

1 **Caspase-dependent activation of Hedgehog-signalling sustains proliferation**
2 **and differentiation of ovarian somatic stem cells.**

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10 **Key words:**

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13 **ABSTRACT**

14 There is increasing evidence associating the role of caspases with the regulation of
15 basic cellular functions beyond apoptosis. However, the molecular interplay between
16 these enzymes and the signalling networks active in non-apoptotic cellular scenarios
17 remains largely uncharacterized. Here, we show that transient and non-apoptotic
18 caspase activation facilitates Hedgehog-signalling in *Drosophila* and human ovarian
19 cells with a somatic origin. Importantly, this novel caspase function controls gene
20 expression, cell proliferation, and differentiation. We also molecularly link this
21 uncovered caspase role with the fine regulation of the Hedgehog-receptor, Patched.
22 Altogether, these findings strikingly suggest that caspase activation can act as a pro-
23 survival factor that promotes the expansion and differentiation of normal healthy
24 cells. These observations have profound implications on our understanding of
25 caspase biology from a cellular, physiological and evolutionary perspective.

26 **MAIN**

27 The transient and moderate activation of caspases in specific subcellular
28 compartments is essential for the regulation of a diverse range of cellular functions
29 beyond apoptosis¹⁻⁴. However, the molecular details underlying these non-apoptotic
30 functions are largely unknown in many cellular scenarios. Addressing this question
31 can provide essential information to fully understand caspase biology, as well as the
32 physiological role of these enzymes in a wide variety of cells, including stem cells.

33 During the last decade, the adult *Drosophila* ovary has been extensively used to
34 investigate stem cell physiology and intercellular communication^{5, 6}. Additionally, it is
35 one of the cellular models that shows widespread non-apoptotic caspase activation
36 in response to environmental stress⁷. Therefore, it appears to be an ideal cellular
37 system to study the interplay between caspases, signalling mechanisms, and stem
38 cell physiology. The early development of *Drosophila* female gametes occurs in a
39 cellular structure referred to as the germarium. The germarium is formed by the
40 germline and the surrounding somatic cells^{5, 6} (Figure. 1a). One of the main genetic
41 cascades coordinating the physiology of somatic and germinal cells is the Hedgehog-
42 signalling pathway⁸⁻¹⁴. The interaction of the Hedgehog (Hh) ligand with its
43 membrane receptor Patched (Ptc) allows the activation of the signalling transducer
44 Smoothed (Smo)¹⁵. This ultimately prevents the proteolytic processing of the
45 transcriptional regulator Cubitus interruptus (Ci)¹⁵, thus eliciting the activation of
46 multiple target genes¹⁵. The main somatic Hh-activating cells in the germarium are
47 the escort cells¹² and the follicular stem cells¹³ (Fig. 1a). Hh-pathway activation in the
48 escort cells controls the signals that emanate to the germline^{9, 12}. This remote system
49 of signalling modulates the timely differentiation of the germline^{9, 12}. In the follicular
50 stem cells, Hh-pathway activation promotes cell proliferation and cell differentiation^{11,}
51 ^{14, 16, 17}, while Hh-deficiency has the opposite effect^{8, 11, 13}. Importantly, the pro-
52 proliferative role of Hh-signalling in ovarian somatic cells is evolutionarily
53 conserved¹⁸⁻²⁰. Furthermore, while an increase in Hh-signalling stimulates tumour
54 growth and metastatic behaviour of ovarian somatic malignancies^{20, 21}, its
55 downregulation through chemical agents or genetic factors compromises cell division
56 and differentiation^{19, 21, 22}.

57 Our work shows that non-apoptotic caspase activation in somatic cells of the
58 *Drosophila* germarium modulates Hh-signalling, autophagy, and the cellular
59 properties of ovarian somatic cells. We also molecularly connect this unexpected

60 caspase functions with the fine-tuning of Ptc levels. Finally, our findings suggest that
61 the caspase-dependent effects on Hh-signalling and autophagy are evolutionarily
62 conserved in human ovarian cells with somatic origin. Altogether, these observations
63 uncover unknown features of caspase biology, whilst conferring a pro-survival role to
64 these enzymes in the ovary.

65

66 **RESULTS**

67 **Non-apoptotic Dronc activation in ovarian somatic stem cells**

68 We recently generated a novel caspase sensor based on a cleavable, but
69 catalytically inactive version of the effector caspase, *Drice* (Drice based sensor QF;
70 DBS-S-QF)²³. Among other applications, this sensor can be used to induce the
71 expression of multiple fluorescent markers with variable perdurance in caspase-
72 activating cells²³. This feature is able to provide a short-term perspective of caspase
73 activation dynamics, as well as a permanent labelling of caspase-activating cells in
74 *Drosophila* tissues²³. Since it has been shown that strong environmental stress
75 (starvation and cold shock) can induce widespread non-apoptotic caspase activation
76 in the *Drosophila* ovary⁷, we sought to investigate whether under moderate stress
77 such activation could exist and follow a stereotyped pattern. The detailed inspection
78 of adult flies maintained at 29 °C showed discrete subsets of escort and follicular
79 cells labelled with the short-lived fluorescent markers induced by our caspase sensor
80 (Fig. 1b). Interestingly, marked cells did not always show signs of cell death such as
81 DNA fragmentation (green positive cells and TUNEL negative in Fig.1b).
82 Furthermore, they often displayed only the fluorescent signature of caspase
83 activation in the past (green signal, Fig. 1b), but not the marker of ongoing caspase
84 activation at the time of dissection (red signal, Fig. 1b). Confirming the presence of
85 transient caspase activation in healthy and proliferative cells, large groups of
86 follicular cells including the follicular stem cells and escort cells were permanently
87 labelled with the DBS-S-QF caspase sensor (Fig. 1c). Furthermore, the number of
88 enduringly labelled germaria with this system increased over time (from 8% to 22% in
89 ovaries dissected at 7 and 14 days after adult eclosion, respectively; Fig. 1d). These
90 results demonstrated the presence of transient and non-apoptotic caspase activation
91 in somatic cells of the germarium, including the proliferative stem cell precursors,
92 under moderate stress.

93 Since DBS-S-QF was designed to specifically report the activation of initiator
94 caspases²³, and *Dronc* (the *Drosophila* orthologue of the mammalian caspase-9) is
95 the main initiator *Drosophila* caspase associated with non-apoptotic functions, we
96 sought to investigate its transcriptional regulation in the ovary by using a *Dronc*^{KO-Gal4}
97 strain generated in the laboratory that recapitulates physiological expression of this
98 gene²⁴. *Dronc*^{KO-Gal4} was able to drive the expression of a UAS-Histone-RFP
99 transgene in the escort and follicular cells of the germarium at 29°C (Fig. 1e, f). This
100 expression pattern was also confirmed through cell lineage-tracing experiments
101 using the same Gal4 driver (Fig. 1g). This data strongly suggested that *Dronc* could
102 be responsible for the caspase activation patterns observed with DBS-QF sensor
103 (compare Fig. 1c, 1f and 1g).

104 ***Dronc* acts as a pro-survival factor that sustains follicular stem cell functions**

105 To determine the biological relevance of caspase activation in the germarium, we
106 took advantage of a conditional allele of *Dronc* generated through genome
107 engineering protocols^{24, 25}. This allele contains a wild-type *Dronc* cDNA flanked by
108 FRT recombination sites followed by a transcriptional activator QF (hereafter
109 *Dronc*^{FRT-Dronc-FRT-QF})²⁴. To test the excision efficiency of the *Dronc* FRT-rescue
110 cassette, we expressed flippase recombinase within the escort and follicular stem
111 cells under the regulation of c587-Gal4²⁶ (Supplementary Fig. 1a). The excision of
112 the cassette allowed the activation of the transcriptional QF factor under the
113 physiological regulation of the *Dronc* promoter, and the subsequent activation of a
114 GFP marker (QUAS-CD8-GFP) within the somatic cells transcribing *Dronc* in all of
115 the analysed germaria (n=20; Supplementary Fig. 1b). These experiments confirmed
116 the transcriptional patterns of *Dronc* in the germarium, whilst demonstrating the
117 suitability of our conditional allele to efficiently target *Dronc* expression in different
118 somatic cell populations, including those with low rates of proliferation (e.g. escort
119 cells). Combining this allele with the 109-30-Gal4 driver, we next preferentially
120 removed *Dronc* expression in the follicular stem cells and their progeny¹³
121 (Supplementary Fig. 1c). This genetic manipulation reduced the expression of the
122 somatic marker Castor (Fig. 2a and 2b), the number of follicular cells, and the size of
123 the 2b region of the germarium (Supplementary Fig. 1 d-e). Additionally, we noticed
124 that these genetic manipulations facilitated the expansion of the area in the
125 germarium occupied by Orb-positive cells (germline cells; Fig. 2c and 2d). These
126 initial results indicated the ability of *Dronc* to regulate the cellular properties of
127 somatic stem cells and the germline in the germarium. In a complementary set of
128 experiments, we preferentially targeted the expression of *Dronc* in escort cells using

129 the *spitz*-Gal4 driver (Supplementary Figure 1f). Castor expression was almost
130 unaffected by these genetic manipulations (Fig. 2b, e), and was only reduced when
131 the *spitz*-Gal4 driver was expressed in follicular stem cells (Supplementary Fig. 1g).
132 However, *Dronc* deficiency in the escort cells led to a pronounced expansion of the
133 germline (Orb-positive cells, Fig. 2d, f). The simultaneous elimination of *Dronc*
134 expression in escort and follicular cells using c587-Gal4 recapitulated all the
135 aforementioned phenotypes in somatic cells and the germline (Castor
136 downregulation and Orb expansion; Figure 2b, d). Confirming the specificity of our
137 results with *Dronc* expression, equivalent results were obtained using a different
138 conditional allele, which expressed a Suntag-HA-Cherry chimeric protein instead of
139 QF upon the FRT-rescue cassette excision ($Dronc^{KO-FRT} Dronc-GFP-Apex FRT-Suntag-HA-Cherry$
140 ²⁴, Fig. 2g). Furthermore, the overexpression of a *Dronc*-RNAi construct under the
141 regulation of $Dronc^{KO-Gal4}$ generated a comparable, but less penetrant, version of the
142 described phenotypes (Supplementary Fig. 1h, i). Together this data confirmed that
143 the non-apoptotic activation of Dronc can act as a pro-proliferative and pro-
144 differentiating factor in the follicular stem cells. Additionally, it can regulate at a
145 distance the cellular properties of adjacent germline cells.

