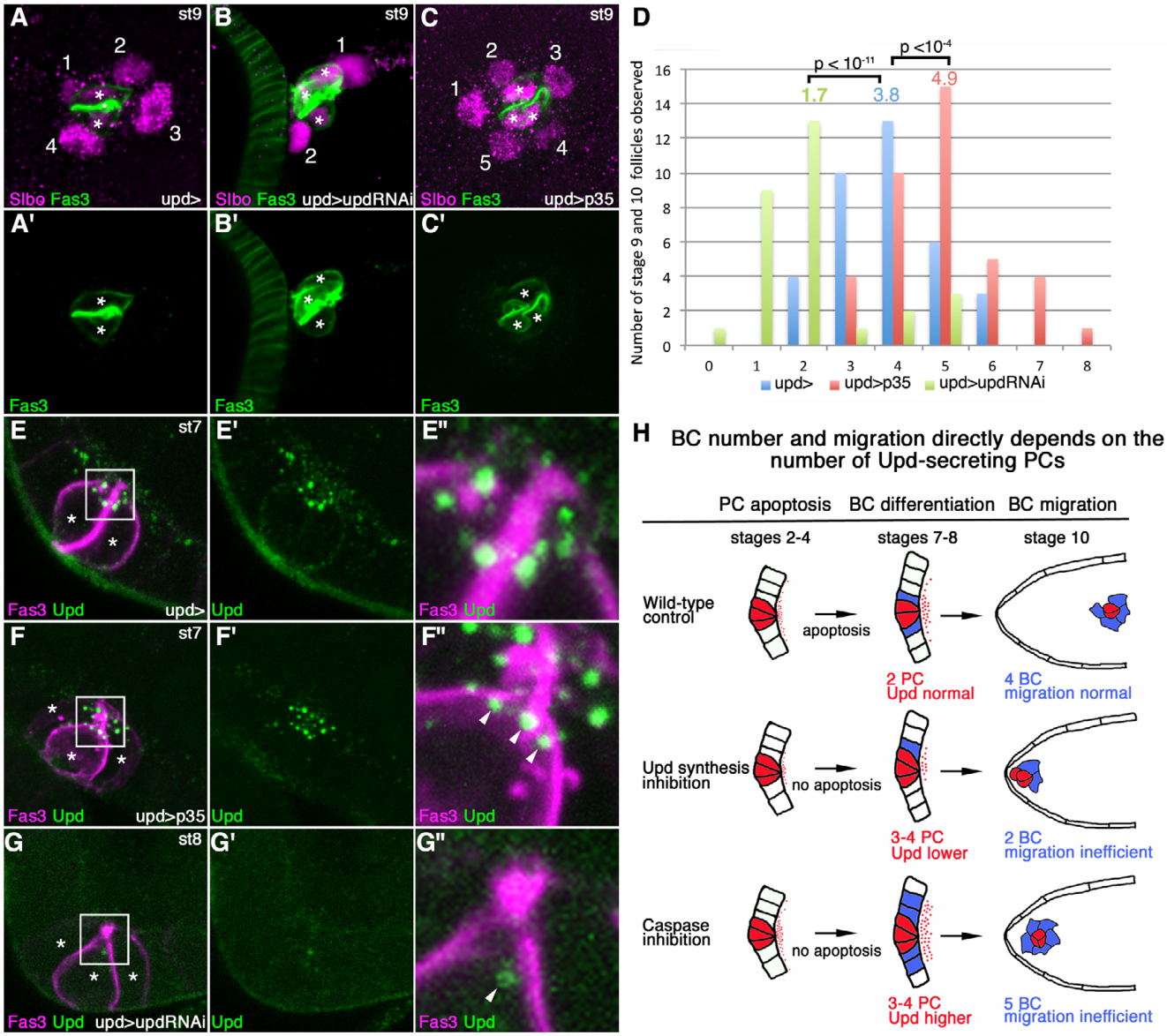

Fig. S3. Supernumerary PCs produced by blocking caspase function recruit excess BCs and produce supplementary Upd signal. (**A-C′**) Confocal images of follicles of the indicated stages for control (A), *Upd* RNAi (B) and P35-expressing (C) ovaries immunostained for Fas3 and Slow border cells (Slbo) which accumulate in PCs and both PCs and BCs, respectively. Anterior to the left. Double- and single-channel (panels with primes) of the same follicles are shown. Each BC is numbered and asterisks correspond to PCs. (**D**) The number of BCs present at the anterior pole of control, *Upd* RNAi-expressing and P35-expressing stage 9-10 follicles. The average number of BCs for each of the genotypes tested is indicated above the bars using the appropriate colour code, as well as the results for ANOVA indicating statistically significant differences (*P*-values) between the results for the control and experimental genotypes. (**E-G″**) Confocal images of follicles of the indicated stages for control (E), *Upd* RNAi (F) and P35-expressing (C) ovaries immunostained for Fas3 and extracellular Upd. Anterior to the top. Double and single-channel (panels with primes) of the same follicles are shown. Double primed panels are magnified views of the boxed regions in the panels labelled with the same letter. In F″, arrowheads indicate extracellular Upd signal associated with a supernumerary PC distinguished from the other PCs by its round