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# 1 **Patterned apoptosis has an instructive role for local growth and** 2 **tissue shape regulation in a fast-growing epithelium**

3  
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## 15 **Abstract**

16 **What regulates organ size and shape remains one of the fundamental mysteries**  
17 **of modern biology. Research in this area has primarily focused on deciphering**  
18 **the regulation in time and space of growth and cell division, while the**  
19 **contribution of cell death has been much more neglected. This includes studies**  
20 **of the *Drosophila* wing, which represents one of the best characterised systems**  
21 **for the study of growth and patterning, and undergoes massive growth during**  
22 **larval stage and important morphogenetic remodeling during pupal stage. So**  
23 **far, it has been assumed that cell death was relatively neglectable in this tissue**  
24 **both during larval and pupal stage and as a result the pattern of growth was**  
25 **usually attributed to the distribution of cell division. Here, using systematic**  
26 **mapping and registration combined with quantitative assessment of clone size**  
27 **and disappearance as well as live imaging, we outline a persistent pattern of cell**  
28 **death and clone elimination emerging in the larval wing disc and persisting**  
29 **during pupal wing morphogenesis. Local variation of cell death is associated**  
30 **with local variation of clone size, pointing to an impact of cell death on local**  
31 **growth which is not fully compensated by proliferation. Using morphometric**

32 **analyses of adult wing shape and genetic perturbations, we provide evidence**  
33 **that patterned death affects locally and globally adult wing shape and size. This**  
34 **study describes a roadmap for precise assessment of the contribution of cell**  
35 **death to tissue shape, and outlines an important instructive role of cell death in**  
36 **modulating quantitatively local growth and morphogenesis of a fast-growing**  
37 **tissue.**

38 **Keywords:** apoptosis, morphogenesis, growth, clone dynamics, quantitative mapping,  
39 *Drosophila*, wing imaginal disc

## 40 **Introduction**

41 The search of cellular mechanisms underlying variation in organ's size and shape is  
42 essential to our understanding of health and evolution. These mechanisms include  
43 changes in cell shape, cell proliferation, oriented cell division, oriented cell intercalation  
44 and also cell death [1]. Programmed cell death and apoptosis are indeed essential  
45 regulators of development and morphogenesis [2]. For instance, cell death is required  
46 to eliminate scaffolding tissues which are not present in the adult body. Apoptosis in  
47 epithelia is also a driving force of morphogenesis which can help to fuse tissues by  
48 generating pulling forces [3] or trigger fold formations by generating apico-basal  
49 traction forces [4, 5]. Finally, apoptosis has been associated with the buffering of  
50 developmental fluctuations which can eliminate miss-specified/miss-patterned cells  
51 through cell competition [6], morphogenetic apoptosis [7, 8], cell sorting and extrusion  
52 [9-11] or more recently, with the buffering of mismatch between the size of the tissue  
53 and the shape of morphogen gradients [12]. Interestingly, while the functions of  
54 apoptosis are numerous, its contribution to the regulation of organ size and growth,  
55 especially in tissues undergoing fast expansion, has been poorly studied. Accordingly,  
56 emphasis has mostly been given to cellular growth regulation including the increase of  
57 cell volume and cell proliferation [13-21]. Moreover, systematic and quantitative  
58 evaluations of the pattern and number of cell deaths remain relatively rare and tedious,  
59 especially in tissues which are not appropriate for long term live imaging [22-24]. As  
60 such, the exact pattern of cell death and its real contribution to the regulation of organ  
61 size and shape remain poorly documented in many situations. This also applies to the  
62 *Drosophila* wing, one of the best studied systems for understanding patterning and  
63 growth regulation [15, 17, 25-28].

64 *Drosophila* wing development can be separated in the larval stage, where the wing  
65 imaginal disc mostly undergoes massive proliferation and patterning, followed by the  
66 pupal stage, where complex morphogenetic movements give rise to the final wing  
67 shape [29]. At larval stages, *Drosophila* wing imaginal discs are epithelial sacs  
68 composed of two epithelia: the peripodial cells (squamous epithelium) and a  
69 pseudostratified epithelium that will form the adult structures [30]. The disc is separated  
70 into a distal domain that will give rise to the adult wing blade (the wing pouch, or wing  
71 proper) and a proximal region called the hinge which will connect the wing to the thorax.  
72 This wing pouch is patterned in different domains prefiguring adult wing structures: the  
73 pro-vein domains L2 to L5 and the dorsal-ventral boundary that will give rise  
74 respectively to the adult wing veins and the adult wing boundaries (**Figure 1A,B**). Wing  
75 imaginal discs undergo massive growth during larval development through several  
76 rounds of cell division (9 to 11 cycles) [31, 32]. This growth phase is followed by  
77 profound remodeling and morphogenesis during the pupal stage [33] including wing  
78 eversion and apposition of the dorsal and ventral epithelial layers [34, 35], the  
79 elongation of the wing proper through distal anchoring and hinge contraction [27, 36],  
80 global tissue growth by cell expansion and folding [37] eventually followed by hatching  
81 and wing spreading[38] (**Figure 1A,B**). All these steps are performed with a high  
82 degree of precision and reproducibility leading to extremely reproducible shape,  
83 specially comparing left and right wings [39].

84 Decades of work sought to dissect the mechanisms controlling growth and size of the  
85 *Drosophila* wing, and have mostly focused on the distribution and regulation of cell  
86 proliferation while neglecting cell death [15-19, 21]. Indeed, seminal works more than  
87 20 years ago found that cell death is relatively minor in the wing disc and that it occurs  
88 sporadically without any noticeable pattern [23]. Similarly, recent systematic tracking  
89 of cells in the pupal wing during elongation (between 16 and 32 hours after pupal  
90 formation, APF) suggested that apoptosis has also a minor role for wing elongation at  
91 the pupal stage [27, 40]. Yet, these works did not exclude more subtle functions of cell  
92 death.

93 Accordingly, apoptosis was proposed to buffer developmental fluctuations to ensure  
94 reproducible wing size [41]. This was assumed to be based on cell competition: the  
95 context dependent elimination of suboptimal cells from growing tissues [42, 43].  
96 Interestingly, while the studies of cell competition are mostly based on the analysis of



97 mutant clone disappearance in the wing disc, we know **very little** about spontaneous  
98 clone disappearance during normal wing development and the prevalence, localisation  
99 and functions of physiological cell competition. Recently, apoptosis was proposed to  
100 fine tune of Dpp gradient shape by eliminating supernumerary cells to match gradient  
101 decay length and tissue size [12]. Alternatively, basal sublethal effector caspase  
102 activity was also proposed to modulate global wing growth and size [44]. These results  
103 call for a reassessment and more thorough characterization of cell death in the wing  
104 and its contribution to growth and tissue shape.

105 Here, we performed systematic mapping of apoptosis using various markers of  
106 caspases and apoptosis, together with spatial registration in the larval wing imaginal  
107 disc. Unexpectedly, we found striking reproducible biases in the distribution of cell  
108 death which outlined hot-spots of apoptosis. These hot-spots correlate with a local  
109 increase of clone disappearance probability and significantly reduce local net-growth  
110 in the wing disc. Using live imaging of the pupal wing, we also showed that these spatial  
111 biases persist at early pupal development and that new spots of apoptosis appear later  
112 during development. Finally, using morphometric analysis, we further demonstrate that  
113 these hot-spots can tune adult wing size and shape. Altogether, we reveal that  
114 apoptosis cannot be neglected for the study of growth/size and is also an essential  
115 modulator of local shape and growth even in a fast-expanding tissue. Our study also  
116 proposes a roadmap for a more systematic characterisation of the contribution of cell  
117 death to clone dynamics and tissue growth.

## 118 **Results**

### 119 **Caspase activity and apoptosis are spatially biased in the larval wing pouch**

120 We first systematically evaluated the distribution of apoptotic cells in the *Drosophila*  
121 wing disc at the larval wandering stage (96 h After Egg Laying - AEL). We used the  
122 live effector caspase reporter GC3Ai [45] driven in the larval pouch and hinge by the  
123 driver GMR11F02-GAL4 (**Figure 1C**). The brightest GFP spots revealed by the  
124 reporter correspond to cell debris located on the basal sides, thus staining cells which  
125 already extruded ([45], **Figure 1D**). We confirmed the accuracy of the GC3Ai marker  
126 as we obtained a similar pattern with cleaved caspase3 staining (cleaved DCP1),  
127 although this was less sensitive and fails to mark the cells with faint GC3Ai signal  
128 (**Figure 1suppE-G'**). Moreover, bright GC3Ai dots used for the analysis colocalized

129 with TUNEL positive cells (**Figure 1suppE-G'**), confirming that they marked late  
130 apoptotic cells. To obtain an averaged map of the spatial distribution of cell death, we  
131 used spatial landmarks, whose positions were defined by the intervein marker  
132 *Drosophila* Serum Response Factor (DSRF, **Figure 1D,E**). The landmarks positions  
133 were used to divide every dissected wing disc into 40 sub-compartments (**Figure**  
134 **1suppA-D**). In addition, we applied procrustes transformation (translation, rotation and  
135 scaling) on landmarks positions to align the discs and superimpose  
136 GC3Ai/Dcp1/TUNEL spatial data from many individuals on a single image (see  
137 **Methods**). The averaged distribution of apoptotic cells revealed a striking non-  
138 homogenous distribution of apoptosis with the strongest "hot spot" located in the  
139 anterior - dorsal quadrant, near the dorsal-ventral boundary (**Figure 1 F,G**,  $p < 0.001$ ,  
140 Moran autocorrelation spatial test, null hypothesis being an homogenous distribution  
141 with no spatial correlation). This bias is not driven by the Gal4 driver or any side effect  
142 of GC3Ai as a similar pattern was obtained in the *w* background with cleaved caspase3  
143 staining (**Figure 1suppH,I**) or with TUNEL staining (**Figure 1suppJ,K**, Moran's  $p$ -  
144 value=0.007). To check whether the spatial bias of GC3Ai signal persists during wing  
145 disc development, we also monitored GC3Ai-positive cells in the early L3 wing disc  
146 (72h AEL). As we could not use the same spatial landmarks (the intervein regions are  
147 not yet defined at this stage) we instead used the location of the 4 compartments  
148 defined by AP and DV compartment boundaries (Anterior, Posterior, Dorsal and  
149 Ventral) as a spatial reference. The number of apoptotic cells was more than two times  
150 higher in the anterior-dorsal compartment than in the others (**Figure 1J-L** pairwise  $t$ -  
151 test  $p$ -values  $\leq 0.056$ ). This suggested that the spatial bias emerges at least during  
152 early L3 stage and persists for more than 24 hours. We then checked whether this  
153 pattern was indeed dependent on core regulators of apoptosis/caspases. Co-  
154 expression of GC3Ai with a dominant negative allele of *Dronc* (*Drosophila* caspase9)  
155 significantly reduced the amount of GC3Ai signal and reduced the spatial bias (**Figure**  
156 **1H**, **Figure 1suppL-N**, **S**). Similarly, depletion of the pro-apoptotic gene *hid* by RNAi  
157 in the wing pouch almost completely abolished the GC3Ai signal (**Figure 1I**, **Figure**  
158 **1suppO-Q**, **S**). Finally, the number of TUNEL positive cells was significantly reduced  
159 in a *dark*<sup>[CD4]</sup> mutant background (*Drosophila* Apaf1/Apoptosome protein[46])  
160 specifically in the apoptosis hotspots regions (**Figure 1suppR,R',T**, compared with  
161 **1suppK**). These observations suggest that the spatial bias is at least in part caspase-  
162 dependent and most likely relies on the expression of the pro-apoptotic gene *hid* and