146 **The function of Dronc in ovarian somatic cells relies on its catalytic activity but** 147 **is largely independent of the apoptosis programme**

148 Most functions of caspases rely on their enzymatic activity, but some of the non-
149 apoptotic roles require only protein-protein interactions^{27, 28}. To determine whether
150 *Dronc* mutant phenotypes were correlated with its enzymatic activity, we used a new
151 conditional allele that contains after the FRT-rescue cassette a *Dronc* allele encoding
152 a catalytically inactive protein²⁴. This inactive version of Dronc contains two
153 mutations, C318A and E352A, which compromise its enzymatic function and
154 proteolytic activation^{29, 30}. This mutant allele behaves as a null in homozygous
155 conditions and is referred hereafter as *Dronc*-FL-CAEA ($Dronc^{KO-FRT} Dronc-GFP-Apex FRT-$
156 $Dronc-FL-CAEA-Suntag-HA-Cherry$)²⁴. Upon excision of the wildtype rescue cassette, the
157 expression of *Dronc*-FL-CAEA mutant protein recapitulated the loss of Castor
158 expression and the cell proliferation defects in the 2b region of the germarium (Fig.
159 2g). Since these experiments indicated a requirement for the enzymatic activity of
160 Dronc, we investigated the potential contribution to the Dronc phenotypes of its
161 primary substrates in many cellular contexts, the effector caspases (drlCE, DCP-1,
162 Decay and Damm)³¹. To avoid a potential functional redundancy between these
163 caspase members³², we simultaneously targeted their expression by overexpressing

164 at the same time validated RNAi constructs against all of them³¹. These genetic
165 manipulations produced weaker phenotypes than the *Dronc* loss-of-function (LOF),
166 but still compromised Castor expression (Fig. 2g). Next, we analysed the role of the
167 main upstream pro-apoptotic factors by targeting their expression via a micro RNA
168 (UAS-RHG miRNA: *rpr*, *hid*, and *grim*)³³. Strikingly, the concomitant elimination of
169 *hid*, *reaper* and *grim* expression did not affect Castor levels (Fig. 2g). Collectively,
170 these results indicate that *Dronc* functions in our cellular scenario demand its
171 enzymatic activity and the subsequent activation of effector caspases, but are largely
172 independent of the apoptotic programme.

173 **Dronc activation promotes Hh-signalling in escort cells and follicular stem** 174 **progenitors**

175 Since Hh-signalling deficiency in escort and follicular stem cells generates
176 phenotypes highly reminiscent of our *Dronc* mutant conditions^{8, 9} (compare Fig. 2
177 with Supplementary Fig. 2a-c), we assessed the expression levels of standard read-
178 outs of Hh-signalling in *Dronc* mutant cells. *Dronc* deficiency in follicular stem cells
179 reduced the levels of the transcriptionally active form of Cubitus interruptus (Ci-155³⁴,
180 Gli in mammals; compare Fig. 3a and 3b). Accordingly, the transcriptional activation
181 of *ptc* was also compromised (compare Figure 3a with 3b; Supplementary Fig. 2d).
182 The downregulation of both markers in *Dronc* mutant cells was a strong indication of
183 Hh-signalling defects. To functionally confirm the crosstalk between caspases and
184 Hh-signalling, we attempted to rescue the *Dronc* mutant phenotypes by
185 overexpressing either a constitutively active form of Smo¹⁵ (Fig. 3c, d) or Ci
186 (Supplementary Fig. 2e, f). The individual overexpression of any of these Hh-
187 components restored Castor expression and the proliferation defects of *Dronc*
188 mutant cells (Fig. 3e). These data directly associated the *Dronc* LOF phenotypes in
189 the germarium with Hh-signalling defects. Furthermore, they genetically placed
190 *Dronc* upstream of *smo*.

191 **Dronc regulates Hh-signalling through the fine-tuning of Ptc**

192 Since our previous experiments placed the activation of *Dronc* upstream of *smo*, we
193 investigated a potential genetic interaction between *Dronc* and *ptc*. A Gal4 P-element
194 insertion in the regulatory region of *ptc* generated a weak LOF allele and a Gal4
195 line³⁵. Unexpectedly, double heterozygous germaria *ptc-Gal4:Dronc^{KO}* (*ptc-Gal4/+*;
196 *Dronc^{KO}/+*) phenotypically resembled the homozygous mutant condition for *Dronc*;
197 Castor expression was downregulated (Fig. 4a-c) and Orb territory was expanded

198 (Fig. 4d-f). These phenotypes were also correlated with Hh-signalling defects, as
199 indicated by Ci downregulation (Supplementary Fig. 2g). To validate this
200 unanticipated interaction, we overexpressed a *Dronc*-RNAi construct under the
201 regulation of the *ptc*-Gal4 driver (Supplementary Fig. 2h), and created double
202 heterozygous flies harbouring a null allele of *ptc* (*ptc*^{S2}) and our *Dronc*^{KO} (*ptc*^{S2/+};
203 *Dronc*^{KO/+}, Supplementary Figure 2i). All of these genetic combinations showed
204 comparable phenotypes. Furthermore, the observed phenotypes were dose-
205 dependent and worsen by fully eliminating *Dronc* expression (*ptc*-Gal4/UAS-*flp*;
206 *Dronc*^{KO}/*Dronc*^{FRT-Dronc-FRT-QF}; Figure 4c, 4f-h). Collectively, these results suggested a
207 clear, but initially counterintuitive, genetic interaction between *ptc* and *Dronc*. Since
208 *ptc* is the receptor of Hh but acts a negative regulator of signalling, one would expect
209 the upregulation of the pathway in *ptc* LOF conditions. Instead, the double
210 insufficiency of *ptc* and *Dronc* severely compromised Hh-signalling.

211 To better understand the interaction between *Dronc* and *ptc*, we investigated the
212 protein levels of the latter. Strikingly, although *ptc* was transcriptionally
213 downregulated in *Dronc* mutant cells (Figure 3b), the protein levels were increased
214 (Fig. 4i,j and Supplementary Fig. 3a). A detailed immunofluorescent analysis also
215 revealed that Ptc positive particles were significantly enlarged in double
216 heterozygous mutant germlaria *ptc*-Gal4:*Dronc*^{KO} (Supplementary Fig. 3b).
217 Confirming the association of *Dronc* phenotypes with Ptc aggregates, we largely
218 rescued the Hh-signalling defects of *Dronc* mutant cells by preventing Ptc
219 accumulation (Supplementary Fig. 3c-e). Recently, it has been described that Ptc
220 can induce autophagy independently of its Hh-signalling regulatory role in
221 mammalian cells and the *Drosophila* ovary^{17, 36}. Therefore, we analysed whether the
222 Ptc aggregates within *Dronc* mutant cells were competent to induce autophagy. To
223 assess the autophagy flux in the germlarium we used as a read-out the levels of
224 Ref2P (the *Drosophila* ortholog of the mammalian p62). p62 is upregulated in cells
225 with the autophagy process compromised, and downregulated upon autophagy
226 activation³⁷. As indicated by the downregulation of Ref2P, the autophagy flux was
227 enhanced in *Dronc* mutant cells (Fig. 4k, 4l and Supplementary Fig. 3f). Collectively,
228 these findings indicated that the non-apoptotic activation of *Dronc* modulates Ptc
229 levels, and secondarily the activation of the Hh-pathway and the autophagy flux.

230 **The caspase-mediated modulation of Hh-signalling and autophagy is**
231 **evolutionarily conserved in human ovarian carcinoma cells**

232 To evaluate the evolutionary conservation of our *Drosophila* findings, we used a
233 human ovarian cell line with somatic origin (OVCAR-3). This cell line, like many
234 others of similar origin, shows a basal hyperactivation of Hh-signalling³⁸. We first
235 downregulated the expression of *caspase-9* using specific siRNAs (Fig. 5a), and then
236 monitored the activation of the Hh-pathway by Q-PCR. A previously validated set of
237 primers³⁹ was used to estimate the transcriptional levels of the universal Hh-target
238 gene *ptch1*. As observed in *Drosophila* cells, *caspase-9* deficiency compromised the
239 transcriptional activation of *ptch1* and therefore we conclude that Hh-signaling is
240 altered in *caspase-9* deficient cells (Fig. 5b). Next, we investigated the impact of
241 caspase deficiency on the autophagy flux, using as marker p62³⁷. The protein levels
242 of p62 did not change in *caspase-9* mutant cells grown in standard cell culture
243 conditions; however, they were consistently reduced in *capase-9*-deficient cells
244 exposed to low concentrations of EtOH (Fig. 5c and 5d). EtOH has been shown to
245 induce cellular stress (mainly reactive oxygen species) and autophagy through
246 multiple molecular pathways in many cell types⁴⁰. Confirming the specificity of the
247 *caspase-9* effects on the autophagy, inhibition of this cellular process with
248 bafilomycin⁴¹ rescued the downregulation of p62 in *caspase-9* mutant cells exposed
249 to EtOH (Fig. 5c and 5d). Collectively, these findings suggest that caspases can also
250 modulate the levels of Hh-signalling and autophagy under moderate stress
251 conditions in human ovarian cells with somatic origin.

252 **DISCUSSION**

253 **Non-apoptotic activation of Dronc acts as a pro-survival factor in ovarian** 254 **somatic stem cells**

255 Our results show that the non-apoptotic activation of caspases modulates Hh-
256 signalling and autophagy in ovarian somatic cells. Furthermore, these non-apoptotic
257 caspase roles ensure the proper implementation of basic cellular functions such as
258 cell proliferation, cell differentiation, and intercellular communication. These findings
259 caution against the generic association of non-apoptotic patterns of caspase
260 activation with the phenomenon of anastasis (pure recovery of caspase-activating
261 cells from the “brink of death”)^{42, 43}. Alternatively, they support the hypothesis that
262 non-apoptotic caspase activation could be essential for regulating signalling events
263 and cellular functions beyond apoptosis during development and adulthood¹⁻⁴.

264 **Molecular basis of the caspase-dependent regulation of Hh-signalling and** 265 **autophagy**

266 At the molecular level, we show that sublethal levels of Dronc activation prevents the
267 accumulation of Ptc receptor, and consequently alleviates its physiological inhibitory
268 role on Hh-pathway (Fig. 5e). However, several factors suggest that Dronc is unlikely
269 to directly cleave Ptc. First, there is no indication *in silico* that Ptc can be directly
270 cleaved by Dronc. Second, the functional targets of Dronc in somatic cells appear to
271 be the effector caspases (Fig. 2g). Third, the genetic interaction between *Dronc* and
272 *ptc* is highly specific to the *Drosophila* germarium (e.g. both factors coexist in many
273 other *Drosophila* tissues without any obvious signs of interaction). Therefore,
274 although we provide the first mechanistic demonstration of the non-apoptotic
275 interplay between caspases and Hh-signalling, unidentified molecular factors must
276 couple their activities in ovarian somatic cells (Fig. 5e). Interestingly, it has been
277 shown in mammalian cells that a set of ubiquitin ligases involved in the degradation
278 of Ptc can also activate caspase-9 (*Dronc* mammalian homolog) and secondarily
279 apoptosis in Hh-signalling deficient cells^{44, 45}. However, it is unlikely that the
280 equivalent *Drosophila* ubiquitin ligase (*smurf*) can explain our phenotypes since its
281 expression and function are neither specific to the ovary or restricted to the Hh-
282 pathway⁴⁶. Nonetheless, our findings indicate that the crosstalk between caspases
283 and Hh-signaling is more complex than anticipated, bidirectional and not specific to
284 apoptosis.

