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

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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 during development 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 supernumeryary 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 supernumeryary PC death. Finally, blocking apoptosis of PCs leads to specification of excess adjacent border cells via excess Upd signalling. Our results therefore show that Upd and JAK/STAT signalling induce apoptosis of supernumeryary 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, which 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 supernumeryary 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 of 10; 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/Apa1 (Apa1-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 supernumeryary 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 supernumeryary 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) (Baks 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-Gaiço 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.

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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 (#38566), 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, Star92EIR/mos-TM3,Sb and FRT82B, Star92EIR/m8-lacZ 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 [a gift from E. Bach (Bach et al., 2007)] 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 test expression in terminal follicle cells except PCs. tubp-Gal80* (7016) from the Bloomington Stock Center was used to avoid embryonic lethality associated with expression of UAS-Stat92EIR. UAS-nsl-GFP allows expression of nuclear GFP. UAS-mCD8:GFP 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 Exp(sy5)yb-lacZ (Kramatschek and Campos-Ortega, 1994; Cooper et al., 2000; Furrillos and Bray, 2001; Vachaspati et al., 2002). 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*, 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, Star92EIR and Stat92EIR/mos-TM3,Sb 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 CO2 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 Dafo.

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, TG10) at 1:20, guinea pig and rabbit polyclonal anti-Hid (gift from D. Ryoos, 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-Notchintracellular (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 gerarium, 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; 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 did not affect upd function in the gerarium 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...
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...
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<sup>398</sup> and Stat92E<sup>681</sup>) 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). In addition, 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
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). frr-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). frr-Gal4 driver expression remains very low or absent in PCs (Fig. 5B,C, arrowheads). In addition, strong frr-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 frr-Gal4 led to the production of late-stage follicle poles with three to five PCs (Fig. 5I,J). At later stages of oogenesis, frr-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. 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/+.

**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’,B’,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 cell 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 gerarium 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 (CaspAct). 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'). 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 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>domeRNAi: fru-Gal4/UAS-domeRNAi
- fru>hopRNAi: fru-Gal4/UAS-hopRNAi
- 7025>nls-GFP: UAS-nls-GFP+;7025-Gal4/+
- 7025>domeRNAi: 7025-Gal4/UAS-dome-RNAi
- 7025>hopRNAi: 7025-Gal4/UAS-hopRNAi

N.O, not observed.
expression of the fra-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 fra-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 expressing 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 fra-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 (\( n=15 \)) (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 fra-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\(^{1061} \) and Stat92E\(^{1061/1081} \) 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 stalks 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 fra-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
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; 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; 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; Xi et al., 2003). Signal transduction via Dome/Hop/Stat92E occurs in nearby TFCs, interfollicular stalks and possibly PC, 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
Interestingly, the characterisation of two other signal transduction in several cell populations at the same time are supernumery PCs, and the effects of removing JAK/STAT TFC clones are more frequently associated with prolonged survival a threshold level of relay signal. In support of this, large stat mutant surrounding TFCs, stalk cells and possibly PCs, for production of apoptosis of supernumery PCs may require participation of all diameters away from PCs are not. In addition, fully efficient supernumery PCs, whereas clones further than three cell diameters away from PCs, are both associated with prolonged survival of PCs, as well as those positioned up to three cell diameters away from PCs, whereas clones further than three cell diameters away from PCs are not. In addition, fully efficient apoptosis of supernumery 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 supernumery 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 supernumery PCs respond to the JAK/STAT-mediated pro-apototic 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 supernumery PCs (supplementary material Fig. S11). 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 supernumery 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 supernumery PCs is physiological necessary for BC 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 organiser 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.

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