163 on the apoptosome. Accordingly, we found a slight but systematic and reproducible  
164 increase of *hid* expression near the GC3Ai hot spot region using a GFP insertion at the  
165 *hid* locus (**Figure 1M,N**). Note however that other factors may also contribute to the  
166 spatial bias as we still observed some heterogeneity in cell death distribution upon  
167 Dronc inhibition or *hid* depletion (**Figure 1suppN, Q, S**) [12]. We also checked the  
168 pattern of expression of other pro and anti apoptotic genes, namely *reaper*, *grim* and  
169 *diap1* (**Figure 2supp A-C'**). While none of them matched specifically the full pattern of  
170 apoptosis, we did find some partial overlap between local increase of apoptosis in the  
171 proximal intervein L3-L4 regions of the disc and *reaper* expression (**Figure 2supp**  
172 **A,A'**). This suggested that the pattern of apoptosis strongly relies on *hid*, but may be  
173 influenced by the pattern of expression of other core regulators of apoptosis.

174 Altogether, we conclude that contrary to what was previously observed [12, 23]  
175 apoptosis is not spatially homogeneous in the wing imaginal disc and that some  
176 regions (anterior and close to the DV boundary, dorsal compartment) undergo higher  
177 rates of apoptosis. These hot-spots are in part explained by a spatial bias in the  
178 expression of the pro-apoptotic gene *hid* but are also likely to be regulated by other  
179 factors.

## 180 **The spatial bias in apoptosis influences clonal disappearance and local net-** 181 **growth.**

182 We next checked whether this spatial bias in apoptosis could have any consequence  
183 on clonal dynamics and local growth. Since *ex-vivo* live imaging of the wing disc does  
184 not allow long-term tracking of cell fate and local growth, we used an alternative  
185 approach based on twin-clone labelling. We used the QMARCM technique [47] to stain  
186 the two daughter cells generated after mitotic recombination and their progeny with  
187 different fluorescent markers using alternate sets of transcription factors (Gal4 or the  
188 Qsystem, **Figure 2A,B**). Since cell movements and cell-cell intercalations are relatively  
189 neglectable in the wing disc [48, 49], the spatial proximity of patches of cells marked  
190 with GFP and RFP can be unambiguously attributed to a twin-clone in conditions of  
191 low frequency of clone induction. We assessed the probability of clone disappearance  
192 by scoring the number of single-coloured clones which are not in the vicinity of the  
193 other sibling clone 48h after clone induction. Such an observation can only be  
194 explained by the early disappearance of the lineage of the other daughter cell, and  
195 thus identifies a clone disappearance event (**Figure 2A**). We first noted that the

196 QMARCM system has an intrinsic bias towards smaller clones and higher probability  
197 of clone disappearance for GFP/Gal4 clones relative to RFP/QF ones (**Figure**  
198 **2suppD**), most likely due to the inherent toxicity of Gal4 [50]. While in this context we  
199 cannot extrapolate on the absolute probability of clone disappearance, this however  
200 should not preclude the analysis of potential spatial biases. By multiplexing a large  
201 number of discs and using the same spatial landmarks as those used in **Figure**  
202 **1suppC (Figure 2B)**, we obtained a coarse-grained spatial map of the probability of  
203 clone disappearance (**Figure 2C**). Strikingly, we observed that similar to caspase  
204 activation in the tissue, the probability of clone disappearance is not spatially  
205 homogenous (Moran's test p-value <0.001). We identified clear hot-spots of clone  
206 death, including the anterior-dorsal region near the DV boundary (~two-fold increase  
207 compared to posterior compartments at similar DV positions, **Figure 2C, 2suppE**), as  
208 already outlined by the caspase positive cells mapping (**Figure 1G**). Note that this  
209 clone strategy reflects the pattern of apoptosis at earlier stages of development (most  
210 likely around the time of clone induction, early L2) compared to the one revealed with  
211 apoptotic markers. Nevertheless, we still observed a significant compartment to  
212 compartment correlation between GC3Ai levels and clone disappearance probability  
213 (weighted Pearson's correlation=0.38, p=0.016). Importantly, clone size was also on  
214 average smaller (estimated through total apical surface and number of cells) in regions  
215 showing high rates of apoptosis and clone disappearance (**Figure 3A, Figure**  
216 **3suppC**). This suggested that spatial biases in caspase activity not only modulate the  
217 probability of clone survival, but also the cumulative local growth in the wing disc.  
218 Indeed, these local differences in clone size match the differences numerically  
219 estimated from an exponential growth model with no spatial difference in proliferation  
220 rate and including the spatial differences in apoptotic rates calculated from our  
221 measurements of clone disappearance (see **Methods, Figure 3B-D**). This indicates  
222 that local differences in clone size could be solely explained by spatial differences in  
223 apoptotic rates. To check whether these spatial differences are indeed driven by  
224 caspases, we repeated the QMARCM clonal assay upon inhibition of caspase in the  
225 Gal4 sibling clones (using UAS-Dronc<sup>DN</sup>). While this did not totally abolish clone  
226 disappearance, the spatial pattern was flattened with no visible hot-spot of clone  
227 disappearance in the anterior side (**Figure 2C', 2suppE**, Moran's test p-value = 0.11,  
228 no significant spatial bias; Pearson's correlation with WT QMARCM single clone  
229 pattern = 0.078, p=0.63) and led to a more homogenous clone size between anterior

230 and posterior compartment (**Figure 3suppD**). To confirm the effect of caspases and  
231 to check whether a similar pattern could be observed in absence of Gal4, we also used  
232 mitotic clones in the H99/+ background (a deletion covering the three pro-apoptotic  
233 genes *hid*, *grim* and *reaper* [51]) generating on the one hand H99 homozygous mutant  
234 clones (resistant for apoptosis) as well as a WT siblings. While we observed globally  
235 less single clones compared to the QMARCM approach (confirming the impact of Gal4  
236 on clone disappearance probability), single H99 clones (loss of WT sibling) were  
237 markedly more frequent near the hotspots of apoptosis (**Figure 2D**, Moran's test for  
238 spatial autocorrelation:  $p=0.003$ , Pearson's correlation with GC3Ai pattern: 0.41,  
239  $p=0.009$ ), and completely biased toward WT clone disappearance. This confirmed the  
240 pattern of clone disappearance observed with QMARCM in absence of any possible  
241 bias caused by Gal4 toxicity. H99 mutant clones were almost systematically larger than  
242 their WT siblings (**Figure 3E**, 30,8% larger on average, t-test  $p$ -value  $< 0.0001$ ), and  
243 this bias was notably high in the hotspot of apoptosis in the margin region at the  
244 intervein L2-L3 region (Ratio=0.24,  $n=72$ ) (**Figure 3F, 3suppE,F**). Induction of neutral  
245 mitotic clones (marked with 2 copies of EGFPnls versus 2 copies of  $\beta$ -gal) on the  
246 contrary shows an almost perfectly symmetric distribution of clone size between the  
247 two siblings with no asymmetry of probability of clone disappearance, and yet a similar  
248 global probability of clone disappearance compared to WT clones in H99/+ background  
249 (7 % versus 8,2 %, **Figure 2 supp F,G, Figure 3 supp G,H**). Moreover, we could also  
250 recapitulate a similar spatial bias of clone disappearance and clone size (**Figure 2**  
251 **Supp G, Figure 3 Supp G**). Altogether, this confirmed that *i*) the spatial bias of clone  
252 disappearance can also be observed in absence of Gal4, *ii*) clone disappearance is  
253 apoptosis dependent and *iii*) apoptosis has a net negative impact on local growth  
254 especially in the apoptotic hotspots regions (**Figure 1**).

255 Altogether, we conclude that the spatial bias in apoptosis generates hotspots of clone  
256 disappearance which also significantly reduces local growth rate mostly in the anterior-  
257 dorsal region near the DV boundary. This suggests that local increases of apoptosis  
258 could play an instructive role in significantly modulating local growth, which therefore  
259 may affect the final shape and size of the tissue.