285 Beyond modulating Hh-signalling and different cellular functions, Ptc aggregates in
286 *Dronc* mutant cells can boost the autophagy levels (Fig. 4 and 5e). Although previous
287 studies have associated *Dronc* with the regulation of autophagy^{47, 48}, our findings
288 establish the first molecular link between this cellular process, Hh-pathway and
289 *Dronc*. In parallel, they also confirm the novel regulatory role of Ptc in the autophagy
290 process¹⁷. Nevertheless, *Dronc* mutant phenotypes can be largely rescued by
291 reactivating the Hh-pathway, and therefore the potential contribution of the
292 autophagy to the *Dronc* phenotypes must be secondary. Interestingly, like in *Dronc*
293 mutant cells, *caspase-9* deficiency appears to enhance the autophagy in human
294 ovarian cells under moderate stress and induced-autophagy conditions (Fig. 5c and
295 5d). Together, these results suggest that caspases are part of the evolutionary
296 conserved genetic network regulating Hh-pathway and autophagy in ovarian somatic
297 cells.

298 **Cellular, physiological and evolutionary implications of non-apoptotic caspase**
299 **activation in ovarian somatic cells**

300 Our findings have shown the stereotyped presence of non-apoptotic caspase
301 activation in somatic cells of the *Drosophila* ovary under physiological or moderate
302 stress conditions. Indeed, this caspase activation appears to promote cell
303 proliferation and differentiation in this context. Consequently, we propose that
304 caspases are at the forefront of the cell survival mechanisms against cellular stress
305 in ovarian somatic cells. Additionally, sustained caspase activation due to persistent
306 signalling defects and/or environmental stress leads to apoptosis⁴⁹. This dual role of
307 caspases, coupled to different signalling pathways, could be an effective mechanism
308 of signalling compensation and cellular selection in multiple cellular scenarios⁵⁰.
309 Future investigations are needed to unravel whether caspase activation to only
310 sublethal thresholds is ensured via either specialised molecular mechanisms in
311 specific subcellular localisations⁵¹, the strength of the activating cues, or a
312 combination of both factors.

313 From a physiological perspective, it is well-described that Hh downregulation
314 triggered by environmental stress restricts egg laying and promotes autophagy in
315 *Drosophila*⁵²⁻⁵⁴. Similarly, Hh deregulation and/or exacerbated autophagy can
316 compromise follicular development in mammalian systems^{55, 56}. Our work suggests
317 that sublethal caspase activation influences Hh-signalling and autophagy, and
318 therefore it is also part of the complex adaptive system that ensures timely egg
319 maturation.

320 Considering the described non-apoptotic roles of ancient members of the caspase
321 family (metacaspases)^{4, 57, 58}, our data may also have evolutionary implications. Since
322 *Dronc* initially plays a pro-survival role in somatic cells, our results support the
323 hypothesis that the primary role of caspases could be for sustaining basic cellular
324 processes, and only inadvertent/persistent activation would lead to cell death⁵⁷. In
325 this view, these pro-apoptotic enzymes would primarily act as pro-survival factors,
326 thus inverting the widely held view of their most primitive function. A greater
327 understanding of the non-apoptotic roles of caspases is needed to test this
328 hypothesis.

329 **Pathological and therapeutic consequences of caspase malfunction in ovarian** 330 **somatic cells**

331 Caspase activation can not only facilitate follicular development (our results), but also
332 degeneration and follicular atresia in mammalian systems⁵⁹. Therefore, caspase
333 deregulation could compromise ovarian function through different mechanisms. On

334 one hand, caspase deficiency can alter the progression of follicular development by
335 compromising the activation of key developmental signalling pathways such as Hh.
336 On the other hand, it can prevent the natural degeneration of redundant follicles⁵⁹.
337 The combination of both effects can lead to ovarian failure, whilst creating tumour
338 prone conditions. Furthermore, since the combined upregulation of Hh-signalling and
339 autophagy is key to explaining the drug resistance of ovarian tumours⁶⁰⁻⁶², caspase
340 malfunctions acting upstream of these factors could compromise the success of
341 therapeutic interventions. Paradoxically, our results also indicate that the therapeutic
342 re-activation of caspases in the ovary also requires a careful calibration since
343 sublethal caspase activation could promote the clonal expansion of somatic
344 malignant cells. Collectively, these considerations illustrate some of the complexities
345 of transferring caspase-modulating molecules into the clinic⁶³.

346 MATERIAL AND METHODS

347 Fly Strains and fly husbandry details

348 All fly strains used are described at www.flybase.bio.indiana.edu unless otherwise
349 indicated. After 24h of egg laying at 25°C, experimental specimens were raised at
350 18°C, thus enabling the repression of Gal4 activity through a Gal80^{ts}. This prevents
351 potential lethality in most of our experimental conditions during larval and pupal
352 stages. After hatching, adults were then transferred from 18°C to 29°C until the
353 dissection time. At 29°C the repression of Gal80^{ts} disappears, and therefore gene
354 expression via Gal4 is elicited within specific cell subpopulations of the germarium.

355

356 Genotypes

357 Full description of experimental genotypes appearing in each figure.

358 Fig. 1

359 **1b.** *Actin DBS-S-QF, UAS-mCD8-GFP, QUAS-tomato-HA/+; QUAS-Gal4/+*

360 **1c and 1d.** *Actin DBS-S-QF, UAS-mCD8-GFP, QUAS-tomato-HA/+; QUAS-FLP*
361 *(BL30126)/+; Actin5C FRT-stop-FRT lacZ-nls/+ (BL6355)*

362 **1f.** *w;; Dronc^{KO-Gal4} / UAS-Histone-RFP (BL56555)*

363 **1g.** *UAS-FLP (BL4539) /+; Dronc^{KO-Gal4} / Actin5C FRT-stop-FRT lacZ-nls (BL6355)*

364

365 Fig. 2

366 **2a and 2c,** left panel. *109-30Gal4 (BL7023)/+; Dronc^{KO} Tub-G80^{ts} (BL7019) /+.* The
367 bars in 2b and 2d referring to this experiment are named as CTRL.

368 **2a** and **2c**, right panel. *109-30Gal4 (BL7023)/ QUAS-CD8-GFP (BL 30002); Dronc^{KO}*
369 *Tub-G80^{ts} (BL7019) / UAS-Flp (BL8209) Dronc^{KO-FRT-Dronc-GFP-APEX-FRT-QF}*. The bars in
370 2b and 2d referring to this experiment are named as *Dronc -/-*.
371 **2e** and **2f**, left panel. *spitz-Gal4 (NP0261)/+; Dronc^{KO} Tub-G80^{ts} (BL7019) / +*. The
372 bars in 2b and 2d referring to this experiment are named as CTRL.
373 **2e** and **2f**, right panel. *spitz-Gal4 (NP0261)/ QUAS-CD8-GFP (BL 30002); Dronc^{KO}*
374 *Tub-G80^{ts} (BL7019) / UAS-Flp (BL8209) Dronc^{KO-FRT-Dronc-GFP-APEX-FRT-QF}*. The bars in
375 2b and 2d referring to this experiment are named as *Dronc -/-*.
376 **2g**. First bar, *109-30Gal4 (BL7023)/ QUAS-CD8-GFP (BL 30002); Dronc^{KO} Tub-*
377 *G80^{ts} (BL7019) / UAS-Flp (BL8209) Dronc^{KO-FRT Dronc-GFP-Apex FRT-Suntag-HA-Cherry}*. Second
378 bar, *109-30Gal4 (BL7023)/ QUAS-CD8-GFP (BL 30002); Dronc^{KO} Tub-G80^{ts}*
379 *(BL7019) / UAS-Flp (BL8209) Dronc^{KO-FRT Dronc-GFP-Apex FRT-Dronc FL-CAEA-Suntag-HA-Cherry}*. Third
380 bar, *109-30Gal4 (BL7023)/UAS-DriceRNAi UAS-DecayRNAi (a gift from Pascal*
381 *Meier); UAS-DammRNAi, UAS-Dcp1RNAi (a gift from Pascal Meier)*. Fourth bar;
382 *109-30Gal4 (BL7023)/ UAS-RHG.miRNA (a gift from Iswar Hariharan)*
383

384 **Fig. 3**

385 **3a.** *109-30Gal4 (BL7023)/ ptc-GFP^{CB02030} (a gift from Isabel Guerrero)*
386 **3b.** *109-30Gal4 (BL7023)/ ptc-GFP^{CB02030} (a gift from Isabel Guerrero); Dronc^{KO} Tub-*
387 *G80^{ts} (BL7019) / UAS-Flp (BL8209) Dronc^{KO-FRT-Dronc-GFP-APEX-FRT-QF}*
388 **3c.** *109-30Gal4 (BL7023)/UAS-smo^{Act} (BL44621); Dronc^{KO} Tub-G80^{ts} (BL7019) / +*
389 **3d.** *109-30Gal4 (BL7023)/UAS-smo^{Act} (BL44621); Dronc^{KO} Tub-G80^{ts} (BL7019) /*
390 *UAS-Flp (BL8209) Dronc^{KO-FRT-Dronc-GFP-APEX-FRT-QF}*
391 **3e.** First bar (*Dronc -/-*), *109-30Gal4 (BL7023)/+; Dronc^{KO} Tub-G80^{ts} (BL7019) / UAS-*
392 *Flp (BL8209) Dronc^{KO-FRT-Dronc-GFP-APEX-FRT-QF}*. Second bar (CTRL1), *109-30Gal4*
393 *(BL7023)/UAS-Ci (BL32571); Dronc^{KO} Tub-G80^{ts} (BL7019)/+*. Third bar (*Dronc -/-*,
394 *UAS-Ci*); *109-30Gal4 (BL7023)/UAS-Ci (BL32571); Dronc^{KO} Tub-G80^{ts} (BL7019) /*
395 *UAS-Flp (BL8209) Dronc^{KO-FRT-Dronc-GFP-APEX-FRT-QF}*. Fourth bar (CTRL2); *109-30Gal4*
396 *(BL7023)/UAS-smo^{Act} (BL44621); Dronc^{KO} Tub-G80^{ts} (BL7019) / +*. Fifth bar (*Dronc -*
397 */-*, *UAS-smo^{Act}*); *109-30Gal4 (BL7023)/UAS-smo^{Act} (BL44621); Dronc^{KO} Tub-G80^{ts}*
398 *(BL7019) / UAS-Flp (BL8209) Dronc^{KO-FRT-Dronc-GFP-APEX-FRT-QF}*. The first and third bars
399 are named in graph as CTRL, while the second and the fourth as *Dronc -/-*.
400

401 **Fig. 4**

402 **4a** and **4d.** *ptc-Gal4^{559.1} (BL2017)/+*
403 **4b** and **4e.** *ptc-Gal4^{559.1} (BL2017)/+; Dronc+/-*

404 **4c** and **4f**. First bar, *ptc-Gal4*^{559.1} (*BL2017*)/+. Second bar, *ptc-Gal4*^{559.1} (*BL2017*)/+;
405 *Dronc*^{KO} +/- . Third bar, *ptc-Gal4*^{559.1} (*BL2017*)/ *QUAS-CD8-GFP* (*BL 30002*); *Dronc*^{KO}
406 *Tub-G80*^{ts} (*BL7019*) / *UAS-Flp* (*BL8209*) *Dronc*^{KO-FRT-Dronc-GFP-APEX-FRT-QF}
407 **4g** and **4h**. *ptc-Gal4*^{559.1} (*BL2017*)/ *QUAS-CD8-GFP* (*BL 30002*); *Dronc*^{KO} *Tub-G80*^{ts}
408 (*BL7019*) / *UAS-Flp* (*BL8209*) *Dronc*^{KO-FRT-Dronc-GFP-APEX-FRT-QF}
409 **4i-l**. First bar, *Dronc*^{KO}/+. Second bar, *ptc-Gal4*^{559.1} (*BL2017*)/ +. Third bar, *ptc-*
410 *Gal4*^{559.1} (*BL2017*)/ + ; *Dronc*^{KO}/+