shape and reduced size. The amount of Upd signal in the p35-expressing clusters is 1.8-fold that of the control (average signal intensities for six to eight clusters of each genotype were compared using the ImageJ SumSlice function). Genotypes: upd>: *upd-gal4/+* ; upd>updRNAi *upd-gal4/+;UAS-updRNAi* ; upd>p35: *upd-Gal4/+;UAS-p35/+*. (**H**) Schematic representations of transverse views of anterior follicle poles at different stage of oogenesis. PCs are in red and BCs in blue. Upon inhibition of Upd synthesis (*Upd* RNAi), excess PCs survive until late stages but the reduced levels of Upd lead to specification of fewer BCs and inefficient migration (see Fig. S1 for BC migration assay results). Upon caspase inhibition (p35 expression), excess PCs secreting Upd survive until late stages leading to specification of excess BCs and inefficient migration (Khammari et al., 2011). The number of BCs recruited is thus directly dependent on the number of surviving PCs if these cells participate to Upd signal production. BC migration defects are thus observed when the amount of Upd secretion, and therefore the number of BCs specified, is either too low or too high.

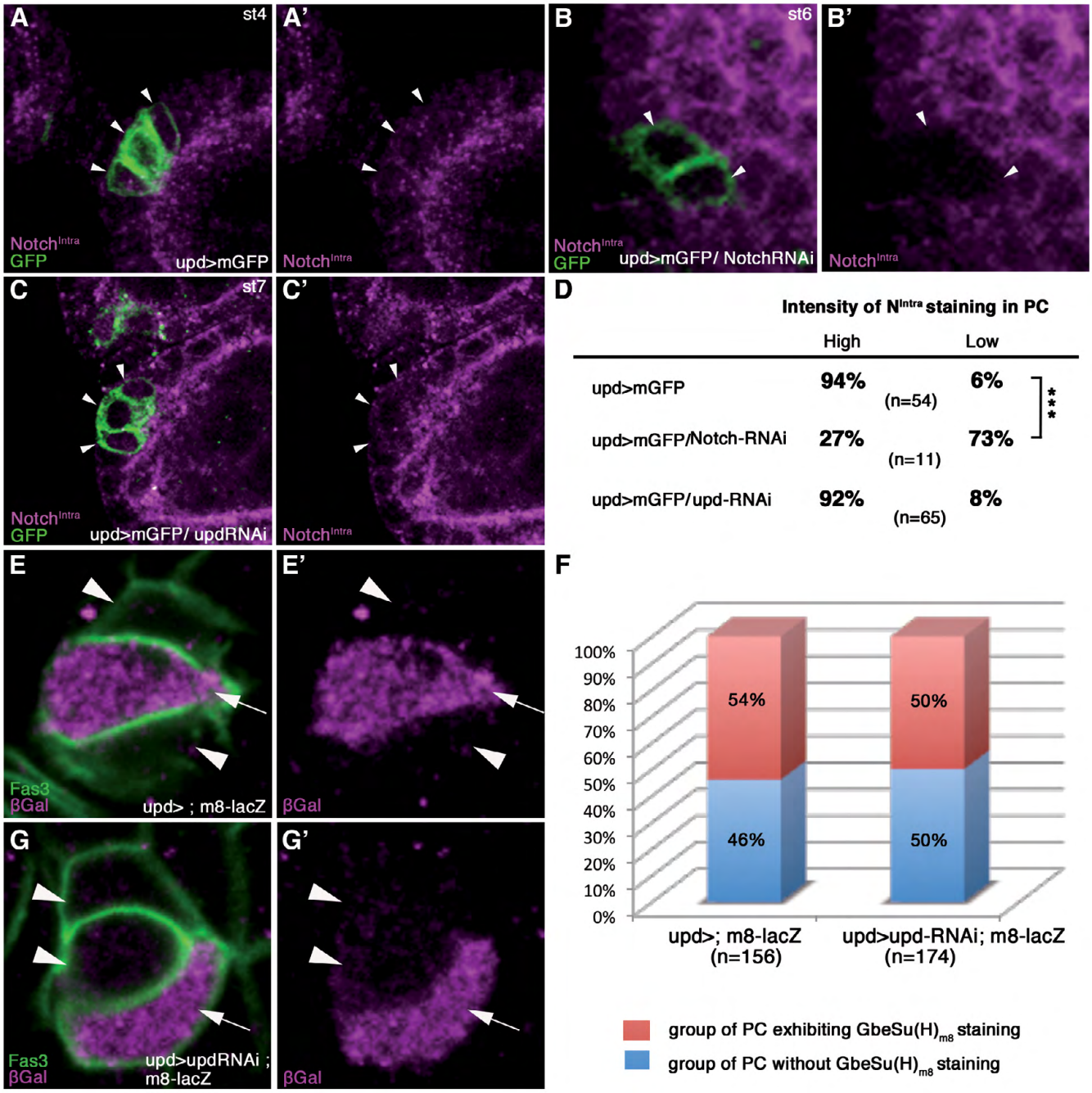

Fig. S4. Upd depletion does not affect Notch activity in PCs. (**A-C**′) Confocal images of control (A), *Notch* RNAi (B) and *Upd* RNAi (C) follicles of the indicated stages immunostained for GFP to identify PCs (arrowheads) and for N^{intra}. A′-C′ are