260 **Patterned apoptosis in the larvae and in the pupae modulate adult wing shape**  
261 **and size**

262 We therefore checked whether local biases in apoptosis could significantly impact adult  
263 wing shape and size. To obtain a precise and quantitative description of adult wing  
264 shape and size, we used a quantitative assay based on semi-automatic wing  
265 segmentation, landmark positioning and procrustes alignment of wings (which includes  
266 translation, rotation and global rescaling) [52, 53] (**Figure 4suppA**). To analyse the  
267 impact of apoptosis in the wing proper, we used the Nab-Gal4 driver, one of the few  
268 drivers well restricted to the pouch both at larval and pupal stage (**Figure 4A**).  
269 Interestingly, inhibition of cell death in the wing tissue using *hid* RNAi or a microRNA  
270 targeting *hid*, *grim* and *reaper* (mirRHG [54]) led to a significant increase in wing size  
271 (**Figure 4B,C** 8.7% and 4.2% respectively, a similar range to what was obtained upon  
272 ectopic overexpression of Dp110/PI3K [55]), suggesting that apoptosis has a net  
273 negative effect on adult tissue size. [Hid-dsRNA and mirRHG effect on hid mRNA levels](#)  
274 [was confirmed by RT-PCR \(see Star Methods\)](#). We also observed significant changes  
275 to adult wing shape, including a relative increase in the size of the most anterior and  
276 posterior domains, as well as a global wing rounding (**Figure 4D**). To check whether  
277 local increases in apoptosis were indeed responsible for the local modulation of shape  
278 and size, we used various drivers to inhibit apoptosis in different wing subdomains  
279 (**Figure 4E**). Accordingly, inhibition of *hid* in the patched domain (*ptc-gal4*, expressed  
280 in a band at the intervein L3-L4, **Figure 4E**) led to a global increase of wing size  
281 characterised by the expansion of the region overlapping the *ptc* domain (**Figure**  
282 **4F,G**). This suggested that apoptosis has a local impact on growth even in regions  
283 other than the apoptosis hot-spots identified at larval stage. We also used drivers  
284 restricted to the DV boundary either in the anterior (*aristaless-GAL4*, encompassing  
285 the caspase hot-spot) or posterior part of the wing disc (*trithorax-GAL4*) (**Figure 4G**).  
286 Interestingly, while *hid* depletion in the posterior-DV boundary domain had a mild effect  
287 on wing size (**Figure 4F,G**, 2.1%, similar to inhibition of *hid* in the *ptc* domain), this  
288 effect was enhanced upon inhibition in the anterior-DV boundary domain (4.1%, **Figure**  
289 **4F,G**). Importantly, the expansion of the most anterior domain of the wing was only  
290 observed upon inhibition of *hid* in the anterior-DV boundary domain (**Figure 4F,G**).  
291 Altogether, this suggests that apoptosis has a local negative effect on wing domain  
292 size which is more significant in regions overlapping hotspots of apoptosis  
293 characterized in the wing imaginal disc.

294 So far, our perturbative approaches did not allow to distinguish the contributions of  
295 apoptosis during larval or pupal stage, where significant tissue size and shape  
296 modulation can still take place [27, 36, 37, 56]. Therefore we also performed  
297 experiments with conditional *hid* depletion under the control of a temperature sensitive  
298 driver (Gal4 combined with Gal80<sup>ts</sup>) to assess the relative contribution of *hid*  
299 expression at larval stage versus pupal stage to the final wing shape and size (**Figure**  
300 **4suppB,C**). Despite the strong confounding effect of temperature on wing size [57],  
301 we found that *hid* depletion at larval stage (up to mid L3) had a significant impact on  
302 adult wing size (**Figure 5A**, larval expression, +7%, t-test p-value < 0.001) while  
303 depletion during pupal stage had a milder effect (**Figure 5A**, pupal expression, +2.9%,  
304 similar to the background effect observed in the negative control, +3.4 %, [the absence](#)  
305 [of significant effect was confirmed by bootstrapping](#)). This suggested that larval  
306 apoptosis had a significant negative contribution to global wing size. We then analysed  
307 the relative contribution of larval and pupal apoptosis to final adult wing shape.  
308 Surprisingly, this revealed that the wing shape modulation observed upon persistent  
309 *hid* downregulation could be partially recapitulated by pupal inhibition of *hid* (compare  
310 **Figure 5B** pupal expression and full expression, to **Figure 4D**), suggesting that pupal  
311 apoptosis also contributes to adult wing shape. We next used PCA (Principal  
312 Component Analysis) to decompose the main contributors of shape variation and  
313 compare unambiguously and quantitatively wing shape between conditions. Removing  
314 the effect of temperature (Principal Component 1) outlined the contribution of shape  
315 variation (Principal Component 3) which is going along the same direction for larval,  
316 pupal and full depletion of Hid (**Figure 5SuppA,B**). Interestingly, the amplitude of  
317 variation along PC3 for Hid full depletion matches the sum of variations observed for  
318 larval and pupal depletion, suggesting that adult wing shape is generated by the  
319 cumulative effect of larval and pupal inhibition of apoptosis, which have similar effects  
320 on shape. Variations along PC3 mostly recapitulates global wing rounding (**Figure**  
321 **5SuppC** wing deformation between the two extreme values along PC3) in good  
322 agreement with the shape modulation observed for Hid depletion throughout  
323 development (**Figure 4B,D**).

324 This prompted us to evaluate the pattern of apoptosis in the pupal wing. We performed  
325 live imaging of pupal wings between 16h and 35h APF (After Pupal Formation)  
326 expressing GC3Ai in the pouch (using the Nab-Gal4 driver). This revealed striking



327 dynamics of apoptosis with an early concentration in the most anterior part of the wing  
328 as well as the junction with the hinge (**Figure 5C, Video S1**). This pattern shows an  
329 interesting correspondence with the hotspots observed at larval stage (**Figure 5C-D'**)  
330 suggesting that the spatial biases present at larval stage are for a good part maintained  
331 during pupal development. Nevertheless, the pattern of apoptosis is dynamic as new  
332 hotspots of apoptosis appear at later (~30 h APF) pupal stage (**Figure 5C,D'**)  
333 Importantly, the domains of high GC3Ai expression and apoptosis match the regions  
334 more significantly deformed in the adult wing upon pupal inhibition of Hid (compare  
335 **Figure 5C-D'' Video S1**). Using ubiquitous expression of GC3Ai driven by *lexA*, we  
336 also confirmed that larval expression of *hid-dsRNA* using *Nab-Gal4* and *Gal80ts* does  
337 not impact caspase activity in the pupal wing blade, while pupal expression of *hid-*  
338 *dsRNA* reduced GC3Ai signal in the pupal wing blade (**Figure 5supp D,E**).

339 Altogether, we conclude that spatial biases in apoptosis distribution in the growing wing  
340 can significantly modify the size and shape of the adult wing, by modulating  
341 morphogenesis either during early larval stages or during later pupal ones. This  
342 suggests that the fine spatial tuning of apoptosis in a fast-growing tissue plays an  
343 instructive role for organ shape and size regulation.

## 344 Discussion

345 In this study, we outlined an unexpected pattern of apoptosis in the growing wing  
346 imaginal disc with a significant upregulation in the anterior and dorsal compartment of  
347 the wing. Part of this pattern is preserved during pupal stage and contributes to pupal  
348 wing morphogenesis. Moreover, our quantitative assessments of clonal growth and  
349 adult wing shape clearly show that this local upregulation of apoptosis has a significant  
350 impact on local net growth and final adult shape and size. Most likely, local upregulation  
351 of apoptosis fine tune wing shape by locally reducing the number of cells and altering  
352 the relative proportion of wing subdomains, although nothing excludes at this stage  
353 more complicated contribution through an apoptosis-dependent modulation of tissue  
354 mechanics and/or signaling pathways. So far, the characterisation of cell death has  
355 never been performed in a systematic manner using spatial landmarks allowing  
356 superimposition of data from many individuals, which may explain why such biases  
357 have been missed [23]. Similarly, the impact of apoptosis on tissue shape and size has  
358 never been studied with such quantitative readouts. Our work emphasises the need



359 for more systematic quantitative assessments of death distribution in order to evaluate  
360 its contribution to organ shape/size regulation. The pipeline used in this study may  
361 easily be applied in other developmental contexts which are not amenable for long  
362 term live imaging, as long as markers can be used for spatial registration and tools are  
363 available for clone generation.

364 Previous work suggested that sublethal caspase activation also contributes to wing  
365 disc growth regulation and adult tissue size, however in this situation basal caspase  
366 activity promotes growth and effector caspase inhibition through p35 overexpression  
367 rather reduces wing size[44]. This effect however is not visible upon inhibition of  
368 upstream regulators of caspases, which was interpreted by the absence of inhibition  
369 of the pro-growth basal effector caspase activity. In this study, we purposively used  
370 upstream inhibitors of apoptosis to avoid this effect, which revealed now an increase  
371 of local growth and wing size upon apoptosis inhibition. Our preliminary observation  
372 in the pupal wing also suggests the existence of a significant proportion of cell death  
373 in the hinge region (**Video S1, Figure 5 C-D**”). Interestingly, our preliminary  
374 observations using hinge specific driver suggests that apoptosis inhibition in the hinge  
375 rather reduces adult wing size (not shown). This is in sharp contrast with the effect we  
376 observed upon inhibition in the pouch and suggest that the interpretation of wing shape  
377 phenotype requires to precisely monitor the domains of perturbations, especially for  
378 commonly used Gal4 encompassing both pouch and hinge region (e.g.: nubbin-Gal4).  
379 While we focused here on tissue autonomous contribution of cell death, future work  
380 will help to decipher the putative contribution of hinge apoptosis to final wing size and  
381 shape through a non-tissue autonomous effect.

382 Compensatory proliferation is one of the best studied processes that relates apoptosis  
383 to the induction of cell proliferation [58]. So far, this process was mostly characterised  
384 either in conditions of massive death induction through irradiation and genetic induction  
385 of apoptosis in large domains [59, 60], or through the perturbation of the core apoptotic  
386 pathway (e.g.: by blocking some of the essential caspases, caspase3 in *Drosophila*  
387 [61], or caspase9 in mammalian epidermis [62]). However, to our knowledge there is  
388 no study that has clearly identified biases in cell proliferation distribution in the vicinity  
389 of physiological apoptosis *in vivo*. Surprisingly, we observed a net negative effect of  
390 the local increase of cell death on local growth and the size of the final adult  
391 compartment, and also outlined a significant effect of physiological death on the final

392 size of the adult wing (**Figure 2** and **Figure 4, Figure 5**). This suggests that  
393 compensatory proliferation is unlikely to occur in the context of physiological apoptosis,  
394 or at least that its contribution is relatively minor and not sufficient to compensate for  
395 cell loss by apoptosis. Further quantitative studies of the coupling between apoptosis  
396 and cell proliferation will be essential to assess its real contribution to physiological  
397 growth regulation. Interestingly, a recent study outlined a significant positive  
398 upregulation of proliferation near dying MDCK cells, however this effect is strongly  
399 context-dependent and is not visible at low stiffness or high density values [63],  
400 conditions that may apply to the wing imaginal disc.

401 Cell competition is the process describing the context-dependent elimination of viable  
402 but suboptimal cells [6]. Studies of clonal elimination in the wing imaginal disc have  
403 thoroughly contributed to our understanding of this process. Interestingly, most of  
404 these studies assumed that clone disappearance in physiological conditions is largely  
405 neglectable. Our results suggest that WT clones can undergo early elimination in the  
406 wing imaginal disc, and that this elimination is spatially biased, although we should  
407 remain cautious when extrapolating the absolute probability of clone elimination  
408 compared to the experimental conditions used in this study. On the one hand, this  
409 opens the possibility for spontaneous competition occurring in the wing imaginal disc,  
410 which so far has not been thoroughly explored (although its existence has been  
411 suggested by indirect genetic evidence [12, 41, 64]). On the other hand, it also  
412 suggests that cell elimination during competition may be influenced by this spatial  
413 pattern of caspase activity and apoptosis. Further quantitative description of clonal  
414 elimination during competition may reveal such spatial bias and would help to study  
415 the influence of pre-existing patterns of caspases/apoptosis on cell competition and  
416 tissue plasticity.