411

412

413

414 **Immunohistochemistry**

415 Adult *Drosophila* ovaries were dissected on ice-cold PBS. Immunostainings and
416 washes were performed according to standard protocols (fixing in PBS 4%
417 paraformaldehyde, washing in PBT 0.3% (0.3% Triton X-100 in PBS). Primary
418 antibodies used in our experiments were: anti-Castor (1:2000; a gift from Alex
419 Gould); rabbit anti-HA (1:1000; Cell Signaling C29F4); mouse anti-betaGal (1:500;
420 Promega Z378B); chicken Anti-βGal (1:200, Abcam AB9361); Anti-FasIII (1:75,
421 Hybridoma Bank 7G10); Anti-Orb (1:75, Hybridoma Bank 4H8), Anti-Ci-155-full
422 length (1:50, Hybridoma Bank 2A1); Anti-Ptc (1:50, Hybridoma Bank Apa1); Anti-
423 Ref2P (1:300, abcam 178440). Conjugated secondary antibodies (Molecular Probes)
424 were diluted in 0.3% PBT and used in a final concentration (1:200): conjugated
425 donkey anti-rabbit Alexa-Fluor- 488 (A21206) or 555 (A31572) or 647 (A31573),
426 conjugated donkey anti-mouse Alexa-Fluor-488 (A21202) or 555 (A31570) or 647
427 (A31571), conjugated goat anti-rat Life Technologies (Paisley, UK) Alexa-Fluor- 488
428 (A21247) or 555 (A21434). DAPI was added to the solution with the secondary
429 antibodies for labelling the nuclei (1:1000; Thermo Scientific 62248). Following
430 incubation in secondary antibodies, samples were washed several times during 60
431 minutes in PBT. Finally, they were mounted on Poly-Prep Slides (P0425-72EA,
432 Sigma) in Aqua-Poly/Mount (Polysciences, Inc (18606)).

433

434 **TUNEL staining**

435 Like in the immunochemistry, follicles from adult *Drosophila* females were dissected
436 in ice-cold PBS and fixed in PBS containing 4% formaldehyde for 20'. After fixation,
437 the samples were washed 3 times for 15' with PBS and subsequently permeabilised
438 with PBS containing 0,3% triton and 0,1% sodium citrate for 8' on ice. 3 PBS washes
439 for 20' with were performed also after permeabilisation. The *in situ* detection of
440 fragmented genomic DNA was performed according to the DeadEnd colorimetric

441 TUNEL (Terminal transferase-mediated dUTP nick-end labeling) system (Promega).
442 Briefly, samples were first equilibrated at room temperature in equilibration buffer (5-
443 10') and then incubated with TdT reaction mix for 1 hour at 37°C in a humidified
444 chamber to obtain the 3'-end labelling of fragmented DNA. The reaction was
445 terminated with 3 washes for 15' in PBS. If necessary, the TUNEL protocol was
446 followed by standard immunofluorescent staining. The detection of TUNEL-positive
447 cells was achieved by an incubation of 45' with streptavidin-fluorophore conjugated
448 dyes.

449

450 **Imaging of fixed and live samples**

451 *Drosophila* ovarioles were imaged using the Olympus Fluoview FV1200 and
452 associated software. Z-stacks were taken with a 40X objective at intervals along the
453 apical-basal axis that ensured adequate resolution along Z-axis (step size 0.5-1.5-
454 µm). The same confocal settings were used during the image acquisition process of
455 experimental and control samples. Acquired images were processed using ImageJ
456 1.52n software⁶⁴ and Adobe Photoshop CC in order to complete the figure
457 preparation.

458

459 **Image quantification**

460 All of the images used in this study were randomised and blindly scored during the
461 quantification process. Images for quantification purposes were processed with
462 ImageJ 1.52n. To estimate the percentage of germaria showing Castor expression
463 defects (Fig 2b, 2g, 3e, 4c and Suppl Fig 2c, 3e), we first projected at least 8 focal
464 planes (maximum projection plug-in included in ImageJ) containing the follicular cells
465 of region 2b forming the arch-like structure around the germline. The germaria were
466 considered mutant for Castor if showing more than two follicular cells negative for
467 this gene in the 2b region.

468 In all other quantifications, all of the focal planes of each ovariole were first merged
469 using the maximum projection plug-in included in ImageJ. The relative area of the
470 germaria occupied by Orb-expressing cells (Fig 2d, 4f) was estimated as follows.
471 First, we delimited with the freehand selection tool of ImageJ the area occupied by
472 Orb-expressing cells and subsequently, we used the corresponding plugin of the
473 software to estimate the area. Following the same workflow, we next estimate the
474 total area of the germarium. The value of area of Orb-expressing cells was finally
475 divided by the total area of the germaria.

476 After applying the thresholding and “Analyse Particles” plug-in from ImageJ, we
477 quantified the number and size of Ptc and Ref2P-positive particles in the regions 1,
478 2a and 2b of the germarium (Fig 4i, 4k, Supp Fig 3b).
479 The “mean grey value” of the GFP channel in regions 1, 2a and 2b of the germaria
480 was used to estimate the intensity of GFP signal derived from the *ptc*-GFP transgene
481 (Supp Fig 2d).

482

483

484 **Western Blot**

485 Adult *Drosophila* ovaries were dissected in ice-cold PBS and snap-frozen in liquid
486 nitrogen. Subsequently, they were homogenised in NP40 buffer [150 mM NaCl, 50
487 mM Tris-HCl pH 7.5, 5% glycerol, 1% IGEPAL CA-630]. Cells were harvested using
488 trypsin/EDTA and centrifuged at 300g for 5'. Pellets were washed in PBS and then
489 treated with RIPA lysis buffer 1x [150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.1 mM
490 EGTA, 0,5 mM EDTA, 1% Triton X-100]. Halt Protease and Phosphatase Inhibitor
491 Cocktail (Thermo Scientific Pierce) and Benzonase (BaseMuncher, Expedeon) were
492 added according to the manufacturer's instructions. Protein content was determined
493 using Bradford reagent (Bio-Rad). Extracts were mixed with NuPAGE LDS Sample
494 Buffer and separated by SDS-PAGE. For performing the SDS-PAGE electrophoresis,
495 lysates were loaded and run in NuPAGE Bis-Tris Gels in NuPAGE MOPS SDS
496 Running Buffer (Thermofisher Scientific). Protein blot transfers were performed using
497 Trans-Blot Turbo Transfer System (Biorad). Nitrocellulose blots were incubated at
498 room temperature for 30' in blocking buffer [Tris-buffered saline with 0.1% Tween
499 containing 5% non-fat dried milk] and then incubated overnight at 4°C in the same
500 blocking solution with the corresponding antibodies. After washing three times for 15'
501 each with Tris-buffered saline containing 0.1% Tween, the blots were incubated with
502 horseradish peroxidase-conjugated (HRP) IgG, followed by washing.
503 Immunoreactive bands were detected using the SuperSignal West Pico PLUS
504 Chemiluminescent Substrate (Thermofisher Scientific). Developed CL-XPosure films
505 (Thermofisher Scientific) were scanned using a flat-bed scanner and the density of
506 the bands was measured using Gel Analyzer plugin in ImageJ software. Primary
507 antibodies used: Anti-Ptc (1:500, Hybridoma Bank Apa1); Anti-Ref2P (1:500, abcam
508 178440); Anti-Actin (1:500, Hybridoma Bank JLA20s); Anti-Ci-155-full length (1:500,
509 Hybridoma Bank 2A1); Anti-Caspase-9 (C9) (1:1000, Cell Signalling 9508); Anti-β-
510 Actin-Peroxidase (1:20000, Sigma A3854), Anti SQSTM1 / P62 antibody (1:5000,
511 GeneTex GTX111393).

512

513 **Cell culture mammalian cells**

514 OVCAR-3 cells were maintained in RPMI (Sigma, R8758), supplemented with 10%
515 FBS (Life Technologies, 10500064) and grown at 37°C in a humidified atmosphere
516 with 5% CO₂. For the experiment shown in Figure 5c and 5d, we replaced the media
517 with fresh media containing either EtOH (0.2%) or EtOH (0.2%) + the inhibitor of
518 autophagy bafilomycin A1 (400nM, Merck Chemicals). Cells were grown in these two
519 different cell culture media during the last 4 hours previous the sample processing.

520

521 **RNA interference**

522 Small interfering RNA (siRNA) specific for Caspase-9 (ON-TARGETplus SMART
523 pool human L-003309-00-0005, 842), PTCH1 (ON-TARGETplus Human PTCH1, L-
524 003924-00-0005, 5727) and non-targeting controls (ON-TARGET plus Non-targeting
525 Pool, D-001810-10-05) were purchased from Dharmacon Inc. (UK). Cells were
526 plated and transfected the day after with Oligofectamine™ Transfection Reagent
527 (Thermofisher 12252) in the presence of siRNAs according to the manufacturer's
528 instructions. Cells were kept in the transfection mix before processing for western
529 blot or Q-PCR at the specified time points (24h and 72h).

530

531 **Gene expression analyses by Q-PCR.**

532 RNA extraction was performed using the Qiagen RNeasy Plus kit (74034). cDNAs
533 were synthesised with Maxima First Strand cDNA synthesis kit (Molecular Biology,
534 Thermofisher, K1642) Q-PCR were performed using QuantiNova SYBR Green PCR
535 Kit (Qiagen, 208054). Detection was performed using Rotor-Gene Q Real-time PCR
536 cyclers (Qiagen).

537 Data was analysed using the Pfaffl method, based on $\Delta\Delta$ -Ct and normalised to actin
538 as the housekeeping gene.

539 Gene expression was estimated with the following primers:

540 Patched1: Forward CCACGACAAAGCCGACTACAT; Reverse GCTGCAGATGGTCCTTACTTTTTC

541 B-actin: Forward CCTGGCACCCAGCACAAT; Reverse GGGCCGGACTCGTCATAC.

542

543 **FIGURE LEGENDS:**

544 **Fig. 1 Non-apoptotic caspase activation in the somatic cells of the *Drosophila***
545 **germarium.**

546 **a**, Schematic drawing of the *Drosophila* germarium. Somatic cells relevant for this
547 study (escort, follicular stem and follicular) are depicted in different colours; germline

548 cells are in white. **b**, Representative confocal image of past (green channel, arrows)
549 and present (red channel) caspase activation in somatic cells using the DBS-S-QF
550 sensor; TUNEL staining indicates apoptosis (grey channel). **c**, Representative
551 confocal image of escort and follicular somatic cells permanently labelled with DBS-
552 S-QF sensor (green channel, arrows); the arrowhead indicates the presence of
553 apoptotic germline cells (red channel, TUNEL staining). Notice the lack of TUNEL
554 signal in DBS-S-QF positive cells in **c**. **d**, Graph bar indicating the percentage of
555 ovarioles permanently labelled with DBS-S-QF sensor at 7 and 14 days; flies were
556 raised at 18°C until eclosion, then shifted to 29°C until the dissection time. **e**,
557 Graphical representation of the *Dronc*^{KO-Gal4} expression pattern in the germarium;
558 cells transcribing *Dronc* are coloured in red. **f**, Representative example of somatic
559 cells in the germarium expressing Histone-RFP (red channel) under the regulation of
560 *Dronc*^{KO-Gal4} at 29 °C; the follicular maker Castor is shown in green. **g**, Representative
561 example of a cell lineage-tracing experiment conducted using *Dronc*^{KO-Gal4}; notice the
562 presence of *Dronc* expressing cells in the escort cell territory and the region 2b of the
563 germarium (green channel shows anti-Bgal staining). Follicular somatic cells are
564 labelled with anti-FasIII (red channel in **g**). Dapi staining labels the nuclei (blue
565 channel) in all the confocal images. Please see full genotype description in the MM
566 section. Scale bars represents 10 µm.

