Physiological apoptosis of polar cells during Drosophila oogenesis is mediated by Hid-dependent regulation of Diap1

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Running title: Hid-mediated regulation of Diap1 for polar cell apoptosis

by

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Abstract

Although much has been learned in recent years about the apoptotic machinery, the mechanisms underlying survival and death choices during development of metazoans remain less clearly understood. During early oogenesis in *Drosophila*, a small excess in the number of specialized somatic cells, called polar cells, produced at follicle extremities is reduced to exactly two cells through apoptosis by mid-oogenesis. We have found that polar cells destined to die first lose their apical contacts and then round-up and shrink progressively until they disappear. Caspases are activated only once the cells have begun to shrink suggesting that they are implicated in this part of the process but not in the initial loss of cell polarity. Loss of function analyses based on mutant, clonal and RNAi approaches show that among the RHG family of pro-apoptotic factors, Hid is specifically necessary for polar cell apoptosis, as well as the initiator caspase Dronc and its adaptor Dark/Apaf-1, and likely several effector caspases, in particular Drice. In addition, we show that Hid protein and transcripts accumulate specifically in polar cells destined to die, while the anti-apoptotic factor Diap1 is downregulated in these cells in a *hid*-dependent manner. Therefore, our results implicate the Hid-Diap1 module as an important regulatory point in a developmental case of apoptosis.

Introduction

In multicellular organisms, control of precise cell number can be crucial for proper tissue morphogenesis and function. In metazoans, apoptosis is widely used during development to reach proper cell number by elimination of targeted individual cells. [1,2] In *Drosophila*, the use of the powerful genetic and molecular tools available has allowed the implication of apoptosis in several specific cell number reduction processes, such as the selection of exactly 3 glial cells from 9 precursors at the ventral midline of each embryonic segment [3], the survival of exactly 6 secondary pigment cells per omatidium allowing formation of the highly-ordered hexagonal lattice of the fly compound eye [4] and patterned cell elimination along the proximal-distal axis of the leg for proper joint formation. [5] The components of the apoptosis machinery have been shown to be highly conserved during evolution [6,7], but the mechanisms underlying these types of exquisitely-precise survival vs. death choices in the context of the whole organism are not as yet fully understood.

Apoptosis is caused by proteolytic cleavage of vital proteins by a group of aspartate-specific cysteine proteases, called caspases. [8] The mammalian genomes encode fourteen distinct caspases, while the *Drosophila* genome bears seven caspase-encoding genes. Caspases are generally classified as initiators or effectors. Initiatior caspases cleave and thereby activate effector caspases and effector caspases are responsible for destruction of the cell. [9,10] Caspase regulation in mammals occurs primarily via their activation through either an extrinsic pathway involving signalling membrane receptors of the Tumor Necrosis Factor Receptor (TNFR) superfamily [11] or an intrinsic pathway involving the mitochondrial membrane integrity regulators of the Bcl2 family. [12] Although both TNFR and Bcl2 members are present in *Drosophila*, these have no or only a relatively minor incidence on characterized cases of apoptosis. Instead, in *Drosophila*, caspases are maintained in an inactive state in virtually all cells by a specific class of proteins called inhibitors of apoptosis (IAPs) that are bound to caspases and inhibit their activity. [13,14] In the presence of apoptotic stimuli, IAP antagonists are activated and competitively bind to IAPs through an IAP-Binding–Motif (IBM) leading to IAP ubiquitination and consequent degradation of both IAPs and IBM-domain proteins. [15,16] In mammals, both IAPs and IBM-domain proteins of the Smac/Diablo and Omi/HtrA2 families are also present, but

they are ubiquitously expressed. In contrast, the IBM-domain proteins Reaper, Hid and Grim (RHG) in *Drosophila* are transcriptionally activated in response to many different hormonal, stress and developmental signals. [17] Posttranscriptionnal regulation has been also reported for Hid. [3,17] Although there have been great advances in understanding the molecular mechanisms that lead to caspase activation and cell death, most of the studies are based on *in vitro* analysis or *in vivo* models in which apoptosis is induced. Thus how the cell death machinery is activated *in vivo* during development is still poorly understood.

In the *Drosophila* ovary, polar cells (PCs) form clusters of specialized somatic cells embedded in the follicular epithelium at anterior and posterior follicle poles and in contact with the germline (Figure 1A). PCs play an important organizer role at several stages during oogenesis (Figure 1A). [18-20] In particular, PCs are the only ovarian cells to secrete Unpaired1 (Upd1), a specific ligand of the JAK-STAT signalling pathway. By activating this signalling pathway in adjacent follicle cells during mid-oogenesis, anterior PCs induce their differentiation into border cells and, subsequently, PCs and border cells migrate together through the egg chamber to attain the oocyte where they will form the micropyle, the sperm entry point into the oocyte (Figure 1A). [21-23]

During early stages of oogenesis, PCs undergo a highly regulated cell death program whereby a small excess of PCs that is produced (1 to 4 supernumerary cells) is eliminated by apoptosis such that by mid-oogenesis 100% of the follicles contain exactly 2 PCs at each anterior and posterior extremity. [24] This restriction fulfils a physiological role since inducing prolonged survival of supernumerary PCs by blocking apoptosis leads to follicular cell patterning defects including recruitment of excess border cells and perturbed migration of these cells. [24] As has been shown under other experimental conditions, an excess in the number of PCs, likely via production of elevated levels of Upd1, leads to overactivation of the JAK-STAT pathway in adjacent follicle cells and recruitment of too many of these cells as border cells. [19,23] PCs thus represent an interesting model to study how cell survival/death choices are made in particular in the context of a whole organ.

We have undertaken a morphological, genetic and molecular analysis of PC apoptosis. We present a model in which cells destined to die are progressively eliminated in an apparently stereotyped sequence involving loss of cell polarity, rounding-up and shrinkage. Our genetic analyses

allowed us to determine which of the known components of the core cell death machinery are involved in PC apoptosis. Elimination of supernumerary PCs requires specifically the RHG family member Hid, the initiator caspase Dronc and its adaptor Dark/Apaf1 and possibly several effector caspases, including Drice. Finally, expression profile analyses reveals that *hid* is transcriptionnally upregulated in PCs destined to die, while DIAP1 protein is specifically downregulated in these cells in a *hid*-dependent manner. Our results thus identify *hid* as an important regulatory point for induction of PC apoptosis and demonstrate that the Hid-DIAP1 cassette regulates apoptosis in a developmental setting.

Results

Ovarian PC death involves apical constriction, rounding-up and exclusion of dying cells during which caspase activation occurs

The number of PCs per follicle extremity between stages 3 and 10 of oogenesis was assayed by immunostaining for Fasciclin III (Fas3) (Figure 1d,e -red). At stage 3, between 20-45% of anterior and posterior follicle poles contained more than 2 PCs in control ovaries depending on the experiment (Tables 1 and 2). Among these, clusters of 3 PCs represented the vast majority, while clusters with 4 PCs were extremely rare (0-2%, Tables 1 and 2). Between stages 3 and 4, PC restriction to 2 cells continued since only 0-14% of follicle poles at stage 4 contained 3 PCs and from stage 5 onwards almost 100% of all follicle poles contained only 2 PCs (Tables 1 and 2). Specific expression of the baculovirus caspase inhibitor p35 in PCs using the *upd-GAL4* driver affected the restriction in PC number in three different ways: 1) The proportion of follicle poles with more than 2 PCs during early stages of oogenesis was much higher than in the control, 2) Clusters of more than 2 PCs persisted into late stages of oogenesis, while these were absent at these stages in the control, and 3) Clusters of 4 PCs, practically absent at stages 3 and 4 in the control, were present at early and later stages of oogenesis (Table 1).

During the process of PC number reduction, the morphology of the individual PCs comprising a cluster was determined by immunodetection of either PC-expressed membrane-addressed mCD8:GFP (*upd-GAL4* driver) or endogenous membrane Fasciclin III (Fas3). During early stage 2, the mCD8:GFP marker indicates that PC clusters (3-5 cells) or PC pairs, at both the anterior and posterior poles, are comprised of flattened cells with their long axis perpendicular to the anterior-posterior axis of the ovariole (Figure 1b',c' - green and data not shown). The Fas3 marker is not specific for PCs during these early stages of oogenesis since adjacent follicle cells are also marked (Figure 1b,c - red). PCs become clearly distinguished using the Fas3 marker by stage 3 (Figure 1d,e – red), upon which they change shape, becoming polarized along the follicular epithelial apical/basal axis with points of convergence between the cells at both the apical and basal poles (Figure 1d',e' -

red). Both PC clusters and pairs show this configuration (Figure 1d',e', respectively) at both the anterior and posterior poles (only posterior shown in Figure 1d',e').

Among PC clusters containing 3 or more PCs at stages 3 and 4, several distinct cell shape configurations were observed. In some groups consisting of 3 PCs, all cells are equivalently-sized and -shaped and the group exhibits radial symmetry, that is, each cell being in close contact with the two others along the radial axis (Figure 1d' and 2A and schematic representation in 2Ka). However, alternate configurations were also observed in which 2 equivalent cells maintain the apical-basal contacts (Figure 2B, white arrowheads), while the supernumerary cell or cells exhibit constriction at the apical contact (Figure 2B,C arrows), rounding up and reduction in size (Figure 2B,C yellow arrows). Importantly, the use of specific antibodies demonstrated that the shrinking PCs specifically accumulate an activated form of caspases (Figure 2B,C green) and are therefore the PCs destined to die. These results indicate that it is possible to observe intermediate steps in the elimination of the supernumerary PCs, which appear to involve release of apical contact with the germline and shrinking, and thus to identify the dying cells before they disappear. Importantly, caspase activity was not detected in groups of 3 PCs before release of apical contact by the cells destined to die had already occurred (Figure 2A). Therefore, the polarity changes in dying PCs may occur independently of caspase activity.

Interestingly, the intermediate steps in the morphological changes in PCs destined to die were even more apparent upon expression of p35 in these cells, possibly because the process of cell elimination is blocked at a downstream step (Figure 2F-J). The analysis of many groups of 3 or more PCs indicated that cell elimination involves progressive lengthening of the apical constriction (Figure 2F-I white arrows), as the dying cell reduces its contacts with the other 2 PCs (Figure 2G-I yellow arrows), while the 2 mature PCs maximize their contacts with each other (Figure 2G-I). The dying PCs round up, diminish drastically in size and occupy more a more basal position (Figure 2D and J, yellow arrows). Even in the presence of p35, some caspase activation was observed in the excluded cells indicating that these are the PCs destined to die (Figure 2D,E, leftmost yellow arrow). Although caspase inhibition in the PC group via p35 expression leads to a high proportion of follicles with supernumerary PCs late into oogenesis (Table 1), the PCs destined to die nonetheless undergo

morphological and polarity changes further suggesting that these processes may be occurring independently of caspase activity. In addition, the morphological and polarity changes in PCs destined to die occurred before significant changes in nuclear integrity, as evidenced by DAPI staining, were observed (Figure 2G-I). A schematic model of the successive morphological and polarity changes, as well as the temporality in caspase activation, in the PC group during progressive elimination of supernumerary PCs is presented in Figure 2K.