417 In this study, we showed that local modulation of *hid* expression can fine-tune the local  
418 level of apoptosis which will impact local growth and adult tissue shape in a subtle and  
419 quantitative way, similarly to the phenotypic changes observed at the  
420 macroevolutionary scale [65]. As such, the evolution of the cis-regulatory elements of  
421 pro-apoptotic genes may constitute an additional lever for shape evolution that could  
422 be used to fine tune adult appendage shape. [It would be important in this perspective  
423 to understand which factors fine tune the expression levels and the pattern of pro-  
424 apoptotic genes and dissect how they might be modulated during evolution.](#)

425 Interestingly, these pro-apoptotic genes are most likely less pleiotropic than pro-  
426 proliferative pathways such as morphogens, RTK signaling pathways, or Hippo  
427 pathways that are classically studied for the regulation of size and growth [15]. As such,  
428 modulating pro-apoptotic genes levels and pattern of expression may constitute a  
429 relatively parsimonious way to evolve wing and appendage shape.

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## 445 **Authors contribution**

446 RL and AMV discussed and designed the project and wrote the manuscript. TC  
447 performed part of the experiments on adult wing shape and twin clones analysis as  
448 well as the wing disc segmentation. FL participated to adult wing dissection, imaging  
449 and segmentation, [performed the RT-PCR and generated the lexAOP-GC3Ai line](#). AD  
450 provided the theoretical calculation of the expected distribution of clone size based on  
451 differences in apoptotic rates. AMV performed all the other experiments and analysis.  
452 Every author has commented and edited the manuscript.

## 453 **Declaration of interests**

454 The authors declare no competing interest

455 **Star Methods**

456 **Resource availability**

457 *Lead contact*

458 Further information and requests for resources and reagents should be directed to and  
459 will be fulfilled by the lead contact, Romain Levayer ([romain.levayer@pasteur.fr](mailto:romain.levayer@pasteur.fr)).

460 *Material availability*

461 All the reagents generated in this study will be shared upon request to the lead contact  
462 without any restrictions.

463 *Data and Code availability*

464 All code generated in this study and the raw data corresponding to each figure panel  
465 (including images) can be shared upon request and will be uploaded to a repository  
466 following acceptance.

467 **Experimental model and subject details**

468 *Drosophila melanogaster* husbandry

469 All the experiments were performed using *Drosophila melanogaster* fly lines (listed in  
470 **Table 1**) breed with regular husbandry techniques. The fly food used contains agar  
471 agar (7.6 g/l), saccharose (53 g/l) dry yeast (48 g/l), maize flour (38.4 g/l), propionic  
472 acid (3.8 ml/l), Nipagin 10% (23.9 ml/l) all mixed in one liter of distilled water. Flies were  
473 raised at 25°C in plastic vials with a 12h/12h dark light cycle at 60% of moisture unless  
474 specified in the legends and in the table below (alternatively raised at 18°C or 29°C).  
475 Females and males were used without distinction for all the experiments, except for  
476 the adult wing shape analysis in which only left wings from female flies were used. We  
477 did not determine the health/immune status of pupae, adults, embryos and larvae, they  
478 were not involved in previous procedures, and they were all drug and test naïve.

479 *Drosophila melanogaster* strains

480 The strains used in this study and their origin are listed in the table 1 below.

Fly line	Chromosome location	Origin (citation)	RRID
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<i>UAS-GC3Ai /TM6b</i>	III	[45], Magali Suzanne	none
<i>w; UAS-GC3Ai/Cyo ; MKRS/TM6b</i>	II	Bloomington and [45]	BDCS_84346
<i>UAS-hid dsRNA</i>	III	VDRRC	GD 8269
<i>UAS-lacZ</i>	II	Bloomington	BDCS_8529
<i>UAS-dronc<sup>DN</sup></i>	II	Boomington	BDCS_58992
<i>GMR11F02-gal4</i>	III	Bloomington	BDCS_48928
<i>w<sup>118</sup></i>	X	Bloomington	BDCS_3605
<i>UAS-dicer2 ; nubbin-gal4</i>	X, II	Bloomington	BDCS_25754
<i>w; ; hid<sup>A.GFP</sup>/TM3</i>	III	[66], Jean-Paul Vincent	none
<i>yw; ET40-QF, QUAS-tdTomato; FRT82B, tub-QS</i>	X, II, III	Bloomington	BDCS_30042
<i>yw, hs-flp22, UAS-GFP ; if/Cyo ; tub-gal4, FRT82B, tub-gal80/TM6b</i>	X, III	This study and Bloomington	BDCS_86311
<i>yw; UAS-mCd2RFP , UAS-dsRNACD8 FRT40A ; TM3/TM6b</i>	II, III	Bloomington	BDCS_56184
<i>yw; UAS-mcd8GFP, UAS-dsRNACD2 FRT40A ; TM3/TM6b</i>	II, III	Bloomington	BDCS_56185
<i>w; ptc-gal4</i>	II	Bloomington	BDCS_2017
<i>w; GMR83F09-gal4</i>	II	Bloomington	BDCS_40367
<i>w; GMR36C06-gal4</i>	II	Bloomington	BDCS_49931
<i>w; tub-gal80ts</i>	II	Bloomington	BDCS_7108
<i>w; UAS-lacZ</i>	II	Bloomington	BDCS_8529
<i>w; ; rpr-LacZ</i>	III	Bloomington	BDCS_58793
<i>w; ;grim-gal4</i>	III	Bloomington	BDCS_29117
<i>w; ; diap1-LacZ</i>	III	Bloomington	BDCS_12093
<i>w; 20xUAS-EGFPx6</i>	II	Bloomington	BDCS_52261
<i>w; ; Df(3L)H99 FRT80B/TM2</i>	III	Andrea Bergman	none
<i>yw hs-flp22; ; arm-βgal FRT80B</i>	III	Bloomington	BDCS_6341
<i>w; ; ubi-EGFPnls FRT80B</i>	III	Bloomington	BDCS_5630
<i>w; ; nab-Gal4</i>	III	DGRC Kyoto	DGRC_104533
<i>w; UAS-miRHG</i>	II	Jae Park[54]	none
<i>yw; p(LacW)Dark<sup>CD4</sup></i>	II	Bloomington	BDCS_23286
<i>w; E-cad::3XmKate(KI)/Cyo GFP</i>	II	Yohanns Bellaïche [67]	none
<i>w; lexAOP-GC3Ai</i>	II	This study	This study

<i>w; act-lexA</i>	II	Bloomington	BDCS_62567
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481 **Table 1** : description and origin of the *Drosophila melanogaster* strains used in this  
482 study.

483 The exact genotype used for each experiment is listed in table 2

Figure	Genotype	Clone induction	Dev. time
1 A-G, J-L	<i>w; + ; GMR11F02-gal4/UAS-GC3Ai</i>	-	96h AEL
1 H	<i>w; UAS-dronc<sup>DN</sup>/+ ; GMR11F02-gal4/UAS-GC3Ai</i>	-	96h AEL
1 I	<i>w; UAS-GC3Ai/+ ; GMR11F02-gal4/ UAS-hid-dsRNA</i>	-	96h AEL
1 J-L	<i>w; + ; GMR-11F02-gal4/UAS-GC3Ai</i>	-	72h AEL
1 M,N	<i>w ; + ; hid<sup>AGFP</sup>/TM3</i>	-	96h AEL
1supp A-G',S	<i>w; + ; GMR11F02-gal4/UAS-GC3Ai</i>	-	96h AEL
1supp H-K	<i>w<sup>118</sup></i>	-	96h AEL
1supp R,R',T	<i>w; dark<sup>(CD4)</sup>/dark<sup>(CD4)</sup></i>	-	96h AEL
1supp L-N,,S	<i>w; UAS-dronc<sup>DN</sup>/+ ; GMR11F02-gal4/UAS-GC3Ai</i>	-	96h AEL
1supp O-Q,S	<i>w; UAS-GC3Ai/+ ; GMR11F02-gal4/UAS-hid-dsRNA</i>	-	96h AEL
2B, 2C, 2suppD,E	<i>yw hs-flp22, UAS-GFP/+; ET40-QF, QUAS-tdTomato/Cyo-lf; FRT82B, tub-QS/FRT82B, tub-gal80</i>	8 min hs, 48h ACI	96h AEL
2D	<i>yw hs-flp22/+; ; arm-βgal FRT80B/ Df(3L)H99 FRT80B</i>	12 min hs, 48h ACI	96h AEL
2supp A,A'	<i>w; ; rpr-LacZ</i>	-	96h AEL
2supp B,B'	<i>w ; ; diap1-LacZ</i>	-	96h AEL
2supp C,C'	<i>W ; UAS-EGFPx6/+ ; grim-Ga4/+</i>		96h AEL
2supp D,E	<i>yw hs-flp22, UAS-GFP/+; ET40-QF, QUAS-tdTomato/Cyo-lf; FRT82B, tub-QS/FRT82B, tub-gal80</i> <i>yw hs-flp22, UAS-GFP/+; UAS-dronc<sup>DN</sup>/ET40-QF, QUAS-tdTomato; FRT82B, tub-QS/FRT82B, tub-gal80</i>	12 min hs, 48h ACI	96h AEL
2supp F,G	<i>yw hs-flp22/+; ; arm-βgal FRT80B/ ubi-EGFPnls FRT80B</i>	12 min hs, 48h ACI	96h AEL
3A-D	<i>yw hs-flp22, UAS-GFP/+; ET40-QF, QUAS-tdTomato/Cyo-lf; FRT82B, tub-QS/FRT82B, tub-gal80</i>	8 min hs, 48h ACI	96h AEL
3E,F	<i>yw hs-flp22/+; ; arm-βgal FRT80B/ Df(3L)H99 FRT80B</i>	12 min hs, 48h ACI	96h AEL
3supp A-C	<i>yw hs-flp22, UAS-GFP/+; ET40-QF, QUAS-tdTomato/Cyo-lf; FRT82B, tub-QS/FRT82B, tub-gal80</i>	8 min hs, 48h ACI	96h AEL
3supp D	<i>yw hs-flp22, UAS-GFP/+; UAS-dronc<sup>DN</sup>/ET40-QF, QUAS-tdTomato; FRT82B, tub-QS/FRT82B, tub-gal80</i>	8 min hs, 48h ACI	96h AEL
3supp E,F	<i>yw hs-flp22/+; ; arm-βgal FRT80B/ Df(3L)H99 FRT80B</i>	12 min hs, 48h ACI	96h AEL
3supp G,H	<i>yw hs-flp22/+; ; arm-βgal FRT80B/ ubi-EGFPnls FRT80B</i>	12 min hs, 48h ACI	96h AEL
4A	<i>w; UAS-EGFPX6/E-cad:mKateX3; Nab-Gal4/+</i>	-	96h AEL and 18hAPF
4B-D	<i>w; UAS-lacZ/+; Nab-Gal4/+</i>	-	adult
4B-D	<i>w; ; UAS-hid dsRNA/Nab-Gal4</i>	-	adult
4E	<i>w; UAS-lacZ/aristaless-gal4</i>	-	96h AEL