567 **Fig. 2 Phenotype characterisation of caspase loss-of-function in the**
568 ***Drosophila* germarium.**

569 **a**, Confocal image comparing the expression of the follicular cell marker Castor (red
570 and grey channels) in either heterozygous (left side of the white dotted line) or
571 homozygous *Dronc* mutant follicular cells (right side) generated using 109-30-Gal4;
572 notice the downregulation of Castor in the mutant condition (arrow). GFP signal
573 labels *Dronc*-transcribing cells (green channel, QUAS-CD8-GFP) after preferentially
574 excising the *Dronc* FRT-rescue-cassette in the follicular stem cells and their progeny
575 (region 2b of the germarium) using the 109-30-Gal4 driver. Dapi labels the nuclei in
576 **a**, **c**, **e**, and **f**, respectively. **b**, Cumulative percentage of Castor-deficient germaria in
577 either heterozygous or homozygous *Dronc* mutant cells using different Gal4 drivers
578 (109-30, *spitz*, and *c587*); the n number for each column in order of appearance
579 n=16, n=17, n=20, n=15, n=34, n=19. **c**, Orb expression (red and grey channels) in a
580 heterozygous (left side) or homozygous *Dronc* mutant background (right side); *Dronc*
581 expression was targeted in the follicular stem cells and their progeny (region 2b of
582 the germarium) using 109-30-Gal4. **d**, Relative area occupied by Orb-expressing

583 cells; Gal4 drivers used were 109-30, *spitz*, and *c587*; n=12, n=20, n=17, n=17,
584 n=18, n=6; statistical significance was established by using an ordinary Unpaired T-
585 test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). **e** and **f**, Castor and Orb expression in a
586 heterozygous (left side) or homozygous *Dronc* mutant escort cells (right side) (red
587 and grey channels in **e** and **f**, respectively) generated using *spitz*-Gal4. **g**,
588 Percentage of Castor-deficient germaria after compromising the expression of *Dronc*
589 (first bar, n=11, *Dronc* *suntag*-HA-Cherry/-), the activation of *Dronc* (second bar,
590 n=15, *Dronc*-FL-CAEA /-), the expression of all of the effector caspases (third bar,
591 n=16, UAS-RNAi) and the expression of proapoptotic factors (fourth bar, n=9,
592 microRNA against the major pro-apoptotic factors *hid*, *grim* and *reaper*). Full
593 genotype description in the MM section. Scale bars represents 10 μm .

594 **Fig. 3 The loss-of function of *Dronc* causes Hh-signalling defects.**

595 **a** and **b**, Representative confocal image showing the expression of Ci-155 (blue and
596 grey channels), *ptc*-GFP (*ptc*-GFP is a *bona-fide* transcriptional read out of Hh-
597 pathway⁶⁵; green and grey channels) and Castor (red and grey channels) in either a
598 control (**a**) or a *Dronc* mutant germarium (**b**); notice the downregulation of *Ci* and *ptc*-
599 GFP expression. **c**, Castor expression (red and grey channels) in follicular cells
600 heterozygous for *Dronc* expressing a constitutively active form of *smo* under the
601 regulation of 109-30-Gal4 driver. **d**, Castor expression (red and grey channels) in
602 follicular cells homozygous for *Dronc* expressing a constitutively active form of *smo*
603 under the regulation of 109-30-Gal4 driver; notice that castor is not downregulated
604 (compare with Fig.2). Dapi staining labels the nuclei in **c** and **d**. **e**, Cumulative
605 percentage of Castor-deficient germaria after expressing Ci or Smo-activated under
606 the regulation of 109-30-Gal4 in either heterozygous or homozygous *Dronc* follicular
607 mutant cells; the n number for each column in order of appearance n=21, n=8, n=24,
608 n=11, n=8. Full genotype description in the MM section. Scale bars represents 10
609 μm .

610 **Fig. 4 *Dronc* modulates Hh-signalling and autophagy through the fine**
611 **regulation of Ptc protein levels.**

612 **a** and **b**. Castor expression (red and grey channels) in either a *ptc* heterozygous
613 germarium (**a**) or double heterozygous *ptc*-*Dronc* (**b**); notice the downregulation of
614 Castor in **b**. Dapi staining (blue channel) labels the nuclei in all the confocal images
615 of the figure. **c**, Cumulative percentage of Castor-deficient germaria in the genetic
616 conditions indicated in the graph; the n number for each column in order of

617 appearance n=20, n=25, n=20. **d** and **e**, Orb expression (red and grey channels) in
618 either a *ptc* heterozygous germarium (d) or double heterozygous *ptc-Dronc* (e). **f**,
619 Relative area occupied by Orb-expressing cells in the genetic conditions indicated in
620 the graph, an ordinary one-way ANOVA Tukey's multiple comparisons test was used
621 to establish the statistical significance (** $p \leq 0.001$, **** $p \leq 0.0001$). **g** and **h**, Castor
622 (red and grey channels in g) and Orb (red and grey channels in h) expression in *ptc*
623 heterozygous-*Dronc* fully mutant germaria; notice the downregulation of Castor (g)
624 and the accumulation of Orb-expressing cells (h). *Dronc*-transcribing cells are
625 labelled with GFP (green channel, QUAS-CD8-GFP) upon excision in the escort and
626 the follicular cells of the *Dronc* FRT-rescue-cassette using the *ptc*-Gal4 driver. **i**,
627 Relative number of Ptc positive particles per germaria; notice the increased number
628 of Ptc particles in double heterozygous germaria (*ptc-Gal4/+;Dronc +/-*), an ordinary
629 one-way ANOVA Tukey's multiple comparisons test was used to establish the
630 statistical significance (** $p \leq 0.001$). **j**, Western blot showing Ptc (upper lane) and
631 Actin (bottom lane, loading control) in different genetic mutant backgrounds; notice
632 the Ptc accumulation in double heterozygous germaria (*ptc-Gal4/+;Dronc +/-*). **k**,
633 Relative number of the Ref2P positive particles per germaria; notice the reduction in
634 the number of Ref2P particles in double heterozygous germaria (*ptc-Gal4/+;Dronc*
635 *+/-*) compared to the (*ptc-Gal4/+*) control; an ordinary one-way ANOVA Tukey's
636 multiple comparisons test was used to establish the statistical significance (** $p \leq 0.01$,
637 **** $p \leq 0.0001$). **l**, Western blot showing Ref2P (upper lane) and Actin (bottom lane) in
638 different genetic mutant backgrounds; notice the Ref2P reduction in double
639 heterozygous germaria (*ptc-Gal4/+;Dronc +/-*) compared to the (*ptc-Gal4/+*) control.
640 Full genotype description in the MM section. Scale bars represents 10 μm .

641 **Fig. 5 The non-apoptotic caspase effects in Hh-signalling and autophagy are**
642 **evolutionarily conserved in ovarian somatic cells with human origin.**

643 **a**, Western blot showing caspase-9 expression (upper lane) and Actin (bottom lane,
644 loading control) in either control or Caspase-9 mutant OVCAR-3 cells (24h and 72h
645 post-transfection of an shRNA against Caspase-9; notice the strong downregulation
646 of Caspase-9 at 72h. **b**, mRNA levels of *ptc* measured by Q-PCR in either control or
647 Caspase-9 mutant OVCAR-3 cells; notice the strong downregulation of *ptc*
648 expression in the Caspase-9 mutant cells at 72h; an ordinary Mann Whitney
649 unpaired T-test was used to establish the statistical significance (** $p \leq 0.01$). **c**,
650 Western blot showing the expression levels of the autophagy marker p62 (upper
651 lane), Caspase-9 (middle lane) and Actin (bottom lane, loading control) in either

652 scrambled or caspase-9 mutant OVCAR-3 cells; the protein levels of the different
653 read outs were measured at 72h after siRNA treatment in cells grown during the last
654 4 h before sample processing in our standard cell culture conditions, in cell culture
655 media containing EtOH (0.2%), and in cell culture media containing EtOH (0.2%) +
656 bafilomycin A1 (400nM). **d**, Quantification of p62 protein levels in the experimental
657 conditions described in d; notice the downregulation of p62 in EtOH treated cells
658 deficient in Caspase-9 (one sample T Wilcoxon test was used to calculate statistical
659 significance, * $p \leq 0.05$, $n \geq 3$. **e**, Model summarising the non-apoptotic effects of
660 caspases in ovarian somatic cells with human origin. Green colour, big font and plus
661 symbol (+) indicate activation, while red colour, small font and negative symbol (-)
662 reflect downregulation. The cellular consequences of caspase activation/deficiency
663 are indicated as Cellular Effects.

664

665 REFERENCES

- 666 1. Aram, L., Yacobi-Sharon, K. & Arama, E. CDPs: caspase-dependent non-
667 lethal cellular processes. *Cell Death Differ* **24**, 1307-1310 (2017).
- 668 2. Baena-Lopez, L.A. All about the caspase-dependent functions without cell
669 death. *Semin Cell Dev Biol* (2018).
- 670 3. Burgon, P.G. & Megeney, L.A. Caspase signaling, a conserved inductive cue
671 for metazoan cell differentiation. *Semin Cell Dev Biol* (2017).
- 672 4. Bell, R.A.V. & Megeney, L.A. Evolution of caspase-mediated cell death and
673 differentiation: twins separated at birth. *Cell Death Differ* **24**, 1359-1368
674 (2017).
- 675 5. Kirilly, D. & Xie, T. The Drosophila ovary: an active stem cell community.
676 *Cell Res* **17**, 15-25 (2007).
- 677 6. Losick, V.P., Morris, L.X., Fox, D.T. & Spradling, A. Drosophila stem cell
678 niches: a decade of discovery suggests a unified view of stem cell
679 regulation. *Dev Cell* **21**, 159-171 (2011).
- 680 7. Tang, H.L., Tang, H.M., Fung, M.C. & Hardwick, J.M. In vivo CaspaseTracker
681 biosensor system for detecting anastasis and non-apoptotic caspase
682 activity. *Sci Rep* **5**, 9015 (2015).
- 683 8. Huang, J. & Kalderon, D. Coupling of Hedgehog and Hippo pathways
684 promotes stem cell maintenance by stimulating proliferation. *J Cell Biol*
685 **205**, 325-338 (2014).
- 686 9. Huang, J., Reilein, A. & Kalderon, D. Yorkie and Hedgehog independently
687 restrict BMP production in escort cells to permit germline differentiation
688 in the Drosophila ovary. *Development* **144**, 2584-2594 (2017).
- 689 10. Vied, C. & Kalderon, D. Hedgehog-stimulated stem cells depend on non-
690 canonical activity of the Notch co-activator Mastermind. *Development*
691 **136**, 2177-2186 (2009).
- 692 11. Zhang, Y. & Kalderon, D. Hedgehog acts as a somatic stem cell factor in the
693 Drosophila ovary. *Nature* **410**, 599-604 (2001).
- 694 12. Rojas-Rios, P., Guerrero, I. & Gonzalez-Reyes, A. Cytoneme-mediated
695 delivery of hedgehog regulates the expression of bone morphogenetic