Among effector caspases, Drice, is specifically implicated in PC apoptosis

The *Drosophila* genome encodes at least 5 effector caspases, Drice, Dcp1, Decay, Damm, and Strica, the latter which can also be classified as an atypical initiator caspase. We first took an RNAi approach to test which of these caspases is specifically involved in physiological PC apoptosis. The UAS-RNAi transgenic lines targeting the genes encoding these caspases had been previously validated upon *hid*-induced apoptosis in the *Drosophila* eye. [25] We induced expression of the UAS-RNAi transgenic constructs specifically in PCs as of stage 2 of oogenesis using either the *neur-Gal4* or the *upd-Gal4* drivers (see Figure 1B-E for the *upd-Gal4* expression profile and data not shown) and determined the number of PCs using the Fas3 marker and expression of a UAS-H2B:YFP transgene. Only RNAi directed against the *drice* gene resulted in prolonged survival of supernumerary PCs (up to 5 cells) at both anterior and posterior follicle poles after stage 6 of oogenesis (Figure 3B,D). At the anterior pole, the presence of an excess number of PCs was associated with a delay in PC/border cell migration at stage 10 (Figure 3B) compared to wild-type (Figure 3A) (see Supplementary Table 1 for quantification of this phenotype). The *upd-Gal4* driver alone gave < 1% (n=150) of stage 7-10 follicle extremities with more than 2 PCs, while expression of a *drice* RNAi transgene using this driver led to 63% (n=171) of follicle extremities with more than 2 PCs (Table 2). A second *drice* RNAi transgenic line also gave high levels of supernumerary PCs with the same driver and a second PC driver, *neur-Gal4*, as well (data not shown). These results suggest that PC apoptosis is dependent, at least in part, on the Drice caspase.

However, we next recovered females homozygous for the amorphic sublethal $drice^{\Delta 1}$ allele and found that dissected ovaries immunostained for Fas3 did not exhibit prolonged survival of more

than 2 PCs after stage 6 of oogenesis (data not shown). It is possible that these mutants compensate for lack of *drice* function by activating alternative cell death pathways or that there is redundancy in caspase function. Indeed, it seemed important to test the latter possibility since the *drice* RNAi transgenic construct used is also able to target *dcp1* sequences (strong off target). The strong hypomorphic $dcp1^{prev}$ allele is homozygous viable and homozygous females did not display supernumerary PC after stage 6 of oogenesis (data not shown). We were not able to recover $drice^{\Delta 1}$; $dcp1^{prev}$ double mutants due to fully penetrant lethality. We therefore recombined the $drice^{\Delta 1}$ allele with an FRT insertion on the same chromosome and generated homozygous $drice^{\Delta 1}$ mitotic clones (FLP-FRT system) in PCs heterozygous for $dcp1^{prev}$. In this genetic context, we did not observe an effect on PC number during late stages of oogenesis (data not shown). The fact that caspases are activated in dying PCs (Figure 2B,C) and that ectopic expression of p35 blocks PC elimination (Figure 2G-J and Table 1) strongly suggests effector caspase function in PC apoptosis. It is possibly necessary to knock-out more fully the function of both *drice* and *dcp1*, and maybe even that of another of the 5 known effector caspases as well, to block PC apopotosis.

The initiator caspase Dronc and its adaptor Dark are required for PC apoptosis

The *Drosophila* genes *dronc* and *dredd* are closely related to genes encoding the mammalian casp8 and casp9 initiator caspases, respectively. Previous studies have shown that *dronc* gene function is involved in apoptosis during *Drosophila* embryonic and eye development [31-32], while evidence supports a role for *dredd* primarily in *Drosophila* innate immunity [57] rather than in developmental apoptosis. We tested the function of these 2 genes in PC apoptosis and only RNAi directed against the *dronc* gene resulted in prolonged survival of excess PCs at both anterior and posterior poles (data not shown).

Since amorphic alleles for the *dronc* gene are available ($dronc^{i29}$ and $dronc^{i24}$), as well as for *dark*, encoding its specific adaptor, ($dark^{G8}$ and $dark^{H16}$), we tested the effect of these mutations on PC apoptosis. These mutations are lethal during development, so we induced homozygous mutant mitotic clones in the ovaries using the FLP-FRT system and loss of ubi-GFP expression as a clone marker.

Under these conditions, we found groups of $Fas3^+$ PCs with more than 2 cells (either 3 or 4) after stage 6 of oogenesis at both anterior and posterior poles and in each case at least one PC in the group exhibited no GFP expression and therefore was homozygous mutant for either *dronc* or *dark* (Figure 4A-F). When only one PC in a group was homozygous mutant for *dronc* or *dark*, this cell often exhibited the morphological characteristics of a PC destined to die, that is diminished apical contacts with the other cells, rounding up and reduced size (Figure 4A,D- yellow arrows). In addition, in all mosaic groups composed of both GFP^+ and GFP^- PCs, the cell or cells in the process of being extruded were always GFP^- (Figure 4A,B,D,E-yellow arrows). These results indicate cell autonomous function of *dronc* and the adaptor-encoding *dark* within the PCs destined die to allow this process to occur. They also suggest that removing *dronc* or *dark* function does not impede the morphological changes observed in PCs destined to die suggesting that initiator caspase activity is not involved in these changes. Finally, in mosaic GFP^+/GFP^- PC groups with supernumerary cells surviving well after stage 6 (Figure 4A,B,D,E), it is interesting to note that GFP^+ PCs which carry at least one wild-type allele of *dronc* or *dark* were not selected for death in place of GFP^- PCs in the same group which are deficient for caspase activity. This result suggests that the selection of PCs destined to survive or die takes place at an early point in the PC development program and that after this point, there is no plasticity between the two states, at least none that can be induced by perturbation of the apoptotic machinery in a subset of the cells.

Among the RHG family members, Hid is specifically required for PC apoptosis

Since the RHG family has been identified as pro-apoptotic in *Drosophila*, we tested the possible function of three family members, *reaper*, *hid* and *grim* using the H99 deletion that covers all three genes which are grouped together on chromosomal arm 3R. Since this deletion is homozygous lethal during development we generated FLP-FRT H99 homozygous mitotic clones marked by the absence of GFP expression. Using this approach, we found groups of PCs with more than 2 PCs (up to 5 cells) marked by Fas3 during late stages of oogenesis at both anterior and posterior poles and in each case at least one PC was homozygous for the H99 deletion (Figure 5A,B). Thus, at least one of the three genes, *reaper*, *hid* or *grim*, is implicated in PC apoptosis. At the anterior pole, the presence of

excess polar cells upon induction of H99 homozygous clones was associated with a significant delay in PC/border cell migration (Figure 5A compared to wild-type in Figure 3A).

We next expressed UAS-RNAi transgenic lines in PCs to test, individually, the functions of *hid*, *grim* and *sickle*, a fourth RHG not covered in the H99 deletion, in PC apoptosis. Only RNAi directed against the *hid* gene, using 2 different RNAi transgenic constructions (Japan and Vienna collections) and two different PC drivers, *upd-Gal4* and *neur-Gal4*, led to prolonged survival of more than 2 PCs at late stages of oogenesis at both anterior and posterior poles (Figure 5C,D and data not shown). Quantification showed that expression of a *hid* RNAi construct led to 35% (n=122) of follicle extremities between stages 7 and 10, including both anterior and posterior, with more than 2 PCs (Table 2). At the anterior pole, the presence of excess PCs was associated with a significant delay in PC/border cell migration (Figure 5C compare to wild-type in Figure 3A and see Supplementary Table 1 for quantification).

We also analyzed both *reaper* and *hid* null escapers for PC number after stage 6 of oogenesis. The *reaper XR38/Df(3L)H99* female escapers showed no PC number phenotype (data not shown), while hid^{P5041}/*Df(3L)R+X1* female escapers displayed more than 2 PCs at 100% of follicle extremities, anterior and posterior, after stage 6 (Figure 5E,F). The *hid* mutant phenotype was therefore highly penetrant, and since the majority of follicle poles had more than 3 PCs (4,5 or 6 PCs, Figure 5E,F and data not shown), highly expressive as well. These results therefore indicate that, among RHG family members, Hid is specifically required for PC apoptosis. In some clusters of 3 or 4 PCs, the supernumerary PCs exhibited loss of apical of apical contact, rounding up and shrinking (Figure 7G,H, yellow arrows) suggesting that *hid* function may not be involved in these upstream events during elimination of these cells.

hid **transcription is specifically activated in PCs destined to die**

Implication of the pro-apoptotic factor Hid in PC apoptosis made it a good candidate as a potential regulatory point in induction of PC apoptosis. We therefore determined the Hid expression profile in the ovary using specific anti-Hid antibodies and marked PCs specifically by Fas3 accumulation. In wild-type ovaries, no Hid protein accumulation was detected in any cell type,

somatic or germline (Figure 6A and data not shown). However, blocking PC apoptosis by overexpression of the p35 caspase inhibitor specifically in PCs led to detectible accumulation of Hid in supernumerary PCs destined to die as determined by their morphological state (Figure 6B, yellow arrow). It was also possible to detect Hid in supernumerary PCs by removing caspase function using RNAi or amorphic mutations directed against *dronc* or *drice* (Figure 6C-F, yellow arrows). In all cases, Hid was detected in PCs shrinking and undergoing exclusion from the group, therefore indicating that Hid is specifically expressed in PCs destined to die. Interestingly, when 2 supernumerary PCs were present, both cells did not necessarily accumulate Hid indicating that the process of PC elimination is not synchronized within the PC group (Figure 6C,E,F, yellow arrows). Taken together with the results of the analysis of *hid* mutants indicating that *hid* is necessary for PC apoptosis, these results strongly suggest that PC apoptosis is induced by specific upregulation of Hid in PCs destined to die

In order to determine whether specific accumulation of Hid in PCs destined to die is regulated transcriptionally, we performed RNA *in situ* hybridization on ovaries using a *hid* riboprobe. The specificity of the *hid* probe was verified in the embryo since the *hid* expression profile has been characterized in this tissue (http://www.fruitfly.org/cgi-bin/ex/insitu.pl and data not shown). In the wild-type ovary, relatively strong *hid* germline signal (equivalent to what is observed in Figure 6G - gc) was observed, as has been previously reported [26] , but no expression was evident in any somatic cells including the PC groups at any stage (data not shown). However, when caspase activity was blocked by overexpression of p35 in PCs then significant accumulation of *hid* transcripts was detected specifically in one somatic cell at poles of follicles from stage 4 onwards (Figure 6G and G', white asterisk). The position of these cells at follicle extremities and the fact that their shape differs from that of other somatic cells in the follicular epithelium in that they display apical constriction and rounding up, strongly suggests that these are PCs destined to die. These results thus allow us to propose that *hid* transcription is specifically activated in PCs destined to die. In the wild-type ovary, the phase of *hid* transcription may be transient and/or cyclic not allowing sufficient accumulation of transcript and protein for detection by the methods employed. Since we have also shown that *hid* function is

necessary for PC apoptosis to occur, then activation of *hid* transcription likely represents an important regulatory point in the induction of PC apoptosis.