4E	<i>w; UAS-lacZ/trithorax-gal4</i>	-	96h AEL
4E	<i>w; UAS-lacZ/ptc-gal4</i>	-	96h AEL
4F,G	<i>w; UAS-lacZ/aristaless-gal4; +</i>	-	adult
4F,G	<i>w; aristaless-gal4/+; UAS-hid dsRNA/+</i>	-	adult
4F,G	<i>w; UAS-lacZ/trithorax-gal4; +</i>	-	adult
4F,G	<i>w; trithorax-gal4/+; UAS-hid dsRNA/+</i>	-	adult
4F,G	<i>w; UAS-lacZ/ptc-gal4 ; +</i>	-	adult
4F,G	<i>w; ptc-gal4/+ ; UAs-hid dsRNA/+</i>	-	adult
4supp A	<i>w<sup>118</sup></i>	-	adult
5A,B	<i>w; UAS-lacZ/tub-gal80<sup>ts</sup>; Nab-Gal4-gal4/+</i>	-	adult
5A,B	<i>w; +/tub-gal80<sup>ts</sup>; Nab-Gal4/UAS-hid dsRNA</i>	-	adult
5C-D	<i>w; E-cad:mKateX3/UAS-GC3Ai; Nab-Gal4/+</i>	-	pupae 15-30h APF
5supp A-C	<i>w; UAS-lacZ/tub-gal80<sup>ts</sup>; Nab-Gal4-gal4/+</i>	-	adult
5supp A-C	<i>w; +/tub-gal80<sup>ts</sup>; Nab-Gal4/UAS-hid dsRNA</i>	-	adult
5 supp D,E	<i>w; act-lexA, lexAOP-GC3Ai/UAS-lacZ; Nab-Gal4-gal4/ tub-gal80<sup>ts</sup></i>	-	pupae ~25h APF
5 supp D,E	<i>w; act-lexA, lexAOP-GC3Ai/tub-gal80<sup>ts</sup>; Nab-Gal4/UAS-hid dsRNA</i>	-	pupae ~25h APF

484

485 **Table 2:** genotype used for each experiment. AEL: after egg laying; hs : heat shock  
486 duration at 37°C; ACI : time after clone induction.

#### 487 **Design of lexAOP-GC3Ai fly line**

488 p13xLexAop2-GC3Ai was generated by PCR-amplifying GC3Ai from pCDH-puro-  
489 CMV-GC3Ai (Addgene 78910) and cloned in pJFRC19-13XLexAop2-IVS-myr::GFP  
490 (Addgene 26224) cut with NotI and XbaI (to excise myr::GFP) and inserted using In-  
491 Fusion assembly method. The construct was checked by sequencing and inserted at  
492 the attp site attp40A after injection by Bestgene.

493

494 The following primers were used for GC3Ai amplication:

495 GC3AI\_F: ATCCTTTACTTCAGGCGGCCGCGAATTGCCACCATGTACCCCTA

496 GC3AI\_R: ACAAAGATCCTCTAGACTTACAGGTCCTCCTCGCTGAT

#### 497 **qPCR validation of Hid depletion**

498 Hid depletion by RNAi and microRNA was validated by RTPCR. Total RNA was  
499 prepared from wing imaginal disc expressing hid-dsRNA or mirRHG under the control  
500 of the Nab-Ga4 driver (non-Tb larvae) using TRIzol reagent (Invitrogen). RNA (1ug)  
501 was retrotranscribed using random primers and Superscript III reverse transcriptase  
502 (Invitrogen). cDNA was analyzed by qPCR using FastStart Universal SYBR Green

503 (Roche) and Actin-42A was used as a reference gene. The primers used for Actin 42A  
504 and Hid amplifications were the following:

505 **Actin 42A\_F:** GAGCGCGGTTACAGCTTCA

506 **Actin 42A\_R:** TCCTTGATGTCGCGCACA

507 **Hid\_F:** GTACCAGAGCCAGCAGAGTG

508 **Hid\_R:** GCGGATGGGGATTTCGAGTTC

509 All assays were performed in triplicate, and mean values were calculated according to  
510 the  $\Delta$ CT quantification method. Results are expressed as the average from at least  
511 three independent experiments normalised to the control condition (Nab-Gal4, UAS-  
512 lacZ). Doing so, we obtained an average Hid mRNA level at 0.59 for mirRHG versus  
513 0.46 for hid dsRNA ( $p=0.02$ ), suggesting a very efficient depletion in both cases, but  
514 more efficient for hid dsRNA.

515

## 516 **Immunostaining**

517 Dissections of larval wing imaginal discs were performed on PBS in ice. Dissected  
518 discs were fixed for 20 min in 4 % formaldehyde (SIGMA F8775), rinsed 3 times in PBT  
519 (PBS 0.4 % Triton), followed by 10 min permeabilisation in PBT. Primary and  
520 secondary antibodies were incubated for 2 h at room temperature (or 12 h at 4 °C)  
521 under rocking agitation. After each antibody incubation, discs were rinsed 3 times in  
522 PBT, followed by 3 washes of 30 min. Discs were mounted in Vectashield® (EUROBIO  
523 SCIENTIFIC / H-1000) and imaged using a confocal spinning disc microscope (Gataca  
524 systems) with a 40X oil objective or a LSM880 equipped with a fast Airyscan using a  
525 40X oil objective. The following primary antibodies were used: rat anti E-cadherin  
526 (1/100, DCAD2 concentrated DSHB), mouse anti DSRF (1/500, gift of Seth Blair), rat  
527 anti Delta (1/1000, gift of François Schweisguth), chicken anti GFP (1/1000, abcam  
528 ab13970), rabbit anti RFP (1/500, abcam 62341), rabbit anti DCP-1 (1/100, Cell  
529 Signaling 9578S), chicken anti Beta-gal (1/1000, Abcam ab 9361), mouse anti  
530 Wingless (1/250, 4D4 concentrated DSHB), mouse anti Patched (1/250, Apa-1  
531 concentrated, DSHB), phalloidin alexa 647 (1/50, Invitrogen). Secondary antibodies  
532 were: anti rabbit alexa 555 (1/500, Invitrogen), anti chicken alexa 488 (1/500,  
533 Invitrogen), ultrapurified anti mouse 405 (1/500, Jackson ImmunoResearch / 715-476-  
534 151), ultrapurified anti mouse Cy3 (1/500, Jackson ImmunoResearch / 715-165-151),



535 ultrapurified anti rat Cy3 (1/500, Jackson ImmunoResearch / 712-165-153),  
536 ultrapurified anti rat alexa 647 (1/500, Jackson ImmunoResearch / 712-605-153).  
537 TUNEL assays for the detection of dying cells with fragmented DNA were conducted  
538 using the *In Situ Cell Death Detection Kit, TMR red* (Roche diagnostics®) and the  
539 *TUNEL dilution buffer* (Roche diagnostics®), following the experimental procedure  
540 described in [68].

#### 541 **Image processing**

542 All images were processed using FIJI[69] . For the twin clones analyses, Z projections  
543 of z-stacks were done using the Fiji LocalZProjector plugin using E-cad or phalloidin  
544 staining as a reference plane[70], allowing to project a limited number of planes around  
545 the apical junction plane while following the local disc curvature. *hid*<sup>AGFP</sup> pattern  
546 measurements were done after fixation and GFP immunostaining and maximum z  
547 projection. The mean GFP intensity was measured in each compartment (defined with  
548 DSRF staining) and normalised by the mean GFP intensity of the full wing pouch.

#### 549 **QMARCM and mitotic clones experiments**

550 Wandering larvae were collected 48 hours after clone induction following a 37°C heat-  
551 shock of 8 or 10 minutes (Table 2). Analysis of twin clones was performed on local  
552 projections of the wing discs after fixation and staining. Only discs with sufficiently  
553 sparse distribution of clones were used (to assign twins unambiguously). To extract  
554 clone position and size, each mitotic recombination figure (twin spot or single clone)  
555 was manually outlined on FIJI and then automatically segmented by applying a  
556 Gaussian blur followed by an automated thresholding (Intermode white method). The  
557 centroid of each mitotic recombination figure was obtained by summing the centres of  
558 each individual patch of cells composing the twin spot, ponderated by their relative  
559 area compared to the total area of the twin clone. Thus, the centroid of each mitotic  
560 recombination figure was given by the following formula:

$$561 \quad Centroid(x, y)_{mitotic\ figure} = \sum_{clone\ i} (Centroid(x, y)_{patch\ i}) \cdot \frac{Area_{patch\ i}}{Total\ Area\ twin\ clone}$$

562 Averaged clone surface was estimated through the segmented surface of GFP or RFP  
563 clones (using the local projection around the apical plane of cells). Estimation of the  
564 clone size in cell number was obtained a posteriori by segmenting (using Tissue

565 analyser[71]) 3 wing discs stained with E-cad (Figure 3suppA') and DSRF to position  
566 landmarks, and by estimating the averaged apical cell area for each compartment. The  
567 3 wing discs segmented for apical cell area shown a very congruent pattern (Figure  
568 3suppA), thus allowing to pool the data from the 3 discs (Figure supp3 B). The average  
569 surface of clone for each compartment was then divided by the local averaged cell size  
570 to obtain an estimation of cell number per clone and to correct for effects driven by  
571 inhomogeneity of cell apical size throughout the disc.