- 696 proteins to maintain germline stem cells in *Drosophila*. *PLoS Biol* **10**,
697 e1001298 (2012).
- 698 13. Sahai-Hernandez, P. & Nystul, T.G. A dynamic population of stromal cells
699 contributes to the follicle stem cell niche in the *Drosophila* ovary.
700 *Development* **140**, 4490-4498 (2013).
- 701 14. Chang, Y.C., Jang, A.C., Lin, C.H. & Montell, D.J. Castor is required for
702 Hedgehog-dependent cell-fate specification and follicle stem cell
703 maintenance in *Drosophila* oogenesis. *Proc Natl Acad Sci U S A* **110**,
704 E1734-1742 (2013).
- 705 15. Briscoe, J. & Therond, P.P. The mechanisms of Hedgehog signalling and its
706 roles in development and disease. *Nat Rev Mol Cell Biol* **14**, 416-429
707 (2013).
- 708 16. Dai, W., Peterson, A., Kenney, T., Burrous, H. & Montell, D.J. Quantitative
709 microscopy of the *Drosophila* ovary shows multiple niche signals specify
710 progenitor cell fate. *Nat Commun* **8**, 1244 (2017).
- 711 17. Singh, T., Lee, E.H., Hartman, T.R., Ruiz-Whalen, D.M. & O'Reilly, A.M.
712 Opposing Action of Hedgehog and Insulin Signaling Balances Proliferation
713 and Autophagy to Determine Follicle Stem Cell Lifespan. *Dev Cell* **46**, 720-
714 734 e726 (2018).
- 715 18. Rosales-Nieves, A.E. & Gonzalez-Reyes, A. Genetics and mechanisms of
716 ovarian cancer: parallels between *Drosophila* and humans. *Semin Cell Dev*
717 *Biol* **28**, 104-109 (2014).
- 718 19. Szkandera, J., Kiesslich, T., Haybaeck, J., Gerger, A. & Pichler, M. Hedgehog
719 signaling pathway in ovarian cancer. *Int J Mol Sci* **14**, 1179-1196 (2013).
- 720 20. Zeng, C., Chen, T., Zhang, Y. & Chen, Q. Hedgehog signaling pathway
721 regulates ovarian cancer invasion and migration via adhesion molecule
722 CD24. *J Cancer* **8**, 786-792 (2017).
- 723 21. Ray, A., Meng, E., Reed, E., Shevde, L.A. & Rocconi, R.P. Hedgehog signaling
724 pathway regulates the growth of ovarian cancer spheroid forming cells.
725 *Int J Oncol* **39**, 797-804 (2011).
- 726 22. Li, H., Li, J. & Feng, L. Hedgehog signaling pathway as a therapeutic target
727 for ovarian cancer. *Cancer Epidemiol* **40**, 152-157 (2016).
- 728 23. Baena-Lopez, L.A. *et al.* Apical caspase reporters uncover unknown stages
729 of apoptosis and enable ready visualization of undead cells. (2018).
- 730 24. Arthurton, L., Nahotko, D., Alonso, J. & Baena-Lopez, L.A. Non-apoptotic
731 caspase-dependent regulation of enteroblast quiescence in *Drosophila*.
732 *bioRxiv* (2019).
- 733 25. Baena-Lopez, L.A., Alexandre, C., Mitchell, A., Pasakarnis, L. & Vincent, J.P.
734 Accelerated homologous recombination and subsequent genome
735 modification in *Drosophila*. *Development* **140**, 4818-4825 (2013).
- 736 26. Jin, Z., Flynt, A.S. & Lai, E.C. *Drosophila* piwi mutants exhibit germline
737 stem cell tumors that are sustained by elevated Dpp signaling. *Curr Biol*
738 **23**, 1442-1448 (2013).
- 739 27. Napoletano, F. *et al.* p53-dependent programmed necrosis controls germ
740 cell homeostasis during spermatogenesis. *PLoS Genet* **13**, e1007024
741 (2017).
- 742 28. Ouyang, Y. *et al.* Dronc caspase exerts a non-apoptotic function to restrain
743 phospho-Numb-induced ectopic neuroblast formation in *Drosophila*.
744 *Development* **138**, 2185-2196 (2011).

- 745 29. Muro, I., Monser, K. & Clem, R.J. Mechanism of Dronc activation in
746 *Drosophila* cells. *J Cell Sci* **117**, 5035-5041 (2004).
- 747 30. Chai, J. *et al.* Molecular mechanism of Reaper-Grim-Hid-mediated
748 suppression of DIAP1-dependent Dronc ubiquitination. *Nat Struct Biol* **10**,
749 892-898 (2003).
- 750 31. Leulier, F. *et al.* Systematic in vivo RNAi analysis of putative components
751 of the *Drosophila* cell death machinery. *Cell Death Differ* **13**, 1663-1674
752 (2006).
- 753 32. Xu, D. *et al.* The effector caspases drICE and dcp-1 have partially
754 overlapping functions in the apoptotic pathway in *Drosophila*. *Cell Death*
755 *Differ* **13**, 1697-1706 (2006).
- 756 33. Siegrist, S.E., Haque, N.S., Chen, C.H., Hay, B.A. & Hariharan, I.K.
757 Inactivation of both Foxo and reaper promotes long-term adult
758 neurogenesis in *Drosophila*. *Curr Biol* **20**, 643-648 (2010).
- 759 34. Motzny, C.K. & Holmgren, R. The *Drosophila cubitus interruptus* protein
760 and its role in the wingless and hedgehog signal transduction pathways.
761 *Mech Dev* **52**, 137-150 (1995).
- 762 35. Shyamala, B.V. & Bhat, K.M. A positive role for patched-smoothened
763 signaling in promoting cell proliferation during normal head development
764 in *Drosophila*. *Development* **129**, 1839-1847 (2002).
- 765 36. Jimenez-Sanchez, M. *et al.* The Hedgehog signalling pathway regulates
766 autophagy. *Nat Commun* **3**, 1200 (2012).
- 767 37. Bjorkoy, G. *et al.* Monitoring autophagic degradation of p62/SQSTM1.
768 *Methods Enzymol* **452**, 181-197 (2009).
- 769 38. Bhattacharya, R. *et al.* Role of hedgehog signaling in ovarian cancer. *Clin*
770 *Cancer Res* **14**, 7659-7666 (2008).
- 771 39. Liao, X. *et al.* Aberrant activation of hedgehog signaling pathway in
772 ovarian cancers: effect on prognosis, cell invasion and differentiation.
773 *Carcinogenesis* **30**, 131-140 (2009).
- 774 40. Li, Y., Wang, S., Ni, H.M., Huang, H. & Ding, W.X. Autophagy in alcohol-
775 induced multiorgan injury: mechanisms and potential therapeutic targets.
776 *Biomed Res Int* **2014**, 498491 (2014).
- 777 41. Mauvezin, C. & Neufeld, T.P. Bafilomycin A1 disrupts autophagic flux by
778 inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-
779 dependent autophagosome-lysosome fusion. *Autophagy* **11**, 1437-1438
780 (2015).
- 781 42. Ding, A.X. *et al.* CasExpress reveals widespread and diverse patterns of
782 cell survival of caspase-3 activation during development in vivo. *Elife* **5**
783 (2016).
- 784 43. Sun, G. *et al.* A molecular signature for anastasis, recovery from the brink
785 of apoptotic cell death. *J Cell Biol* **216**, 3355-3368 (2017).
- 786 44. Mille, F. *et al.* The Patched dependence receptor triggers apoptosis
787 through a DRAL-caspase-9 complex. *Nat Cell Biol* **11**, 739-746 (2009).
- 788 45. Fombonne, J. *et al.* Patched dependence receptor triggers apoptosis
789 through ubiquitination of caspase-9. *Proc Natl Acad Sci U S A* **109**, 10510-
790 10515 (2012).
- 791 46. Liang, Y.Y. *et al.* dSmurf selectively degrades DPP-activated MAD and its
792 overexpression disrupts imaginal disc development. *J Biol Chem* (2003).

- 793 47. Martin, D.N. & Baehrecke, E.H. Caspases function in autophagic
794 programmed cell death in *Drosophila*. *Development* **131**, 275-284 (2004).
- 795 48. Daish, T.J., Mills, K. & Kumar, S. *Drosophila* caspase DRONC is required for
796 specific developmental cell death pathways and stress-induced apoptosis.
797 *Dev Cell* **7**, 909-915 (2004).
- 798 49. Fadeel, B. & Orrenius, S. Apoptosis: a basic biological phenomenon with
799 wide-ranging implications in human disease. *J Intern Med* **258**, 479-517
800 (2005).
- 801 50. Moreno, E., Basler, K. & Morata, G. Cells compete for decapentaplegic
802 survival factor to prevent apoptosis in *Drosophila* wing development.
803 *Nature* **416**, 755-759 (2002).
- 804 51. Williams, D.W. & Truman, J.W. Remodeling dendrites during insect
805 metamorphosis. *Journal of neurobiology* **64**, 24-33 (2005).
- 806 52. Huey, R.B., Wakefield, T., Crill, W.D. & Gilchrist, G.W. Within- and between-
807 generation effects of temperature on early fecundity of *Drosophila*
808 *melanogaster*. *Heredity (Edinb)* **74 (Pt 2)**, 216-223 (1995).
- 809 53. Terashima, J. & Bownes, M. Translating available food into the number of
810 eggs laid by *Drosophila melanogaster*. *Genetics* **167**, 1711-1719 (2004).
- 811 54. Terashima, J., Takaki, K., Sakurai, S. & Bownes, M. Nutritional status
812 affects 20-hydroxyecdysone concentration and progression of oogenesis
813 in *Drosophila melanogaster*. *J Endocrinol* **187**, 69-79 (2005).
- 814 55. Pepling, M.E. Hedgehog signaling in follicle development. *Biol Reprod* **86**,
815 173 (2012).
- 816 56. Zhou, J., Peng, X. & Mei, S. Autophagy in Ovarian Follicular Development
817 and Atresia. *Int J Biol Sci* **15**, 726-737 (2019).
- 818 57. Dick, S.A. & Megeney, L.A. Cell death proteins: an evolutionary role in
819 cellular adaptation before the advent of apoptosis. *Bioessays* **35**, 974-983
820 (2013).
- 821 58. Lee, R.E., Brunette, S., Puente, L.G. & Megeney, L.A. Metacaspase Yca1 is
822 required for clearance of insoluble protein aggregates. *Proc Natl Acad Sci*
823 *U S A* **107**, 13348-13353 (2010).
- 824 59. Hurst, P.R., Mora, J.M. & Fenwick, M.A. Caspase-3, TUNEL and
825 ultrastructural studies of small follicles in adult human ovarian biopsies.
826 *Hum Reprod* **21**, 1974-1980 (2006).
- 827 60. Zeng, X. & Ju, D. Hedgehog Signaling Pathway and Autophagy in Cancer.
828 *Int J Mol Sci* **19** (2018).
- 829 61. Song, X. *et al.* Activation of hedgehog signaling and its association with
830 cisplatin resistance in ovarian epithelial tumors. *Oncol Lett* **15**, 5569-5576
831 (2018).
- 832 62. Zhu, L. *et al.* Autophagy is a pro-survival mechanism in ovarian cancer
833 against the apoptotic effects of euxanthone. *Biomed Pharmacother* **103**,
834 708-718 (2018).
- 835 63. Yang, C. & Novack, D.V. Anti-cancer IAP antagonists promote bone
836 metastasis: a cautionary tale. *J Bone Miner Metab* **31**, 496-506 (2013).
- 837 64. Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. NIH Image to ImageJ: 25
838 years of image analysis. *Nat Methods* **9**, 671-675 (2012).
- 839 65. Buszczak, M. *et al.* The carnegie protein trap library: a versatile tool for
840 *Drosophila* developmental studies. *Genetics* **175**, 1505-1531 (2007).
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845 **CONTRIBUTIONS**