Hid controls Diap1 post-translational downregulation in PCs destined to die

In order to determine the expression pattern of Diap1 protein in the ovary, we used specific antibodies directed against Diap1 and membrane GFP expression to identify PCs (UAS-GAL4 system). In the wild-type ovary, we observed Diap1 accumulation in all follicle cells (Figure 7A,B',C), in mature PC pairs (Figure 7D",D"', white arrowheads) and in germline nurse cells (Figure 7A). The specificity of the Diap1 antibodies was confirmed by testing them on ovaries expressing an RNAi construct targeting *diap1* sequences specifically in PCs. Under these conditions, staining was absent only in PC clusters and pairs indicating that the antibodies detect Diap1 specifically (see Supplementary Figure 1). Expression of this *diap1* RNAi construct was also associated with progressive disappearance of PCs during mid-oogenesis such that 92% (n=69) of stage 7-10 follicle poles had no PCs (Supplementary Figure 1). These females were sterile, for although they laid eggs, no larvae hatched (data not shown). It is likely that the eggs remained unfertilized due to the absence of PCs and consequent lack of a micropyle. These results show that Diap1 in mature PCs is necessary for their survival and consequently for fertility.

We next examined the expression of Diap1 in clusters of 3 PCs during early oogenesis in wild-type controls. In all cases 2 PCs displayed high Diap1 levels, but three different situations were encountered for the supernumerary PC. In 50% of the clusters of 3 PCs, 1 out of the 3 PCs presented no detectible Diap1 protein (Figure 7A",A"' lower white arrowhead), while in 31% of the clusters Diap1 was present at low levels in 1 out of the 3 PCs (Figure 7B",B"' lower white arrowhead) and in only a minority of cases (19%) all 3 PCs of a cluster exhibited similar high levels of Diap1 protein (Figure 7C",C"' white arrowheads)(Table 3). Upon ectopic expression of the anti-apoptotic factor p35, a high proportion of follicle poles present PC clusters with 3 or 4 PCs (Table 1). Under these conditions, 100% of PC clusters with more than 2 PCs after stage 6 (n=85) exhibited 2 $Diap1^{+}$ PCs (Figure 7E",E"', two top and leftmost arrowheads) and 1 or 2 PCs with no detectible or extremely low Diap1 accumulation (Figure 7E",E"' two bottom and one top and rightmost arrowheads). In these

cases as well, the Diap1^{-} supernumerary PCs were often round and basally positioned indicating that they are the PCs that were destined to die (Figure 7E",E"' two bottom arrowheads). Taken together, these results strongly suggest that Diap1 is differentially regulated in the PC cluster such that 2 PCs accumulate Diap1 affording them protection from apoptosis, while supernumerary PCs undergo downregulation of Diap1 thereby allowing caspase activation and death to occur.

We next tested whether Hid might be implicated in Diap1 regulation by assaying Diap1 accumulation in PC clusters in which *hid* function is knocked down using the UAS-GAL4 system to drive expression of a RNAi construct directed against this gene (*upd-Gal4* PC driver). Under these conditions, clusters of 3 (or 4) PCs survived after stage 6 of oogenesis (Table 2), and this was associated with a greater proportion of clusters exhibiting Diap1 accumulation in all cells of the cluster compared to wild-type (Table 3). Expression of *hid* RNAi lead to a majority of clusters from stage 7-10 with 3 Diap1^{+} PCs (47% compared to only 19% in the wild-type control, Figure 7F), and only a minority exhibited complete Diap1 downregulation in the supernumerary PC(s) (15% compared to 50% in the wild-type control). These results implicate *hid* function in downregulation of Diap1 in PCs destined to die.

In order to assay Diap1 accumulation in *hid* mutant escapers, we worked out a sequential immunodetection protocol to be able to detect Fas3 and Diap1 antibodies, both generated in mouse, in two different colors (green and red, respectively). Two different genotypes were tested, $hid^{P[5014]}/Df(3L)H99$, which removes one copy of *reaper* as well as affecting both copies of *hid* and $hid^{P[5014]}/Df(3L)R+X1$ which only affects *hid* and similar results were obtained in both cases. Almost all follicle extremities after stage 6 exhibited groups of more than 2 PCs as detected by membrane accumulation of Fas3 (Figure 7G, 3 PCs and Figure 7H,H', 4 PCs observed in two different focal planes) and Diap1 protein was never absent in the supernumerary PCs (Figure 7G',H,H', yellow arrows), in contrast to the situation in controls in which Diap1 was often low or absent in these cells (Figure 7A,B,E). These results therefore indicate that *hid* function is necessary for downregulation of Diap1 in supernumerary PCs destined to die. In addition, since supernumerary PCs devoid of *hid* function and presenting Diap1 accumulation nevertheless exhibited loss of apical contact, rounding up

and shrinkage (Figure 7G, H, yellow arrows), it would seem that these processes do not require *hid* function or Diap1 downregulation.

A *diap1-lacZ* reporter construct was used to determine whether *diap1* (*thread*) transcription might be regulated in the PC cluster. This reporter construct has been shown to be regulated in other tissue contexts in *Drosophila*. [27] In the ovary, in a wild-type context, *diap1-lacZ* expression was always observed in all cells comprising a PC cluster during early stages of oogenesis even when one PC was already showing signs of apical detachment and shrinking (Figure 7I,J). Therefore, downregulation of Diap1 in PCs destined to die likely occurs post-transcriptionally. In addition, we found that overexpression of *diap1* using an *UAS-diap1* transgene and PC-specific Gal4 drivers did not lead to prolonged survival of excess PCs (data not shown). This result can be explained if the Diap1 produced by this system is downregulated post-transcriptionally such that no significant accumulation of the protein occurs. RHG members, notably Hid and Reaper, have been shown to bind Diap1 and promote its auto-ubiquination and consequent degradation, both *in vitro* and *in vivo* upon ectopic expression of Hid or Reaper. During oogenesis, we show that Hid is implicated in the downregulation of Diap1 in PCs for subsequent apoptosis of these cells.

Discussion

Due to the power of the genetic and molecular tools available, studies in *Drosophila* have made it possible for us to gain a great deal of understanding as to the mechanistic interactions and regulations between the components of the core apoptotic machinery. However, the vast majority of the studies have been carried out under conditions of induced apoptosis via ectopic expression of pro-apoptotic factors, or *in vitro*, at the biochemical level and physiological cases of apoptosis during development have not been thoroughly characterized. Here, we have carried out genetic and molecular analysis of a case of developmental apoptosis occurring during early stages of oogenesis which eliminates supernumerary specialized somatic cells, polar cells (PCs), which constitute an important organizing center in the ovary. Our results indicate that induction of apoptosis in supernumerary PCs requires the specific function of the RHG family member, Hid, Hid-mediated downregulation of Diap1, the activity of the initiator caspase Dronc and its specific partner Dark and among effector caspases, most likely the function of several caspases including Drice.

PC apoptosis therefore involves a canonical apoptotic pathway identical to one of the pathways described by Leulier *et al*. (2006) [25] in the *Drosophila* eye upon ectopic expression of Hid and consequent apoptosis. Our study therefore provides the confirmation that this pathway is functional in a physiological case of developmental apoptosis. Leulier *et al*. (2006) also found evidence for a second pathway involving Hid-induced activation of the caspases Strica and Decay, independent of Diap1. Using the same genetic tools they used (transgenic RNAi lines), however, we found no involvement of Strica or Decay functions in PC apoptosis. This difference might reflect a different level of Hid expression in the two systems. The high level of Hid induced in the eye by transgene expression allows activation of Strica and Decay, while the physiological level of Hid in supernumerary PCs may not be sufficient to do so.

A role for effector caspases in PC apoptosis is strongly suggested by p35-induced survival of supernumerary PCs and specific caspase activation in PCs destined to die as revealed by cross reactivity with antibodies directed against activated human caspase3. In addition, among the transgenic RNAi lines targeting 4 effector caspase-encoding genes, *drice*, *dcp1*, *decay* and *damm*

(previously validated by Leulier et al., 2006 [25]), only RNAi directed against *drice* led to prolonged survival of excess PCs. However, when females homozygous for the null $drice^{\Delta 1}$ allele were analyzed, no PC number defect was observed. Since the *drice* sequence used for the RNAi construct also targets *dcp1*, we also analyzed females homozygous for the strong hypomorphic allele $dcp1^{prev1}$, as well as $drice^{\Delta 1/\Delta 1}$; $dcp1^{prev1/+}$ females and $drice^{\Delta 1/\Delta 1}$ PC mitotic clones in a $dcp1^{prev1/+}$ background and found that PC apoptosis proceeded normally in all these genetic contexts. It is perhaps necessary to remove the function of both *drice* and *dcp1* completely ($dcp1^{prev1}$ is not amorphic) since redundant function between these two genes has been reported in several cell types [28,29] or to remove the function of additional caspases in addition to Drice and Dcp1. Taking into consideration these points, the best interpretation for our results is that *drice* and *dcp1* (and possible *decay* and/or *damm* as well) exhibit redundant function for PC apoptosis.