572 For mitotic clones (H99 and WT sibling clones, [ubi-GFP](#) and [arm-β-gal](#) WT neutral  
573 [mitotic clones](#)), E-cad and phalloidin signals were used to project locally the β-gal  
574 signal ([the absence of LacZ](#) marking the H99 allele) using the local z-projection Fiji  
575 plugin [70] and combined with DSRF staining. [The same procedure was applied to](#)  
576 [GFP and β-gal for neutral mitotic clones, except that the projection was performed](#)  
577 [around the nucleus plane](#). A 3D gaussian blur filter was applied on the β-gal and GFP  
578 signal (1px width in X,Y,Z). [For H99 mitotic clones](#), homozygous mutant (no β-gal  
579 signal) and WT siblings (2 copies of β-gal) were recognised manually, and their  
580 contours drawn by hands on Fiji. The same procedure was applied to neutral mitotic  
581 clones (comparing clones with 2 copies of β-gal without GFP, and clones with two  
582 copies of GFP without β-gal). We only used discs with sufficiently sparse clones and  
583 proper β-gal/GFP signal and counted single clone only when there was no ambiguity.  
584 Twin clone localisation was then assigned using the centroid estimation described  
585 above.

### 586 **Spatial maps of wing discs (twin clones and GC3Ai).**

587 Positions of 36 landmarks were manually set on Z projections of wing discs using FIJI.  
588 Landmarks were positioned according to the pattern of DSRF revealed by  
589 immunostaining, along the veins, margin and folds of the wing pouch (**Figure 1supp**  
590 **A,B**). Two methods were used to characterise a spatial map of the tissue based on  
591 landmarks position. For the first one, landmarks were geometrically aligned within each  
592 genotype using the General Procrustes Analysis (GPA) [72] as implemented in R  
593 geomorph package [73]. GPA translates the set of landmarks of each wing disc to the  
594 same origin, scales them to size, and rotates them until the coordinates of  
595 corresponding points align as closely as possible. GPA allows to superimpose as  
596 closely as possible several wing discs, thus allowing superimposition of the signal from

597 many individuals (**Figure 1F, Figure 1supp,H,J,M,P,R**). For the second mapping  
598 method, 14 additional landmarks were added (landmarks 37 to 50) at the centre of  
599 segments defined by other landmarks (for example landmark 37 was set as the centre  
600 of the segment defined by landmarks 21 and 22, **Figure 1suppC**). The resulting 50  
601 landmarks were thus used to define polygons dividing the wing disc tissue into 40  
602 compartments (**Figure 1suppC**). With this later approach, clones and apoptotic bodies  
603 (defined by GC3Ai or cleaved DCP-1 figures) were assigned to a compartment  
604 according to the coordinates of the centre of mass of the clone/apoptotic body. In order  
605 to account for uncertainty in clone/apoptotic body localisation (e.g., because of clone  
606 movement between the time of induction and imaging), as well as for uncertainty in  
607 landmark positioning (e.g., because of immunostaining variability or user error), clone  
608 assignment included consideration of an error margin. We used the *st\_buffer* function  
609 implemented in the *sf* package of R software to add an error margin at a distance of  
610 11.8  $\mu\text{m}$  (40 pixels) around compartment margins (**Fig. 1suppD**). As a result, buffered  
611 boundaries of neighbour compartments overlap within each other and a given position  
612 in the tissue could be assigned to belong to more than one compartment. This results  
613 in a smoothing of the spatial map allowing to account for uncertainty in cells and  
614 landmarks positions.

615 To analyse the spatial pattern of GC3Ai in discs at 72 h AEL, the anti DSRF staining  
616 could not be used to draw the landmarks because at this stage the DSRF patterning is  
617 not yet established. Instead we stained the wing discs for Ptc and Wg to detect AP and  
618 DV compartment and subdivide the wing disc in 4 quadrants (Anterior-Dorsal; Anterior-  
619 Ventral; Posterior-Dorsal; Posterior-Ventral).

## 620 **Adult wing shape and size analyses**

621 Crosses were done by placing 50-100 couples in egg laying cages with juice-agar  
622 plates kept at 25 °C. Freshly hatched larva from the agar plates were transferred to  
623 *Drosophila* vials with fly food as described above at a density of 30 individuals / vial,  
624 and grown at 18 °C, 25 °C or 29 °C until emergence of adults. Flies were collected and  
625 stored in ethanol 70 % at room temperature until dissection. Only left wings from  
626 females were taken, and mounted dorsal side up on a glass slide in a solution of 1:1  
627 ethanol 80% and lactic acid 90%. Imaging was carried out on a ZEISS Discovery V8  
628 stereomicroscope using a ZEISS Axiocam ICc 5 camera. All wings were imaged in the

629 same orientation and with the same imaging parameters. For the experiments using  
630 Gal80ts (**Figures 4 and 4supp**), flies were crossed in cages supplied with agar petri  
631 dishes and allowed to lay eggs for 6 to 12 h. Freshly hatched larva were transferred  
632 to regular vials at a density of 30 individuals / tube, and placed at 18 °C or 29 °C. Once  
633 arrived at the wandering larval stage, individuals where switched temperature (from 18  
634 °C to 29 °C and vice-versa). Upon hatching, flies were collected for wing dissection.

635 Wing measurements were done using a semi-automated procedure for estimating the  
636 positions of 12 landmarks and 37 semi-landmarks along the wing outline and veins  
637 (**Figure 4suppA**). This was done using Wings4 software [52, 53] which fits a B-spline  
638 model to the wing from which the coordinates of landmarks and semi-landmarks are  
639 extracted. Wings4 outputs were examined using CPR software[53] which allows to  
640 screen for outliers and to generate a consolidated dataset of landmarks and wing  
641 areas.

642 Landmarks data were geometrically aligned within each experiment using the General  
643 Procrustes Analysis (GPA)[72] as implemented in R geomorph package[73]. GPA  
644 translates all wing images to the same origin, scales them to unit-centroid size (centroid  
645 size is a measure of specimen size computed as the square root of the sum of squared  
646 distances of all the landmarks from the specimen's centroid) and rotates them until  
647 the coordinates of corresponding points align as closely as possible. Differences in  
648 landmarks coordinates resulting from the GPA represent shape differences between  
649 wings. Wing shape of each individual is characterised by the value of 96 variables,  
650 coming from the Procrustes transformation of x and y coordinates of the 48 landmarks  
651 and semi-landmarks. To reduce dimensionality of the data, wing shape variation was  
652 analysed by Principal Components Analysis as implemented in the function  
653 *plotTangentSpace* (now deprecated and replaced by *gm.prcomp*) of the R *geomorph*  
654 package. Visualisation of the wing shape variation among the principal component  
655 axes was done by comparing wing shape of the individual presenting the lowest value  
656 along the axis, with the individual presenting the highest value. To enable visualisation  
657 of local growth differences in the adult wing upon apoptosis inhibition, we used the  
658 program Lory[74] to show one pattern of relative expansion or contraction that can  
659 transform mean shape of control genotype into mean shape of genotypes where  
660 apoptosis was inhibited.

## 661 **Live imaging of pupal wing**

662 Pupa were collected at early fluid stage and kept at 29°C for 7 h, resulting in pupa aged  
663 of ~15 h APF (after pupal formation). Pupal wings were prepared for live imaging as  
664 described in [75]. Briefly, pupa were glued on their lateral side on a support made of  
665 three layers of tape (one layer of single-side tape, a second layer of double-side tape,  
666 and a third layer of single-side tape), and a window was opened using a pair of fine  
667 scissors (Fine Science Tools) in the pupal case to expose the pupal wing tissue. The  
668 wing was covered with halocarbon 10S oil, and the tape with the pupa on it was  
669 mounted on a glass bottom dish of 1,5 µm thickness (Miltenyi Biotec). Imaging was  
670 done on a confocal spinning disc microscope (Gataca systems, Metamorph software)  
671 with a multi-immersion 20X objective with oil (N.A. 0.75). The dorsal epithelial layer of  
672 the wing was scanned on a mosaic of two (x,y) tiles, and Z-stacks of 50 µm (20 slices  
673 of 2.5 µm), every 15 min at room temperature (~ 22 °C). Z projections were done using  
674 the Fiji LocalZProjector using E-cad plane as a reference [70].

## 675 **Estimation of clone size differences in an exponential growth regime**

676 The aim of this analysis is to predict spatial differences of clone size under an  
677 exponential growth regime given the estimated spatial differences of apoptosis rate  
678 based on the twin-clone experimental assays. For the sake of simplicity, we assume  
679 that division rate and apoptosis rate are constant over time.

### 680 *Estimation of the clone extinction probability*

681 Upon recombination, two daughter cells of different colours are generated and  
682 continue to grow, divide and die. After a given time  $T$  (time of observation), there are  
683 four possible outcomes: we can recover the two daughter clones, a single colour clone,  
684 green or red (the other clone died before time  $T$ ), and finally both clones disappeared  
685 before time  $T$  (which cannot be measured experimentally). For this estimation, we  
686 assumed that both lineages (green and red) have the same proliferation and death  
687 rates.

688  $n(t)$  is the size (number of cells) of a clone at the time  $t$ , and it obeys to? a stochastic  
689 process that follows the same probability function for every clone. For every clone, we  
690 have  $n(0) = 1$ . The probability of clone disappearance before time  $t$ . *i.e.* the extinction  
691 probability,  $p_0(t)$  is defined as

692 
$$p_0(t) = P(n(t) = 0).$$

693 Experimental data gives access to the number of single clones  $N_s$  and to the number  
 694 of twin clones  $N_d$  that we observe at the time  $T$ . From this, we can estimate the  
 695 probability of extinction at the observation time  $T$ ,  $p_0 = p_0(T)$ .

696 The expected number of single clones is  $N_s = 2p_0(1 - p_0)$

697 The expected number of twin clones is  $N_d = (1 - p_0)^2$

698 which gives the estimator of  $p_0$ ,

699 (1) 
$$\hat{p}_0 = \frac{q}{2-q}$$

700 where 
$$q = \frac{N_s}{N_s + N_d}$$

701

702 *Estimation of the clone size and relationship with apoptosis and proliferation rate*

703 Let  $\mu(t)$  be mean value of  $n(t)$ , clone size in number of cell at time  $t$ . In theory,

704 
$$\mu(t) = p_0(t) \cdot 0 + p_1(t) \cdot 1 + \dots + p_i(t) \cdot i + \dots,$$

705 where  $p_i(t)$  is a probability to observe a clone of size  $i$  at the given time  $t$ , or  $p_i(t) =$   
 706  $P(n(t) = i)$  and  $\sum p_i(t) = 1$  for all  $t$ . In order to estimate  $\mu(t)$  from the observations we  
 707 use:

708 
$$\hat{\mu} = \frac{k_0}{N} \cdot 0 + \frac{k_1}{N} \cdot 1 + \dots + \frac{k_i}{N} \cdot i + \dots,$$

709 where  $N$  is a total number of clones (including clones of size "0"),  $k_i$  is a number of  
 710 clones of size  $i$ , and  $\sum k_i = N$ .