846 L.A.B-L. was responsible for the initial conception of the work and original writing of
847 the manuscript. The experimental design was elaborated by A.G and L.A.B-L. A.G
848 was responsible for most of the experimental work. D.I. performed some
849 experiments under the supervision of A.G. The figure preparation was made by A.G
850 and L.A.B-L. All co-authors have provided useful criticisms and commented on the
851 manuscript before submission.

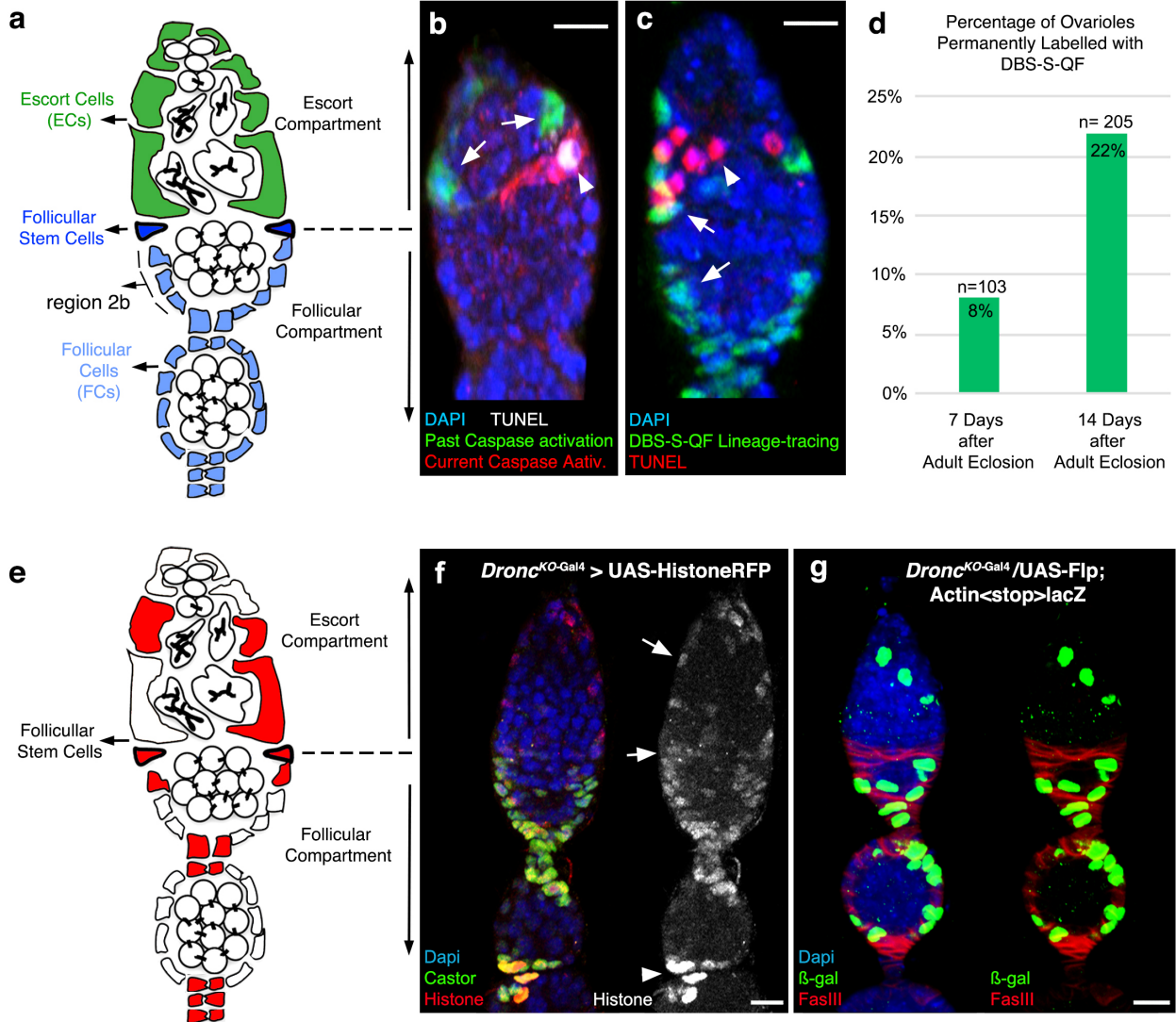
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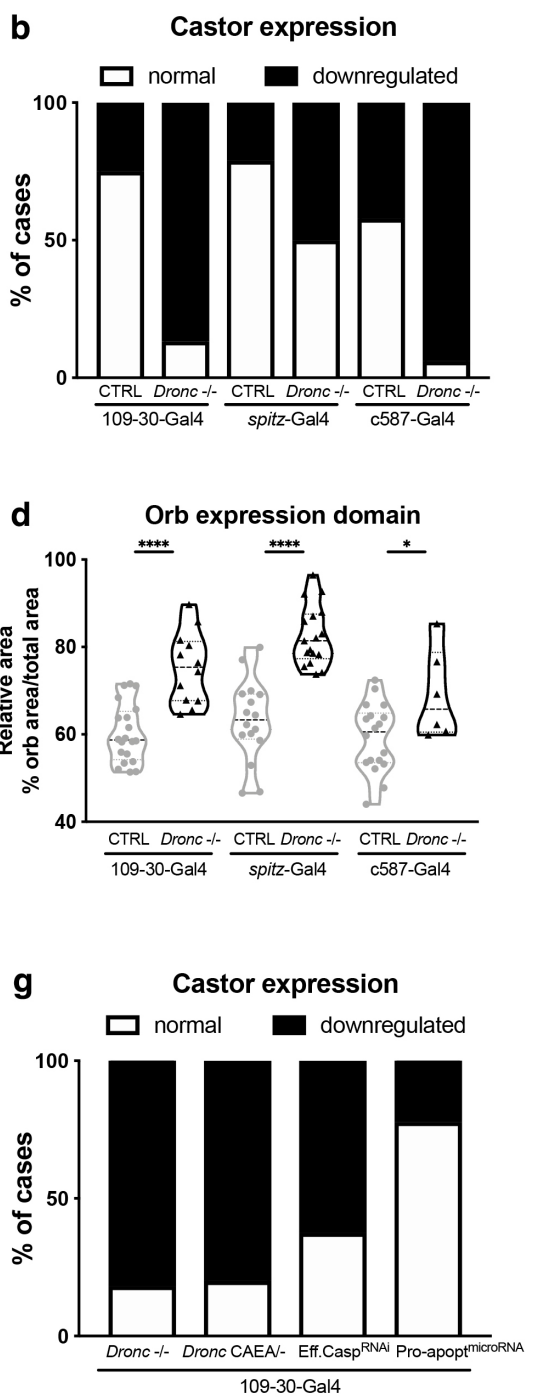
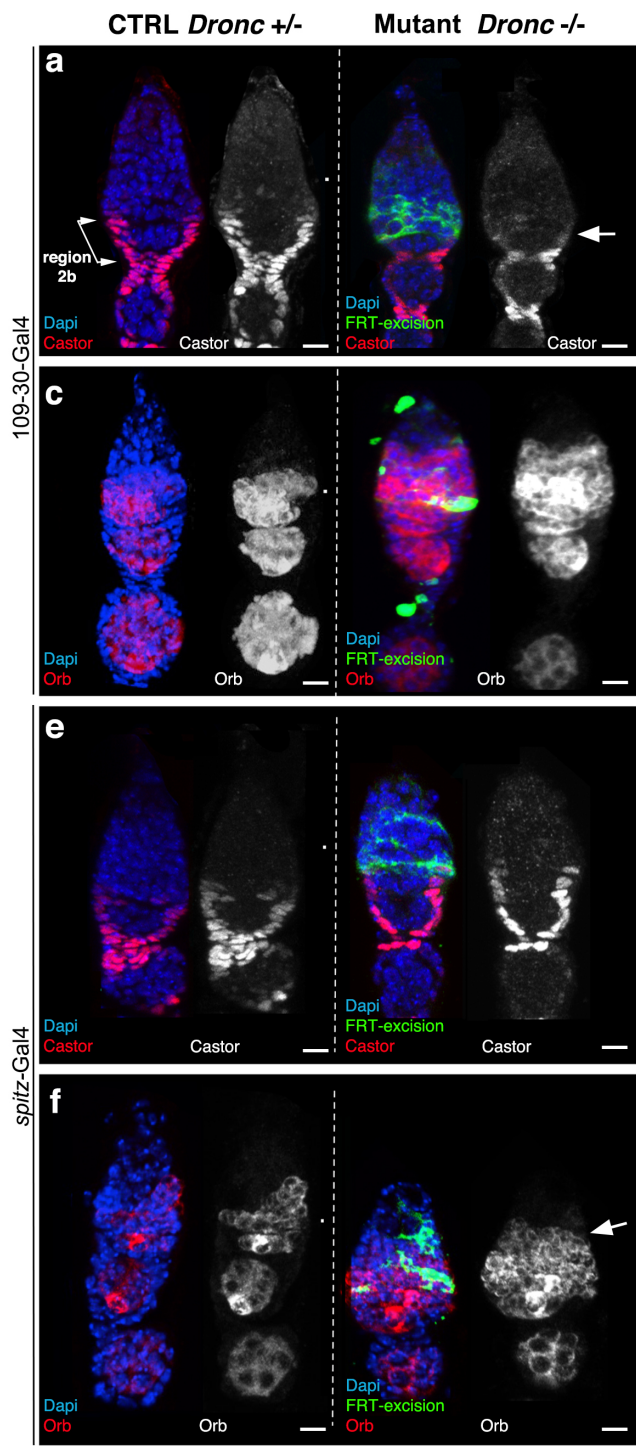
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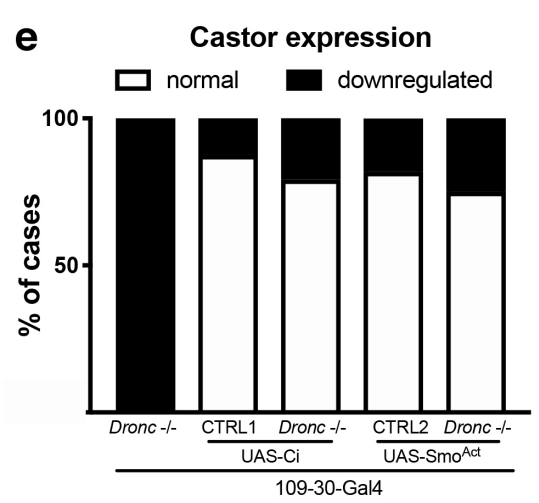
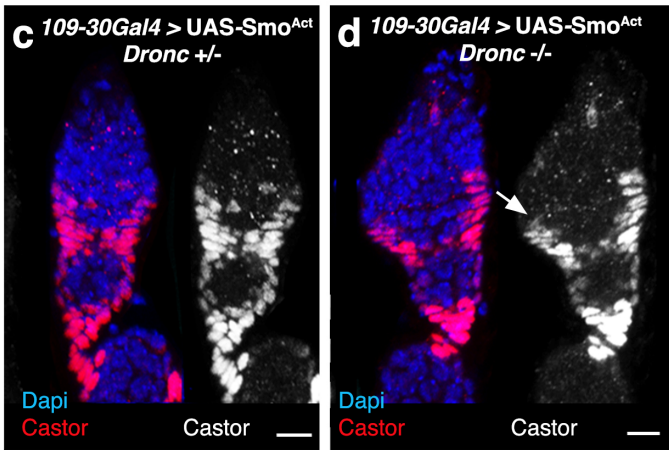
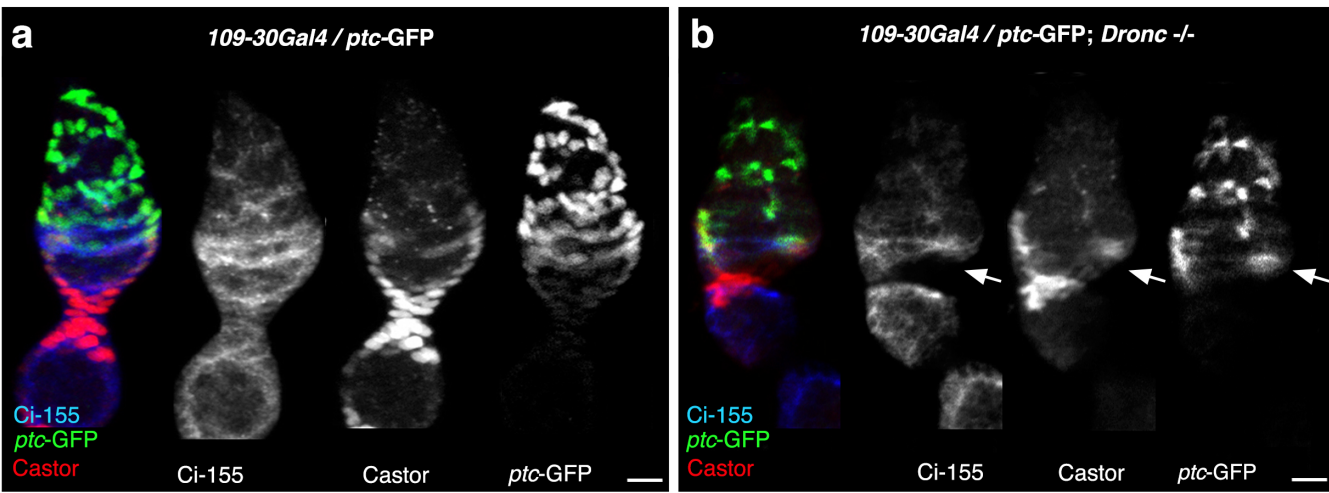
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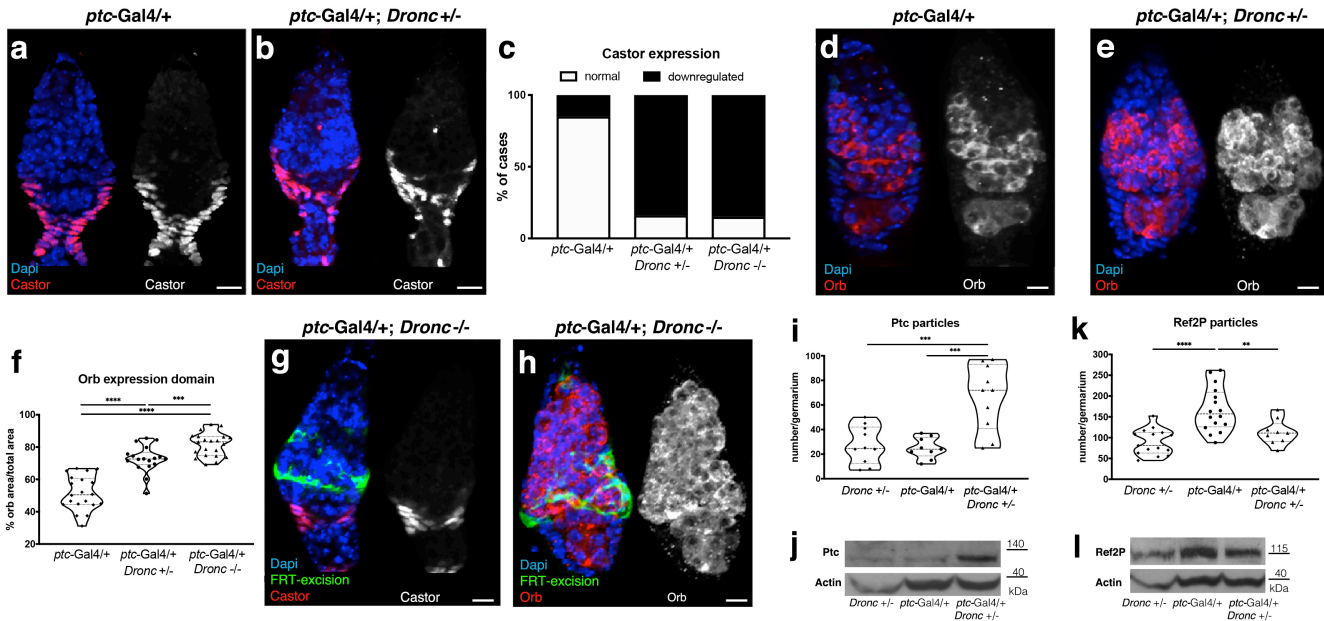