Among transgenic RNAi lines targeting genes encoding Dredd, Dronc and Strica which resemble inititator caspases, [25] only *dronc* RNAi showed an excess number of PCs. Loss of function mutations of *dronc* have shown that Dronc is required for many of the known developmental cell death programs, with specific exceptions, as well as in radiation-induced apopotosis. [30-32] Importantly, the shared phenotypes and results of genetic interaction studies indicate that Dronc function involves activation via Apaf-1/Dark as has been shown in *C.elegans* and mammals. [30,33,34] Our analysis shows that induction of both *dronc* and *dark* null mutant clones leads to prolonged survival, late into oogenesis, of clusters of PCs with more than 2 (3-5) cells. In addition, in mosaic clusters of 3-5 PCs containing both wild-type and mutant PCs, PCs destined to die, identified by their morphological changes, were in each case homozygous mutant for either *dronc* or *dark*. These results therefore indicate that both *dronc* and *dark* are necessary for PC apoptosis and that their function is cell autonomous in PCs destined to die. The partnership between Dronc and Dark thus seems also maintained in the case of PC apoptosis further supporting the idea that Dronc and Dark are universal partners for induction of apoptosis.

Control of caspase activation in PCs likely involves Diap1 function for several reasons. Indeed, Diap1 protein, present in all other germline and somatic cells of the ovary, is specifically downregulated in PCs destined to die. In particular, during early stages of oogenesis, in clusters of 3 or

more PCs, 2 PCs always showed Diap1 expression, while supernumerary PCs in the majority of cases exhibited lower levels of Diap1 or absence of Diap1 suggesting progressive downregulation of Diap1 accumulation in these cells. This regulation is likely to be post-translational since we show that expression of a *diap1-lacZ* enhancer trap is ubiquitous even in clusters where supernumerary PCs are undergoing elimination. In addition, overexpression of *diap1* in PCs using the UAS-GAL4 system did not protect supernumerary early PCs from death, which might be expected if Diap1 accumulation is regulated at the protein level (data not shown). Finally, inactivation of *diap1* by RNAi specifically in PCs lead to progressive degradation and demise of PC pairs, indicating that Diap1 is necessary for survival of mature PCs. In view of the known function of Diap1 in inhibition of caspase activity [13], our results allow us to propose that specific downregulation of Diap1 in PCs destined to die leads to caspase activation in these cells and consequent apoptosis.

We have also implicated a specific member of the RHG family of known Diap1 antagonists in PC apoptosis. [35] Induction of homozygous mutant PC clones of the H99 deletion, uncovering the three RHG genes, *reaper hid* and *grim*, led to prolonged survival of supernumerary PCs during late oogenesis. Among these three genes, we show that the function of *hid* is specifically necessary for PC apoptosis in several ways. PC expression of RNAi constructs targeting *hid*, but not *reaper*, lead to a significant number of follicle poles with excess PCs after stage 6 of oogenesis. In addition, females in which *hid* is specifically absent (hid^{P5041}/ *Df(3L)R+X1* and hid^{P5041}/ *Df(3L)H99* exhibited 100% of follicle poles with excess PCs after stage 6, while females specifically mutant for *reaper* (*XR38/Df(3L)H99*) did not show any PC number phenotype. The functions of *reaper*, *hid* and *grim*, have been shown to be largely redundant in developmental cell death, though some specific phenotypes are associated with single gene mutations. [36-39] Our results clearly indicate a specific, essential function for *hid* in PC apoptosis and thus distinguish individual developmental roles for members of the RHG family. However, a role for some of the other RHGs, in particular Reaper, though possibley redundant or secondary, is not to be excluded. Indeed we have previously shown that a *lacZ* transcriptional reporter for the *reaper* gene is activated in PCs destined to die when PC apoptosis is blocked by expression of p35. [24] It is thus possible that *hid* function is necessary for

primary induction of PC apoptosis and that *reaper* function participates to a secondary amplification loop that insures completion of PC elimination.

In addition, we have been able to establish a link between *hid* function and Diap1 regulation in PC apoptosis. Significantly, upon RNAi knockdown of *hid* function specifically in the PC group, or in *hid* mutant escapers (*hid*^{P5041}/ *Df(3L)R+X1* and *hid*^{P5041}/ *Df(3L)H99*), the supernumerary PCs that survive until late stages of oogenesis show derepressed expression of Diap1 likely explaining their prolonged survival. Although RHG family regulation of Diap1 levels by promoting Diap1 autoubiquitination and degradation has been shown through biochemical studies *in vitro* and upon ectopic overexpression of RHG proteins, [27,35,40,41,42] this is the first demonstration, combining both genetic evidence and analysis of protein accumulation *in situ*, of Hid-mediated Diap1 downregulation in a case of developmental apoptosis involving physiological levels of both of these proteins. Some studies report a significantly higher capacity for Reaper than for Hid in blocking Diap1 activity, [41] but our results show a specific role for Hid in Diap1 regulation for PC apoptosis.

Finally, we identify *hid* as an important regulatory point in the apoptotic cascade responsible for PC death. The accumulation of Hid has been shown to be regulated at both the transcriptional and post-transcriptional level depending on the tissue. [43] In PCs, both Hid protein and *hid* transcripts accumulate specifically in supernumerary PCs destined to die indicating upregulation of *hid* transcription and/or stabilization of *hid* mRNA. It is important to note however that detection of *hid* expression in PCs was not possible in a wild-type context. It was necessary to block PC apoptosis by knocking down caspase function (ectopic p35 expression or mutations in caspase-encoding genes) in order to reveal the presence of Hid protein or *hid* transcripts in supernumerary PCs normally destined to die. Therefore, in the wild type, both *hid* transcripts and Hid protein are likely short-lived and/or transient, and induction of prolonged survival of supernumerary PCs by blocking caspase activity may allow increased accumulation, and thus detection, of these gene products. The accumulated Hid protein in supernumerary PCs induced in these genetic contexts appears to be functional since these cells exhibit highly efficient downregulation of Diap1.

The PC developmental program during oogenesis involves determination of a pool of precursors in the germarium, differentiation of PCs during follicle budding from the germarium (expression of

specific markers), elimination of excess PCs by apoptosis during stages 2-4 and migration as a group along with border cells to reach the oocyte for micropyle formation. During the PC elimination process, PCs destined to die exhibit apical constriction followed by apical detachment from the germline and reduction in the contact with the other PCs leading to progressive shrinking of these cells. These morphological and polarity events may be caspase-independent since they seem to occur before caspase activation as revealed by the fact that cross-reacting human activated caspase3 antibodies label supernumerary PCs only once they have already decreased in size significantly. In support of this, caspase loss of function (mutants, RNAi or p35 ectopic expression) blocks PC elimination after apical constriction and detachment. In addition, *hid* function is not likely involved in these early processes either. The *hid* escapers recovered exhibited almost 100% of follicle poles with more than 2 PCs after stage 6 and at many poles the supernumerary PCs showed apical detachment, rounding up and shrinking (see Figure 7G and H for two examples).

Polar and border cell migration is also significantly delayed by mutations affecting apoptotic effectors. We quantified the migration defects in several genetic contexts affecting the apoptotic machinery (*hid* RNAi, *drice* RNAi, ectopic p35) and similar results were obtained irrespective of the molecule targeted for inactivation (Supplementary Table 1). In addition, in all three genetic contexts tested, clusters with 4 PCs were more perturbed in their migration than clusters with 3 PCs (Supplementary Table 1). These results suggest that these migration defects are likely a consequence of the elevated number of PCs surviving into late stages of oogenesis, rather than reflecting a role for these molecules (caspases, for instance) in migration *per se*.

Final disappearance of supernumerary PCs occurs when shrinking PCs are reduced to one small point and other types of structures resembling apoptotic corpses have not been observed (data not shown). PC apoptosis therefore involves an atypical mechanism for complete elimination of the cellular contents. There are at least two possibilities, elimination of the contents from within the dying cells themselves or take-up of the contents by neighboring mature PCs, follicular cells, germline cells or even circulating hemocytes, which would not involve formation of apoptotic corpses. It will be interesting to develop an *in vivo* real-time approach which may reveal important aspects of PC apoptosis, as has been useful for the study of other aspects of *Drosophila* oogenesis. [44,45]

Materials and Methods

Fly strains

The following strains were generated for polar cell specific expression of GFP or YFP: the enhancer trap *upd-Gal4* X (provided by S.Noselli) combined with *UAS-mCD8:GFP* III or with *UAS-H2B:YFP* III (provided by M.Gho) and *neu*^{p72}*-Gal4; UAS–H2B:YFP.* [46] The following chromosomes were used in this study: *UAS-p35,* [47] *FRT80B dronc* ^{i29} and *FRT80B dronci*^{24}, [32] *FRT42D dark* ^{H16}, *FRT42D dark*^{G8}, [48] *FRT79D Df(3L)H99,* [49] *P (PZ) hid* ^{0514} , [36] *hid* $^{WR+X1}$, [50] *XR38*, [37] yw ;th^{j5C8}/TM3Sb (*lacZ* enhancer trap in *thread/diap1*) [27], *UAS-diap1IR , tub-Gal 80*^{ts} (provided by F.Leulier), *drice*$^{\Delta 1}$, [51] *drice*^{17}, [29] *dcp1*^{prev} [52] and strains carrying RNAi constructs from NIG-FLY and VDRC collections : *UAS-driceIR* (NIG 7788R1, R2) , *UAS-hidIR* (NIG 5123 R2, R3 and VDRC 7912, 8269) , *UAS-dammIR* (NIG 118188 R1), *UAS-decay-IR* (NIG 14902 R2), *UAS-dcp1IR* (NIG 5370R and VDRC 34328, 34330), *UAS-stricaIR* (NIG 7863R) and *UAS-dronc* (VDRC 23033, 23035). FRT^{82B}, *drice*$^{\Delta 1}$ recombinants were generated between the *P(neo FRT)82B* (Bloomington stock number 2004) and *drice*$^{\Delta 1}$ chromosomes. Flies carrying the recombinant chromosomes were selected for the presence of the neomycin-resistance gene by using fly medium supplemented with 0.1 mg/ml G418 (Gibco BRL Life Technologies, Inc) and tested for the presence of the deletion corresponding to the *drice*$^{\Delta 1}$ sequence by PCR (primers: 5'-CAT GGA CGC CAC TAA CAA TG-3' and 5'-GAG AAT CAA AGA GGC GCA AG-3').