711 However, since we cannot observe clones of size 0 we cannot measure  $k_0$  nor  $N$ . We  
 712 therefore use  $p_0 = \frac{k_0}{N}$  (the probability of clone extinction, see above), and the total  
 713 number of observed clones  $N_{obs} = N - k_0$ . From these two we can estimate  $N = \frac{N_{obs}}{1-p_0}$ .

714 Hence, we estimate  $\mu$  with

715 (2) 
$$\hat{\mu} = (1 - \hat{p}_0) \left( \frac{k_1}{N_{obs}} \cdot 1 + \dots + \frac{k_i}{N_{obs}} \cdot i + \dots \right).$$

716 Each clone observed is a result of a stochastic birth-death process, that is fully  
 717 described by its death rate,  $a$ , which is a probability of an individual cell to die per unit  
 718 time and its birth rate,  $b$ , which is a probability of an individual cell to divide per unit  
 719 time. While the size of the clone at the given time,  $n(t)$ , is a stochastic process, the  
 720 mean size  $\mu(t)$  can be approximated by a deterministic process and is given by the  
 721 following exponential function:

$$722 \quad (3) \quad \mu(t) = e^{(b-a)t}$$

723 Furthermore, the probability to be extinct at the time  $t$  ( $n(t) = 0$ ) can be expressed as  
 724 follows [76]:

$$725 \quad (4) \quad p_0(t) = \frac{a - ae^{-(b-a)t}}{b - ae^{-(b-a)t}}$$

726

727 *Estimating the apoptosis and proliferation rates for each compartment.*

728 From the twin clone experimental data, we retrieve for each compartment the  
 729 proportion of single clone occurrence (**Figure 2C**) and the averaged clone size (**Figure**  
 730 **3A**) which, together with (1), allows us to estimate  $p_0$ , and  $\mu$  for each of the 40  
 731 compartments of the wing disc. These estimates can then be used to estimate  
 732 parameters  $a$  and  $b$  (apoptosis and division rate). For the fixed  $t$ , based on (3) and (4)  
 733 we have:

$$734 \quad (5) \quad b = \frac{\ln(\mu)}{t} + a$$

735 and

$$736 \quad (6) \quad p_0 = \frac{a - \frac{a}{\mu}}{b - \frac{a}{\mu}}$$

737 Hence

$$738 \quad (7) \quad a = \frac{\ln(\mu)}{t \left[ \frac{1}{\mu} + \frac{\mu-1}{\mu p_0} - 1 \right]}$$

739 *Estimating the expected differences of clone size between compartments assuming*  
 740 *constant proliferation rate*

741 We use the fixed value of a birth rate,  $b = 0.0657$  (average of the estimated  $b$  from (5)),  
742 as a fixed value for every compartment. We look for a death rates  $a$  for each  
743 compartment using the expression for  $p_0(a)$  (4). To the best of our knowledge it is not  
744 possible to express explicitly  $a$  from the given formulation, so we use numerical  
745 approach to calculate it. We plot function of  $p_0$  as  $p_0(a)$  and look for the point of  
746 intersection with the measured value  $\hat{p}_0$ . In this way we estimate value of  $a$ . Then we  
747 use  $a$  and the fixed value of  $b$  to predict  $\mu$  (6), that we then compared with the estimated  
748 value  $\hat{\mu}$  from the experimental data (2) correcting for the non-observable disappeared  
749 clones (see **Figure 3B**). We overall found a good correlation between the estimated  
750 clone size and the prediction, (correlation coefficient  $\rho = 0.6$  , **Figure 3D**) suggesting  
751 that the spatial differences in apoptosis are to a good approximation sufficient to  
752 explain the spatial differences in average clone size.

### 753 **Statistics**

754 Data were not analysed blindly. No specific method was used to predetermine the  
755 number of samples. The definition of  $n$  and the number of samples is given in each  
756 figure and associated legend. Error bars are standard error of the mean (s.e.m.) or  
757 confidence interval 95%. Test for spatial autocorrelations in apoptosis patterns were  
758 conducted using Moran's statistics implemented in the *moran.mc* function from the  
759 *spdep* R package[77]. This tests for spatial clustering of apoptosis levels, by testing  
760 the hypothesis that the levels of apoptosis are randomly distributed across the 40  
761 compartments, versus the hypothesis that the level of apoptosis of a given  
762 compartment is correlated with the levels of the neighbouring compartments. Tests for  
763 spatial correlation across compartments between log transformed GC3Ai levels and %  
764 of single clones occurrences were done using the function *wtd.cor* from *weights* R  
765 package (v. 1.0.4), which produces a Pearsons correlation test and uses weights for  
766 calculation of the correlations. In this case, we weighted the values for the % of single  
767 clones by the number of mitotic events over which the percentage was estimated within  
768 each compartment, thus giving higher weights to the compartments for which  
769 percentages were estimated over a large number of events. [Bootstrapping analysis](#)  
770 [was used to assess the distribution of differences between Hid-dsRNA and LacZ adult](#)  
771 [wing size at different temperatures upon larval and pupal induction at 29°C \(Figure](#)  
772 [5A\)](#). 50 random resamplings of each dataset was performed and used to look at the  
773 [percentage of variation of the difference obtained for each condition between the lacZ](#)



774 and Hid-dsRNA flies. We then assessed the overlap of the distribution and confirmed  
775 that the difference between LacZ and Hid-dsRNA at 18° (control) is similar to the  
776 difference between LacZ and Hid-dsRNA induction during pupal stage (18°C-29°C),  
777 while the differences were higher than the control for all the other conditions.

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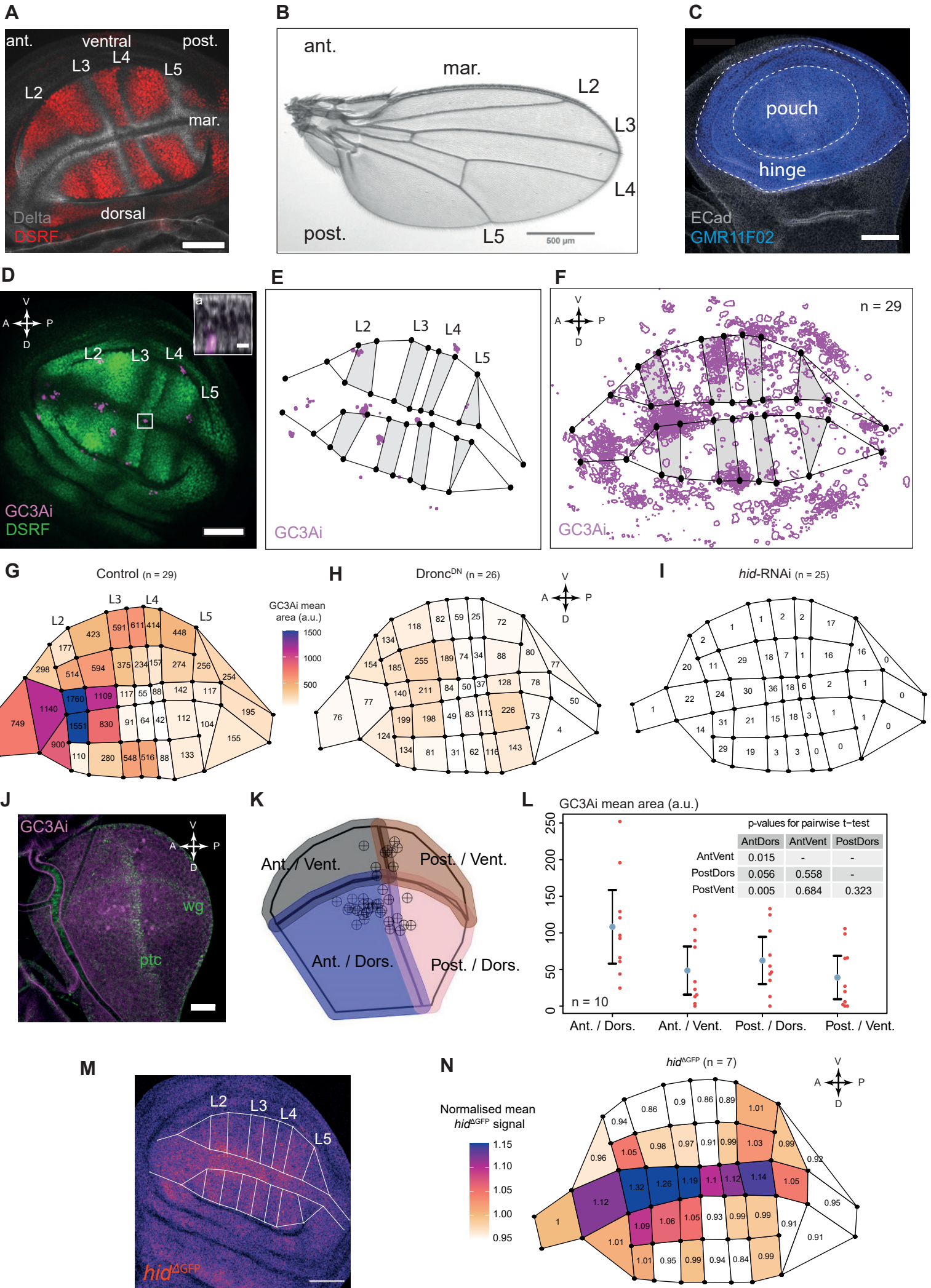
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959