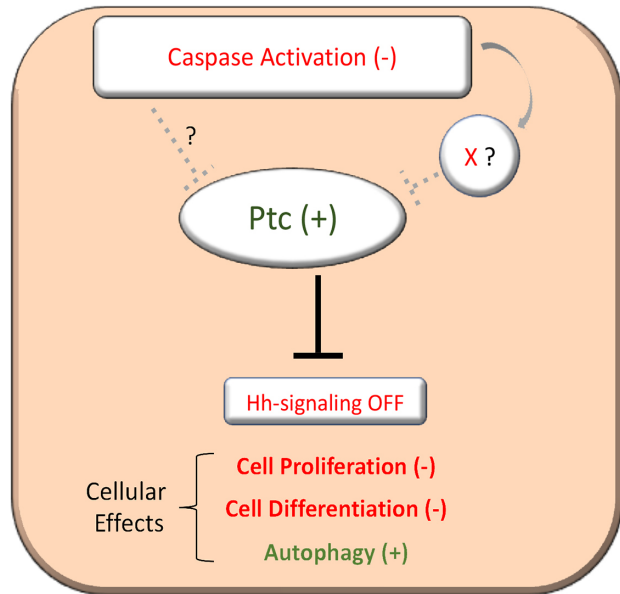
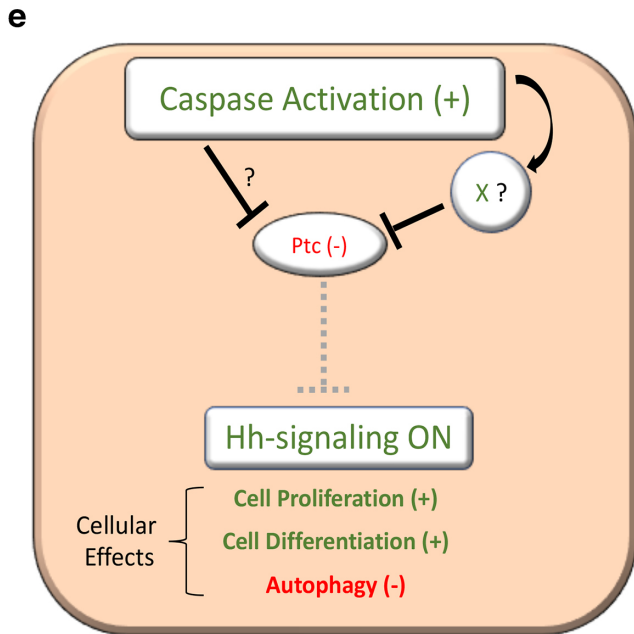
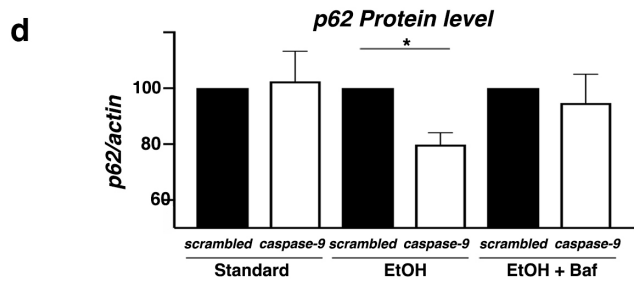
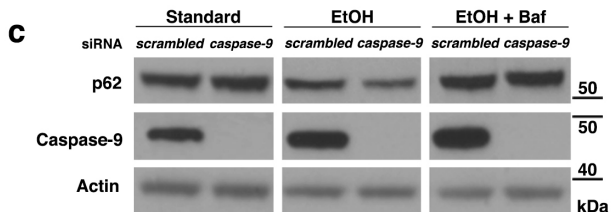
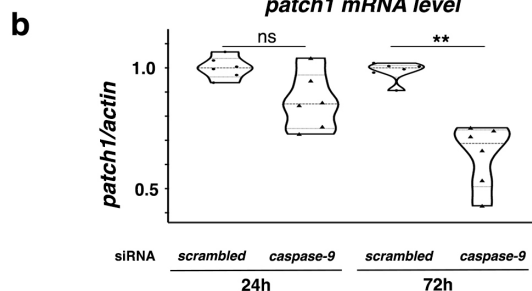
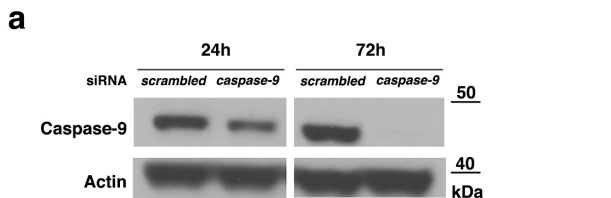
Galasso et al Figure 2



Galasso et al Figure 3



Galasso et al Figure 4



Galasso et al Figure 5