Egg chamber staining procedure

Ovaries were dissected in PBS1X, then fixed for 20 minutes in 4% formaldehyde and rinsed three times in PBT (0.3% Triton X-100). The following primary antibodies, diluted in PBT, were used in this study: mouse monoclonal anti-Fasciclin III (1:20-Developmental Studies Hybridoma Bank-DSHB), rabbit polyclonal anti-β-galactosidase (1:200-DSHB), rabbit anti-GFP (1:500-Interchim), rabbit anti-human cleaved caspase 3 (1:20-Ozyme), guinea pig polyclonal anti-Hid (1:100 [53]) , mouse

monclonal anti-Diap1 (1:200 [27]), mouse monoclonal anti-Singed (1:20 - DSHB). Fluorescence conjugated secondary antibodies were purchased from Invitrogen. All were used at a 1:200 dilution. In experiments in which anti-Fasciclin III and anti-Diap1 mouse monoclonal antibodies were used on the same tissue sample, first anti-Fasciclin III immunodetection was carried out with anti-mouse Alexa-488 secondary antibodies for fluorescent detection in green, samples were then thoroughly rinsed and anti-Diap1 immunodetection was next carried out with anti-mouse Cy3 secondary antibodies for fluorescent detection in red. All samples were mounted in cytifluor (Kent).

The *hid* riboprobe was generated using the 5A1 cDNA clone (provided by J. Abrams). [36] In situ hybridization with *hid* riboprobe was performed as described in Suter and Steward, 1991 [54], with the following changes : Ovaries were dissected and fixed with 4% paraformaldehyde in PBST ; temperature of hybridization was 65°C and sheep serum was used instead of bovine serum albumin. Detection was performed using the Dig Nucleic Acid Detection Kit (Roche).

RNAi analysis

Crosses between RNAi lines and the *upd-Gal4* driver were carried out at 25° for 2 days, then progeny was transferred to 29° for the rest of development as well as for adulthood. Females were dissected 4-5 days after eclosion.

upd-Gal4/ + ; UAS-diap1IR , tub-Gal 80^{ts} / females were maintained at 18° until eclosion, at which point they were shifted to 29°C and ovaries were dissected 5 days later.

Clonal analysis

For clonal analysis, females of the following genotypes were analyzed: *hsp-flp*/+;; *FRT80B dronc^{i24,29}* / *FRT80B ubi-GFP*, *hsp-flp*/+ ;; *FRT42D dark^{G8,H16}*/ *FRT42D* ubi-GFP and *hsp-flp*/+;; *FRT79D H99*/ *FRT79D ubi-GFP*. Females raised at 25°C were heat-shocked at mid pupae, upon eclosion and at two days of age for one hour at 37°C each time, and ovaries were dissected 4 days later.

Microscopy and image processing

Immunolabeled ovary Z-stack images were taken on a Leica Sp2 confocal microscope housed by the Imagif Platform (https://www.imagif.cnrs.fr) and Z- projections were made using ImageJ.

Figure Legends

Figure 1. Polar cells are produced in excess and restrict to a pair of cells during oogenesis. (**a**) Schematic drawing of an ovariole showing the anteriorly-positioned germarium from which follicles (or egg chambers) are formed, subsequently maturing progressively as they move towards the posterior (stage 2 through 9 follicles shown of 14 total stages). [55] Each follicle is composed of 15 nurse cells (nc) and one posteriorly-positioned oocyte (o) all of germline origin, surrounded by a monolayered epithelium of somatic follicular cells (fc). Specialized somatic cells are indicated by different colors, pola cells in green, interfolicular cells in red and border cells in blue. Somatic and germline stem cells present in the germarium (not shown) give rise to all the different cell types that constitute each follicle. [56] Groups of supernumerary polar cells restrict to only a pair of cells between stages 2 and 5. As of stage 7, anterior polar cells induce differentiation of adjacent border cells and during stage 9, the border/polar cell group delaminates from the follicular epithelium and migrates between the nurse cells to reach the oocyte. (**b-e**) Confocal images of *upd-Gal4; UAS-mCD8:GFP* follicles in which polar cells are identified by membrane mCD8:GFP whose expression is driven only in these cells (green) and immunodetection of endogenous Fas3 (red). b'-e' panels are magnified views of boxed areas in the corresponding b-e panels. Polar cells are marked with asterisks. The anterior of all follicles is to the left and the apical side of polar cells in c'-e' also to the left. (**b,c**) At early stage 2, the membrane mCD8:GFP marker allows specific identification of polar cells, while the Fas3 marker is expressed in both polar cells and surrounding follicular cells. At this stage, polar cells are flattened with their long axis perpendicular to the antero-posterior axis of the follicle. Some follicle extremities contain more than 2 polar cells (4 in b) or 2 polar cells (c). (**d**) At stage 3, Fas3 and mCD8:GFP both show specific polar cell accumulation. At this stage, polar cells in clusters of 3 cells acquire a more rounded shape, are equivalent in size and organized around an axis of radial symmetry, all maintaining apical contact with the germline (arrowhead), as well as basal contacts with the basement membrane. (**e**) At stage 6, follicles present 2 equivalent polar cells at each extremity, each contacting the germline apically (arrowhead).

Figure 2. Supernumerary PCs die by apoptosis in a stereotyped manner. Confocal images of control *upd-Gal4/+* follicles (**A-C**) and *upd-Gal4/+; UAS-p35/+* follicles in which PCs express the baculovirus caspase inhibitor, p35 (**D-J**). The apical side of PCs is always to the top. In all images, except E, the morphology of PCs is revealed by immunostaining specifically for membrane Fas3 (red). Immunodetection of activated caspases (green), which confirms the identity of dying polar cells, was carried out in **A-E.** D and E are views of the same group of PCs. DAPI staining was used to reveal nuclei in **F-I. F-J** represent a series of different groups of 3 PCs which illustrate what may be the temporal progression of morphological changes occurring during PC elimination, in particular, initial apical constriction (F,G-white arrows), followed by apical detachment (H,I-white arrows) as the supernumerary PC rounds up, reduces in size and takes a basal position (H-J-yellow arrows). Immunodetection of activated caspases (green) was carried out in **A-E** which confirms the identity of some of the dying polar cells in B-D. DAPI staining was used to reveal nuclei in **F-I.** (**K**) Model for supernumerary PC elimination. PCs are in red and adjacent follicle cells in white. (a-e) Represent successive steps during elimination of one cell in a group of 3 PCs. (a) Group of equivalent cells. The white vertical line indicates the axis of radial symmetry around which the group of equivalent cells is organized. The shrinking supernumerary PC progressively loses its apical contact (b-d) and becomes spherical (c-e). (c-e) The green color indicates the timing of caspase activation detection in the dying cell.

Figure 3. Drice is implicated in PC apoptosis. Confocal images of control *upd-Gal4/+;UAS-H2B:YFP/+* (**A,C**) and *upd-Gal4/+; UAS-driceIR/+ ; UAS-H2B:YFP/+* (**B,D**) stage 10 ovarian follicles stained with DAPI (white), Fas3 antibodies (red) and GFP antibodies (green) that respectively label all nuclei, PC membranes and PC nuclei. Anterior is to the left or to the top, as well as the apical side of PCs in A,C,D. The PCs and surrounding border cells are migrating between nurse cells in B.

A'-D' and A"-D" are magnified views of the boxed areas in the corresponding A-D panels. Note that in the control, pairs of PCs are present at a given extremity of stage 10 follicle (**A",D"** – white arrowheads**)**, while expression of a *drice* RNAi transgene leads to the presence of more than 2 PCs at both anterior and posterior poles at the same stage (**B",D"** – white arrowheads and yellow arrows to indicate the supernumerary PCs that have begun to shrink and shift basally). Also note that upon expression of a *drice* RNAi transgene, excess anterior PCs surrounded by border cells are delayed in their migration towards the oocyte at stage 10 (**B**,**B'**) compared to control PC pairs that have already reached the oocyte at the same stage (**A,A'**).

Figure 4. The initator caspases Dronc and its adaptor Dark are required for PC apoptosis. Confocal images of stage 7-10 *hsp-flp/+; +/+; FRT42D dronc$^{i24/i29}$/FRT42D P[ubi-GFP]* (**A-C**) and *hsp-flp/+; +/+ ; FRT42D dark^{G8}/FRT42D P[ubi-GFP]* (**D-F**) ovarian follicles stained with Fas3 antibodies (red) to label PC membranes and GFP antibodies (green) to identify homozygous *dronc* and *dark* mutant clones (absence of green staining). The apical side of the follicular epithelium in which the PCs are embedded is towards the top. White arrowheads and yellow arrows mark PCs destined to survive or die, respectively, as indicated by the round shape, smaller size and more basal position indicating progressive exclusion of the latter from the group. The presence of PC groups with more than 2 cells (3 or 4) is always associated with the presence of at least 1 or 2 *dronc* or *dark* homozygous mutant PCs.

Figure 5. Among the members of the RHG family, Hid is specifically necessary for PC apoptosis. (**A,B**) Confocal images of *hsp-flp/+;; FRT79D H99/FRT79D P[ubi-GFP]* stage 10 ovarian follicles stained with DAPI (white), Fas3 antibodies (red) and GFP antibodies (green) that respectively label nuclei, PC membranes and homozygous H99 mutant clones (absence of green), respectively. A',B' and A",B" are magnified views of the boxed areas in the corresponding A,B panels. A GFP$^-$ clone of 5 anterior Fas3$^+$ PCs is visualized in A',A"(asterisks) and a GFP$^-$ clone of 4 posterior Fas3$^+$ PCs is visualized in B',B"(asterisks). (**C,D**) *upd-Gal4/+;; UAS-hid-IR/UAS-H2B:YFP* stage 10 ovarian follicles stained with DAPI (white), Fas3 antibodies (red, only in D') and GFP antibodies (green) that respectively label nuclei, PC membranes and PC nuclei. C',D' are magnified views of the boxed areas in the corresponding C,D panels. Note that in C, a cluster of 3 PCs within the boxed area (each indicated by an asterisk) has not delaminated from the anterior end of a stage 9-10 follicle. In D, 3 PCs are present at the posterior end of a stage 9 follicle. The two PCs that have maintained apical contact with the germline are indicated with asterisks, while the third PC (yellow arrow), that has lost apical contact and is positioned basally is presumably that supernumerary cell destined to die. (**E,F**) *hid$^{PI(5014)}$/ Df(3L)$^{R+X1}$* ovarian follicles after stage 6 stained with Fas3 antibodies which indicate the presence of 4 (E) and 6 (F) PCs each marked with an asterisk. In these groups of more than 3 PCs, the supernumerary PCs destined to die are not clearly identifiable.