single-channel views of A-C. N^{intra} accumulates in PCs in the control (A). *Notch* RNAi in PCs significantly reduces N^{intra} accumulation in these cells (B), whereas *Upd* RNAi in these cells does not affect N^{intra} accumulation (C). (**D**) Percentage of PC clusters presenting high or low levels of N^{intra} staining in control, *Notch* RNAi, and *Upd* RNAi follicles. The total number (n) of clusters analysed is indicated. A statistical difference between results for control and Notch RNAi is indicated with asterisks according to the code in Fig. 1. (**E**,**E**′,**G**,**G**′) Confocal images of control (E) and *Upd* RNAi (G) follicles immunostained for Fas3 to identify PCs and β-galactosidase (β-Gal) to detect Notch reporter activity [m8-lacZ:GbeSu(H)m8-lacZ]. E′,G′ are single-channel views of E,G. Notch reporter activity is present within some PCs of given clusters (arrow), but not in all (arrowheads). (**F**) Percentage of PC clusters of more than two PCs presenting Notch reporter activity (red) or not (blue) in control and *Upd* RNAi follicles. A second Notch reporter, *E*(*spl*)*m*β-*lacZ*, was also analysed and similar results were obtained (data not shown). Genotypes: upd>: *upd-Gal4*/+;*UAS-mCD8:GFP*/+ ; upd>mGFG/NotchRNAi: *upd-Gal4*/+;*UAS-mCD8:GFP/UAS-NotchRNAi*/+; upd>mGFP/updRNAi: *upd-Gal4*/+;*UAS-mCD8:GFP/UAS-updRNAi*/+; upd>;m8-lacZ: *upd-Gal4*/+; $GbeSu(H)_{m8}$-lacZ/+; upd>updRNAi;m8-lacZ: *upd-Gal4*/+; $GbeSu(H)_{m8}$-lacZ/ *UAS-updRNAi*.