Figure 6. Hid is specifically expressed in supernumerary PCs. Confocal images of ovarian follicles stained with Fas3 antibodies to identify PCs (**A-F**), Hid antibodies (**A-F**), and anti-GFP antibodies to identify homozygous mutant *dronc* clones (absence of GFP) (**D-F**). The apical side of the follicular epithelium in which the PCs are embedded is towards the top. White arrowheads and yellow arrows mark PCs destined to survive or die, respectively, as indicated by the round shape, smaller size and more basal position indicating progressive exclusion of the latter from the group. (**G**) Image using light micrcoscopy of a stage 4-5 follicle stained with an antisense riboprobe complementary to *hid* mRNA. G' is a magnified view of the boxed area in the corresponding G panel in which the position of 3 PCs is indicated by asterisks. The white asterisk indicates the PC destined to die that has already lost apical contact, is smaller in size than the other two PCs and accumulates *hid* signal. The gemline cells of this follicle are indicated by gc. (**A**) *upd Gal4/+*. (**B,G**) *upd-Gal4/+; UAS-p35*. (**C**) *upd Gal4/+; UAS-drice-IR*. (**D**) *hsp-flp/+;;FRT 82B dronc^{i29}/FRT 82B P[ubi-GFP*]. (**E,F**) *hsp-flp/+;; FRT82B dronc^{i24}/ FRT 82B P[ubi-GFP]* and (**G**) *upd-Gal4/+; UAS-p35*. In the wild-type control, Hid protein is never detected (A), while inducing prolonged survival of supernumerary PCs by inhibiting caspase function leads to accumulation of Hid protein (B-F) and transcripts (G) in PCs destined to die.

Figure 7. Hid controls Diap1 downregulation in PCs destined to die. Confocal images of ovarian follicles stained with DAPI to label nuclei (white), GFP antibodies to label PC membranes (green), and Diap1 antibodies (red) (**A-F**) or with Fas3 antibodies to label PC membranes (green) and Diap1 antibodies (red) (**G,H**) or with Fas3 antibodies to label PC membranes (red) and βgalactosidase antibodies (green) to detect *diap1-lacZ* enhancer trap expression (**I,J**). The apical side of the follicular epithelium in which the PCs are embedded is towards the top. A'-F', A"-F", and A"'-F"' are magnified views of the boxed areas in the corresponding A-F panels. In A'-F', each PC is indicated by an asterisk. In A"-E" , A"'-E"' and I,I', it is not possible to distinguish between PC destined to survive or die so all PCs are indicated by white arrowheads. In F",F"',G,G',H,H',J,J', white arrowheads and yellow arrows mark PCs destined to survive or die, respectively, as indicated by the round shape, smaller size and more basal position indicating progressive exclusion of the latter from the group.
(**A**-**D**) Control: *upd-Gal4/+; UAS-mCD8:GFP/+*, (**E**) *upd Gal4/+ ; UAS-mCD8:GFP/UAS-p35* , (**F**) *upd-Gal4/+; UAS-mCD8:GFP/+; UAS-hid-IR/+*, (**G,H**) $hid^{P(5014)}$*/ Df(3L)* ^{H99} (G) and $hid^{P(5014)}$*/* $Df(3L)^{R+XI}$ (H) escaper and (**I**, **J**) yw ;th^{j5C8}/TM3Sb ovaries.

Supplementary Figure 1. Absence of Diap1 leads to loss of mature PCs and border cell recruitment and migration defects. Confocal images of ovarian follicles stained with GFP antibodies (green - **A,F,G**) labeling PC membranes (A,F) or nuclei (G), Diap1 antibodies (red - **A**), Fas3 antibodies labeling PC membranes (red - **B**-**E**), and Singed antibodies labeling border cell membranes (red - **F,G**). Anterior of follicles is to the top or left. (A'1,A'2), (F',F") and (G',G") are magnified views of the boxed areas in the corresponding A, F and G panels. (A-E) *upd-Gal4/+; UAS-diap1-IR, tub* $Gal80^{ts}$ */ UAS-mCD8:GFP*, (F) Control : *upd-Gal4/+; UAS-mCD8:GFP/+* and (G) *upd-Gal4/+; UAS-diap1-IR, tub-*$Gal80^{ts}$*/+; UAS-H2B:YFP/+*. (A) When a transgenic RNAi construct targeting *diap1* was expressed in PCs, Diap1 was absent only in PCs, at both anterior and posterior follicle poles (arrrowheads). (B-E) In ovarioles expressing an RNAi construct targeting *diap1* in PCs, PCs were present in early stage 2-3 follicles (B), but as of stage 4, follicle poles with one PC (C-arrow), with remnants of PCs (D-arrow) or no PCs (E) were observed. At stage 10, in the control, mature PC pairs surrounded by border cells have reached the nurse cell-oocyte interface (F-boxed area). When Diap1 was removed from PCs, most stage 10 follicles had no anterior PCs and no or a small number of border cells (1-3 compared to approximately 7 in wild-type). In the example shown in (G), one PC is present and 3 surrounding border cells, which have not migrated from the anterior of an advanced stage 10 follicle.

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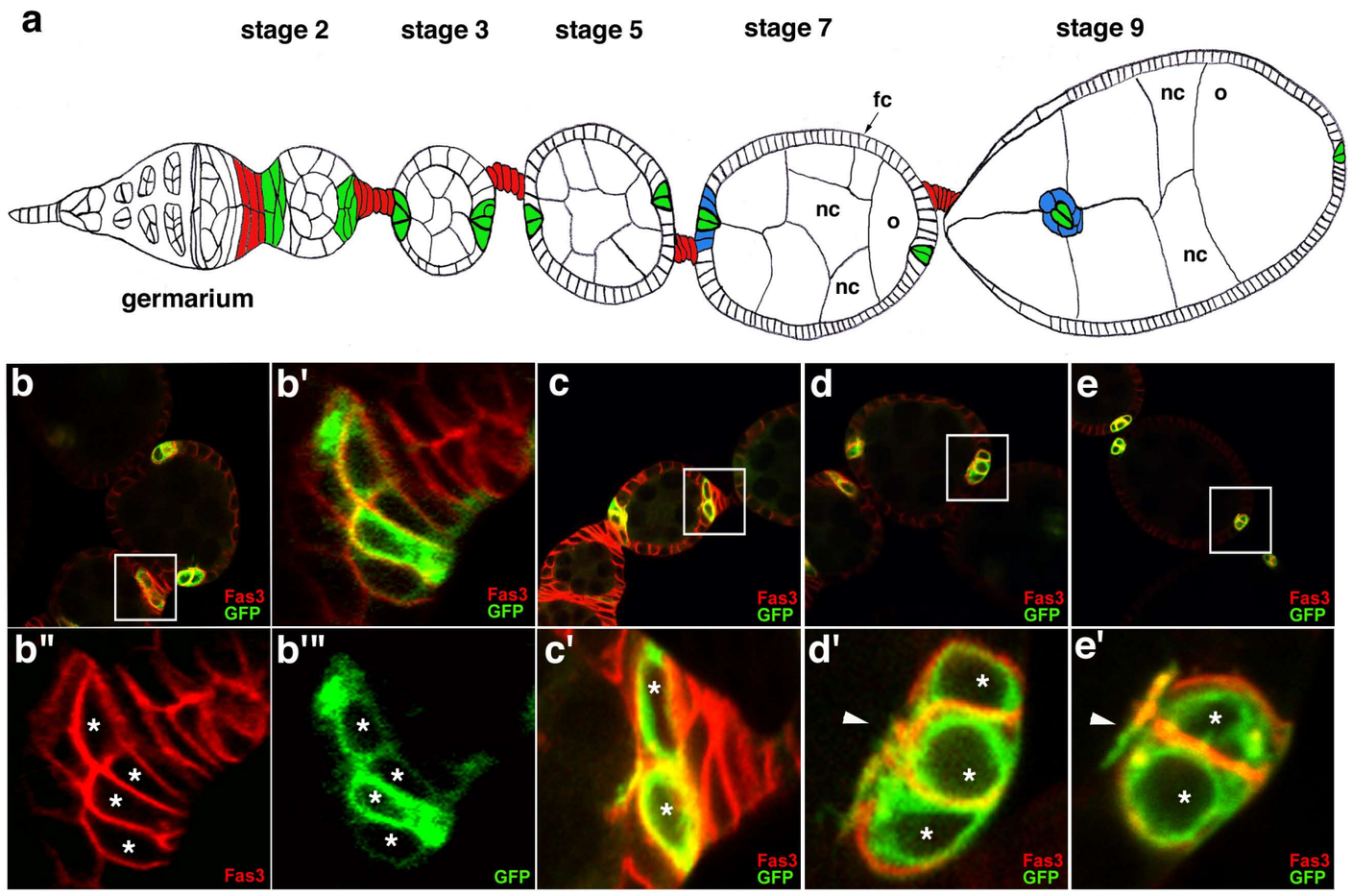
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stage 2
stage 3
stage 5
stage 7
stage 9
germarium
fc
nc
o
nc
nc
o
nc
b
b'
c
d
e
b"
b'''
c'
d'
e'
Fas3
GFP
Fas3
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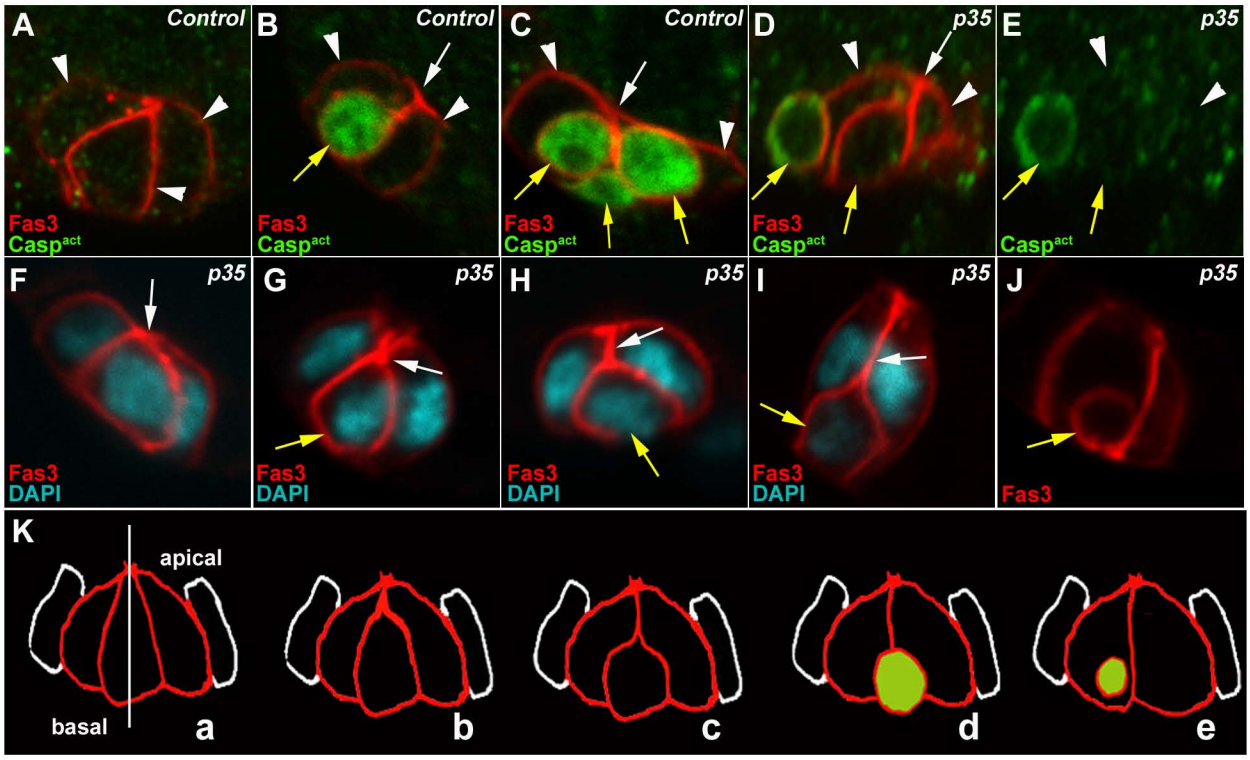
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Fas3
Casp^act
B
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Casp^act
C
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D
p35
Fas3
Casp^act
E
p35
Casp^act
F
p35
Fas3
DAPI
G
p35
Fas3
DAPI
H
p35
Fas3
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K
apical
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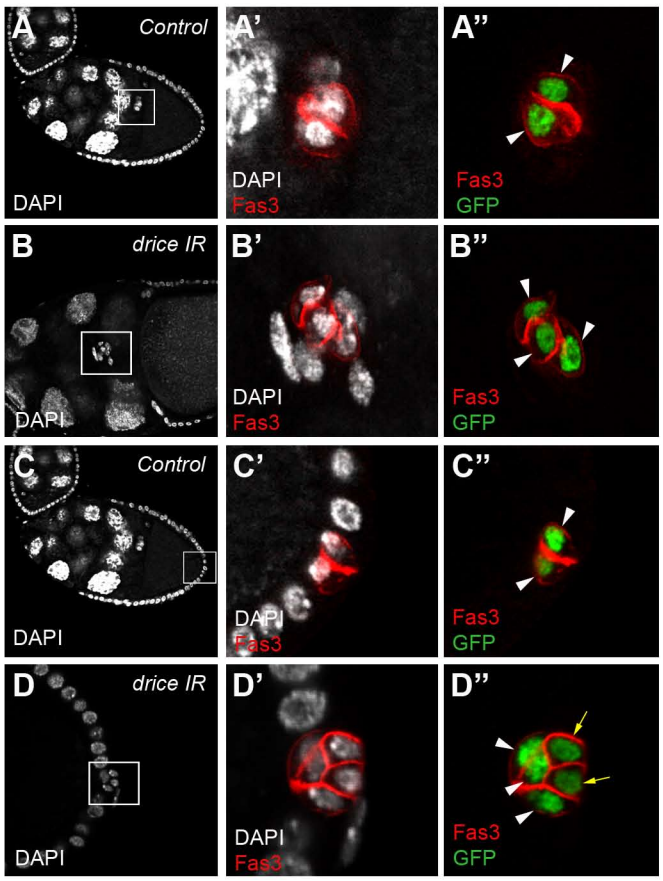
b
c
d
e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GFP
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drice IR
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B''
Fas3
GFP
C
Control
DAPI
C'
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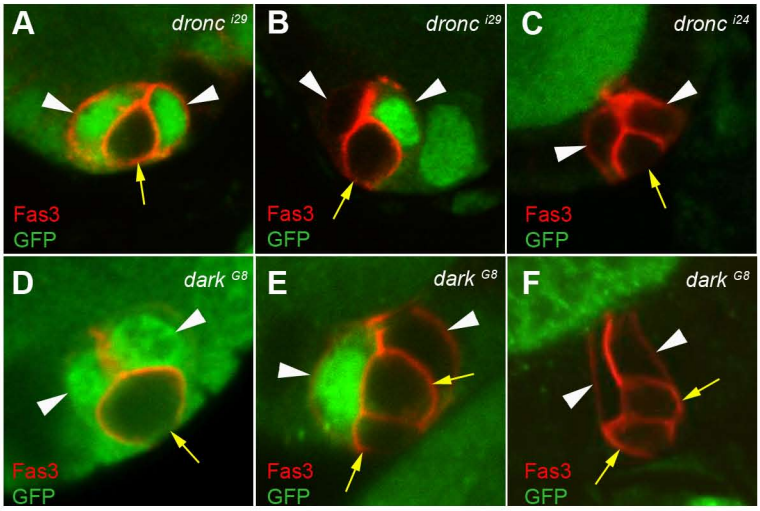
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C
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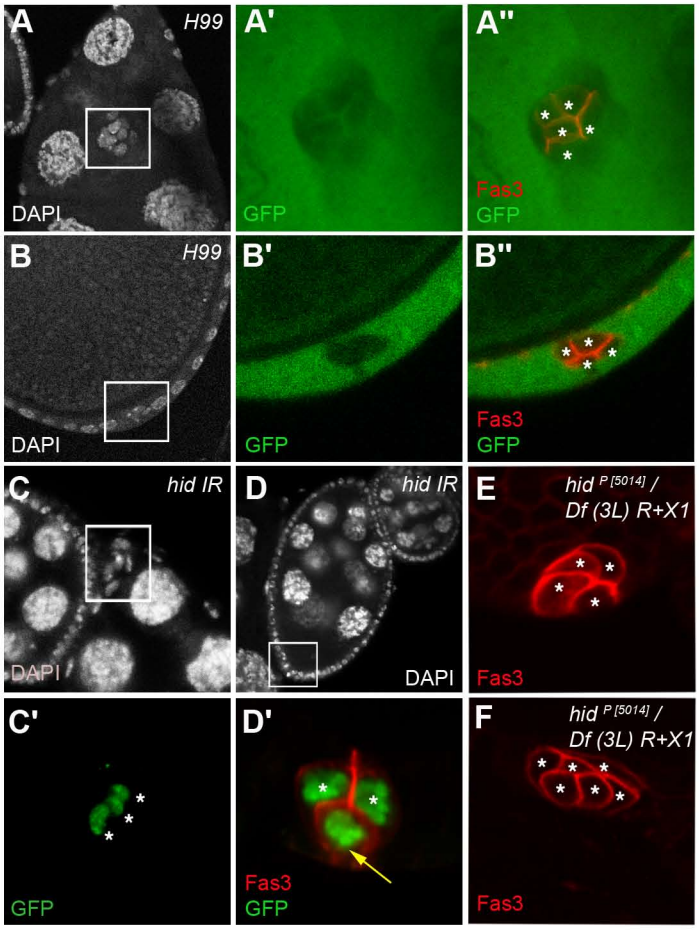
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C
hid IR
DAPI
D
hid IR
DAPI
E
hid P [5014] /
Df (3L) R+X1
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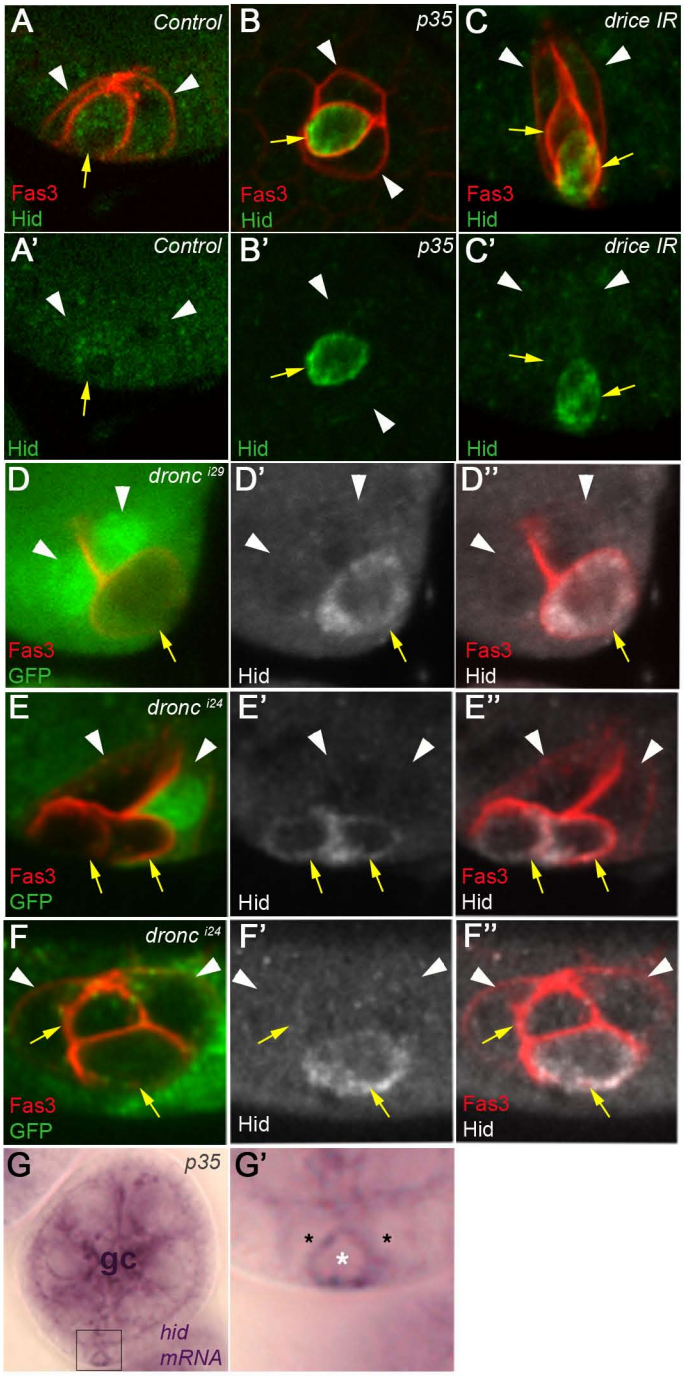
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p35
Hid
C'
drice IR
Hid
D
dronc i29
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GFP
D'
Hid
D''
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Hid
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GFP
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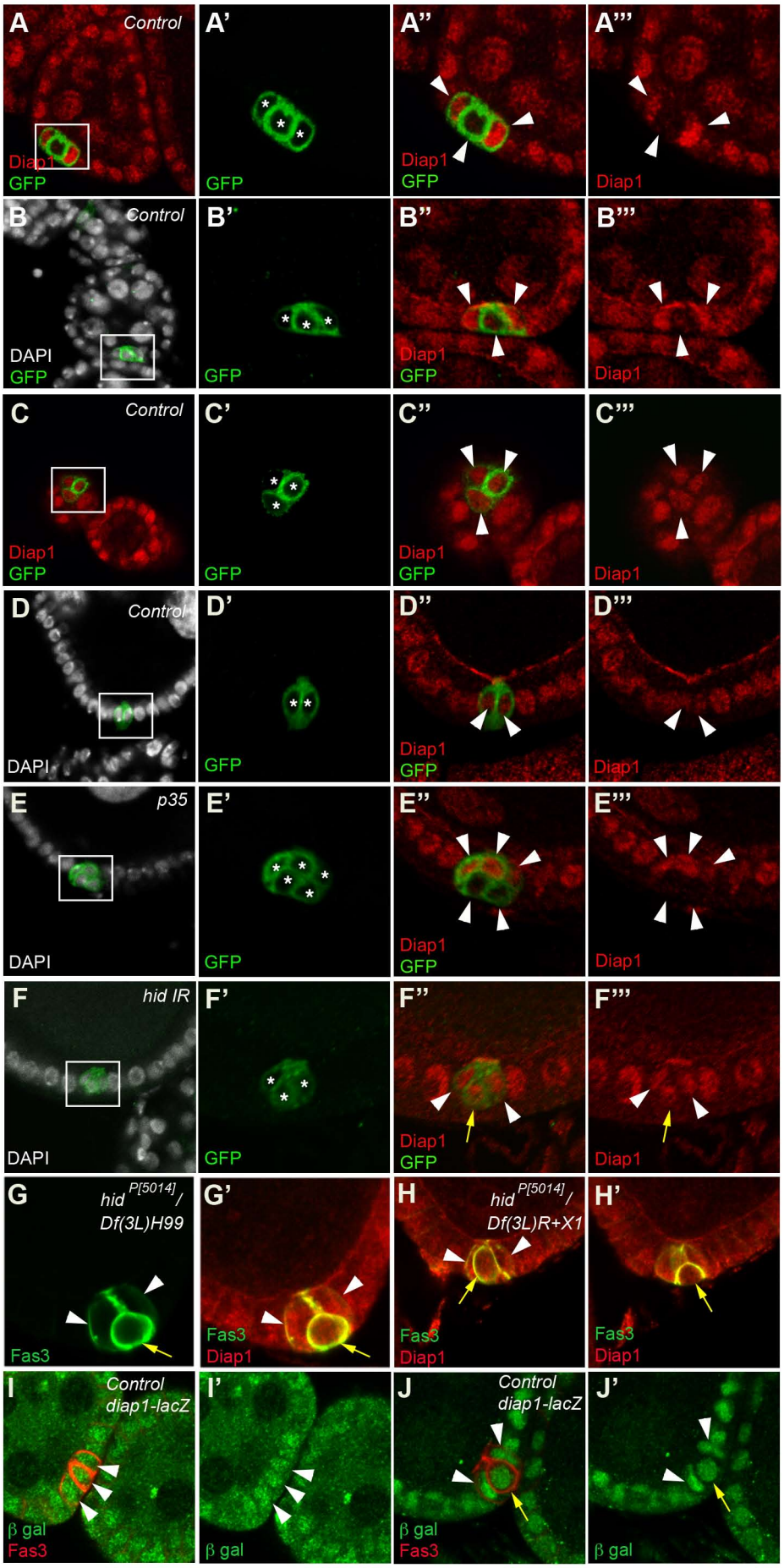
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GFP
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Diap1
GFP
C'''
Diap1
D
Control
DAPI
D'
GFP
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Diap1
GFP
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p35
DAPI
E'
GFP
E''
Diap1
GFP
E'''
Diap1
F
hid IR
DAPI
F'
GFP
F''
Diap1
GFP
F'''
Diap1
G
hid P[5014] / Df(3L)H99
Fas3
G'
Fas3
Diap1
H
hid P[5014] / Df(3L)R+X1
Fas3
Diap1
H'
Fas3
Diap1
I
Control
diap1-lacZ
β gal
Fas3
I'
β gal
J
Control
diap1-lacZ
β gal
Fas3
J'
β gal

Table 1. An excess in polar cell number is produced in egg chambers upon overexpression of the baculovirus caspase inhibitor p35 in these cells

	stage 3		stage 4		stages 5-6		stages 7-8		stages 9-10		stages 7-10	
	A	P	A	P	A	P	A	P	A	P	(A + P)	
genotype												
	n=51		n=48		n=71		n=50		n=60		n=220	
upd-gal4/+; UAS-mCD8:GFP/+												
chambers with 2 PCs	78%	67%	100%	98%	100%	100%	100%	100%	100%	100%	100%	
chambers with 3 PCs	20%	33%	0%	2%	0%	0%	0%	0%	0%	0%	0%	**0%**
chambers with 4 PCs	2%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	
average nb of PCs	**2.2**	**2.4**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	
	n=49		n=21		n=45		n=40		n=23		n=126	
upd-gal4/+;UAS-p35/UAS-mCD8:GFP												
chambers with 2 PCs	21%	57%	29%	24%	38%	36%	30%	30%	27%	45%	32%	
chambers with 3 PCs	73%	37%	62%	62%	53%	55%	55%	55%	73%	55%	58%	
chambers with 4 PCs	6%	6%	9%	14%	7%	7%	15%	13%	0%	0%	8%	**68%**
chambers with 5 PCs	0%	0%	0%	0%	2%	0%	0%	2%	0%	0%	1%	
chambers with 6 PCs	0%	0%	0%	0%	0%	2%	0%	0%	0%	0%	1%	
average nb of PCs	**2.9**	**2.5**	**2.8**	**2.9**	**2.7**	**2.8**	**2.8**	**2.8**	**2.7**	**2.5**	**2.7**	

In females ectopically-expressing the baculovirus caspase inhibitor p35 specifically in polar cells, the number of polar cells (PCs), as indicated by Fas3 immunostaining, both at the anterior (A) and posterior (P) ends of each chamber, was determined from stages 3 to 10 of oogenesis. Results are expressed as the percentage of total chambers (n=) of a given genotype with 2, 3, 4, 5, or 6 polar cells. The average number of polar cells at each pole for each stage is indicated at the bottom of each column.

Table 2 : An excess in polar cell number is produced in egg chambers upon expression of RNAi constructs targeting either a member of the RHG family (*hid*) or the effector caspase (*drice*)

	stage 3		stage 4		stages 5-6		stages 7-8		stages 9-10		stages 7-10	
	A	P	A	P	A	P	A	P	A	P	A+P	
genotype												
	n=31	n=31	n=37	n=37	n=41	n=41	n=38	n=38	n=37	n=37	n= 150	
upd-gal4/+ ;; UAS-H2B:YFP/+												
chambers with 2 PCs	58%	55%	86%	86%	97%	100%	100%	100%	97%	100%	99%	
chambers with 3 PCs	42%	45%	14%	14%	3%	0%	0%	0%	3%	0%	<1%	
average nb of PCs	**2.42**	**2.45**	**2.13**	**2.13**	**2.02**	**2.0**	**2.0**	**2.0**	**2.02**	**2.0**	**2.01**	
	n=18	n=21	n=29	n=29	n=38	n=38	n=42	n=42	n=40	n=47	n= 171	
upd-gal4/+;UAS-driceIR*/+ ; UAS-H2B:YFP/+												
chambers with 2 PCs	17%	57%	14%	21%	37%	26%	47%	32%	34%	34%	37%	
chambers with 3 PCs	61%	37%	62%	55%	60%	55%	47%	52%	55%	47%	50%	
chambers with 4 PCs	22%	6%	17%	24%	3%	19%	5%	14%	11%	19%	12%	**63%**
chambers with 5 PCs	0%	0%	7%	0%	0%	0%	0%	2%	0%	0%	1%	
average nb of PCs	**3.05**	**3.0**	**3.17**	**3.17**	**2.66**	**3.02**	**2.67**	**2.86**	**3.4**	**2.85**	**2.76**	
	n=21	n=21	n=25	n=25	n=29	n=29	n=29	n=29	n=32	n=32	n=122	
upd-gal4/+ ;; UAS-hidIR */ UAS-H2B:YFP												
chambers with 2 PCs	10%	62%	52%	32%	66%	52%	52%	76%	69%	63%	65%	
chambers with 3 PCs	76%	33%	40%	64%	31%	45%	45%	24%	25%	37%	33%	**35%**
chambers with 4 PCs	14%	5%	8%	4%	3%	3%	3%	0%	6%	0%	2%	
average nb of PCs	**3.04**	**2.43**	**2.56**	**3.17**	**2.38**	**2.52**	**2.52**	**2.24**	**2.38**	**1.95**	**2.37**	

*The strains carrying RNAi constructs used were obtained from NIG-FLY.
UAS-hidIR : 5123R2
UAS-driceIR : 7788R1

In females ectopically expressing the RNAi transgenes *UAS-driceIR* and *UAS-hidIR* specifically in polar cells, the number of polar cells (PC), as indicated by Fas3 and YFP immunostaining, both at the anterior (A) and posterior (P) ends of chambers was determined from stages 3 to 10 of oogenesis. Results are expressed as the percentage of total chambers (n=) of a given genotype with 2, 3, 4, or 5 polar cells. The average number of polar cells at each pole for each stage is indicated at the bottom of each column.

	 1 Diap1^{-} PC	 1 Diap1$^{+/-}$ PC	 All Diap1^{+} PCs
Control (st 2 to 6)	8/16 **50%**	5/16 **31%**	3/16 **19%**
hid IR (st 2 to 6)	4/11 **36%**	5/11 **45%**	2/11 **18%**
hid IR (st 7 to 10)	2/13 **15%**	5/13 **33%**	6/13 **47%**

Table 3. Diap1 protein accumulation in polar cells during oogenesis in control and *hid* RNAi contexts.

Diap1 protein accumulation in clusters of 3 polar cells (PCs) was assessed using immunodetection. All clusters contained 2 Diap1^{+} cells, but three different categories were encountered with respect to the supernumerary polar cell.

(1 Diap1^{-} PC) No Diap1 was detected in the supernumerary polar cell.

(1 Diap1$^{+/-}$PC) Low levels of Diap1 weredetected in the supernumerary polar cell compared to the two other polar cells.

(All Diap1^{+} PCs) All three polar cells in the cluster presented equivalent amounts of Diap1.

The number of cases (and the percentage of the total) for each category between stages 2-6 and stages 7-10 is indicated for control flies and flies expressing a UAS-hidIR transgene in polarcells. Note that in control flies there are no clusters of 3 polar cells between stages 7 and 10 to be assayed.