**Figure 1**

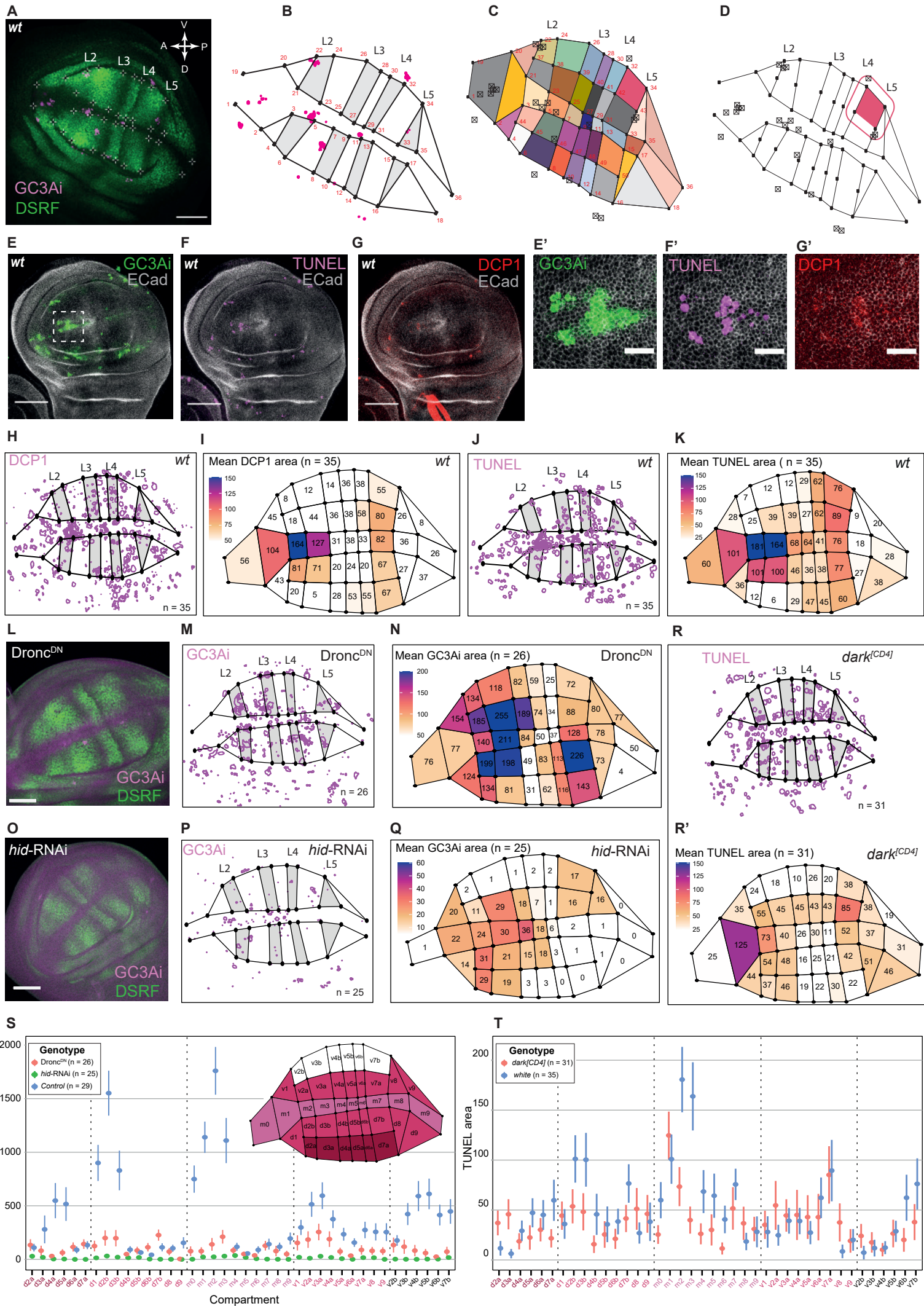




## Figure 1: Apoptosis is spatially biased in the developing wing tissue.

**A:** Wing disc at ~ 96 h after egg laying (AEL) showing veins (L2 to L5), interveins (red), dorsal, ventral, margin (mar.), anterior (ant.) and posterior (post.) territories. Red: anti-DSRF immunostaining. Grey: anti-Delta immunostaining. Orientation is the same for all wing discs images and schemes of this article, with the anterior compartment leftwards, and the dorsal compartment downwards. **B:** Female adult wing on dorsal view, with longitudinal veins (L2 to L5), margin (mar.), anterior (ant.) and posterior (post.) territories. **C:** Wing disc at ~ 96 h AEL showing the GMR11F02-GAL4 expression domain (blue). Gray: anti E-Cadherin immunostaining. **D:** z-projection of a wing disc showing caspase-3 activity using the GC3Ai reporter (magenta) expressed under the control of GMR11F02-GAL4 at ~ 96 h AEL. The inset is an orthogonal view of the white square, showing that the GC3Ai signal (magenta) points to apoptotic bodies located basally in the epithelium. a: apical. Grey: E-Cadherin. Inset scale bar: 5  $\mu$ m. **E:** Segmentation output from the disc in **D**, showing the DSRF pattern and the GC3Ai segmented signal (magenta). Veins regions are grey. **F:** Superimposition of the segmentation data from 29 discs scaled, rotated and aligned using the General Procrustes Analysis (GPA), showing a spatially heterogeneous signal for GC3Ai (magenta). The map shown for vein / intervein territories is the average map from the 29 discs. **G-I:** Heat-maps showing the average GC3Ai positive area on each of the 40 compartments of the wing disc for the *control* (**G**, n = 29), *Dronc<sup>DN</sup>* (**H**, n = 26), *hid-RNAi* (**I**, n = 25) genotypes. Numbers within each compartment show the average value for GC3Ai positive area (arbitrary units). These values are also shown as a colour coded heat-map which has the same scale for three maps shown in G-I. **J:** Visualisation of caspase-3 activity using the GC3Ai reporter (magenta) expressed under the control of GMR11F02-GAL4 at ~ 72 h AEL. The wing disc is divided into four quadrants (Anterior-Dorsal; Anterior-Ventral; Posterior-Dorsal; Posterior-Ventral) using the immunostaining anti Wingless (wg) and antiPatched (ptc) marking compartment boundaries. **K:** Segmentation of the disc shown in **J**. The circled black crosses denote the centroids of the segmented GC3Ai signal. The black line was manually drawn using anti-Wingless and anti-Patched immunostainings to delineate the four quadrants, which are coloured. In order to account for uncertainty in centroid location and manual drawing of the contours, an overlap between the quadrants was allowed. **L:** Mean GC3Ai positive area for 10 discs at ~ 72 h AEL. One red dot represents one disc, blue circles are means and error bars are 95 % confidence intervals. The inset shows the p-values for all pairwise t-tests between quadrants. **M:** Pattern of *hid<sup>AGFP</sup>* (KI) on a single wing disc (LUT fire, Gaussian blur 0.75). White lines denote the vein/intervein territories extracted from the anti-DSRF immunostaining (not shown). **N:** Heat-map showing the mean *hid<sup>AGFP</sup>* signal on each of the 40 compartments over n = 17 discs. To allow comparison of intensities among discs, the GFP signal intensity was normalised within each disc (i.e., within each disc, the value of each pixel was divided by the mean signal intensity over all the pixels of the disc). All scale bars are 50  $\mu$ m length excepted in **B**, 500  $\mu$ m, and in **J**, 25  $\mu$ m.

# Supplementary figure 1



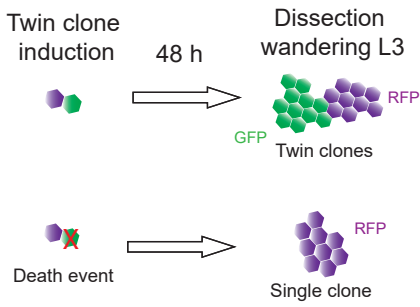
## Supplementary figure 1 (related to Figure 1)

**A:** Wing disc from **Fig. 1-A** showing the 36 landmarks (white crosses) that were manually positioned at the intersections between veins (L2 to L5), folds and margins of the tissue, visible thanks to DSRF (green). **B:** Map of the wing disc shown in **A**, drawn from the landmark positions (black dots, numbered 1 to 36), and showing the segmented GC3Ai signal (magenta). Vein regions are in grey and intervein/margin regions in white. **C:** Division of the wing disc map shown in **B** into 40 compartments based on polygons (plain colours) defined with the landmarks. To this aim, 14 additional landmarks (numbered 37 to 50) were added at the middle of segments made by previously defined landmarks (see **Methods**). Boxed black crosses show the centroid of the GC3Ai segmented signal shown in **B**. **D:** Illustration of the error margin of the compartment boundaries used to account for uncertainty in signal localisation and landmark position. For each compartment, the margin was extended by 11.8  $\mu\text{m}$  in order to encompass surrounding regions. In this example, the compartment defined by landmarks 32;34;43;42 (coloured pink) has its margin extended and as a result the three GC3Ai centroids located around the compartment (boxed black crosses) will be considered as being also part of this compartment. **E-G':** Co-localisation of GC3Ai (green), TUNEL (magenta) and cleaved DCP-1 (*Drosophila* caspase3, red). E-cad in grey. Close-up view of the white dotted square are shown in **E'-G'**. **H-K:** Superimposition of cleaved DCP-1 (**H**, magenta) and TUNEL (**J**, magenta) signals from 35 *w118* discs superimposed using the General Procrustes Alignment (GPA) based on the 36 landmarks positions. Heat-maps showing the average cleaved DCP-1 and TUNEL positive area for each of the 40 compartments are shown in **I** and **K** respectively. Numbers within each compartment show the average value also shown as a colour coded heat-map. **L-Q:** Pattern of GC3Ai in *UAS-Dronc<sup>DN</sup>* background (**L-N**) and *hid RNAi* background (**O-Q**) driven by GMR11F02-GAL4, single representative wing disc (**L**, **O** DSRF green, GC3Ai magenta), superimposition of 26 *Dronc<sup>DN</sup>* discs (**M**) and 25 *hid-RNAi* disc (**P**) using GPA, and heat maps showing the averaged GC3Ai positive area for each genotype(**N,Q**). Note that contrary to the maps shown in **Figure 1 H,I**, the colour-scale is adapted for each genotype to better show spatial differences. **R:** Superimposition of TUNEL positive particles from 31 wing discs homozygous mutant for Dark (*dark<sup>CD4j</sup>*, *Drosophila Apaf1*). **R':** Heat map showing the averaged TUNEL area for the 31 *dark<sup>CD4j</sup>* discs. The scale is the same as the map shown in **Figure 1suppK** for TUNEL area in *w118* background, which is the corresponding control. **S,T:** Mean GC3Ai positive area for three genotypes (**S**, Control, *hid-RNAi*, *Dronc<sup>DN</sup>*) and TUNEL positive area in two genotypes (**T**, *dark<sup>CD4j</sup>*, *white=w118*) for the 40 compartments. The top-right inset in **S** shows a wing disc diagram with the 40 compartments. Error bars show s.e.m.. All scale bars are 50  $\mu\text{m}$  length excepted in **E'-G'**, 10  $\mu\text{m}$ .

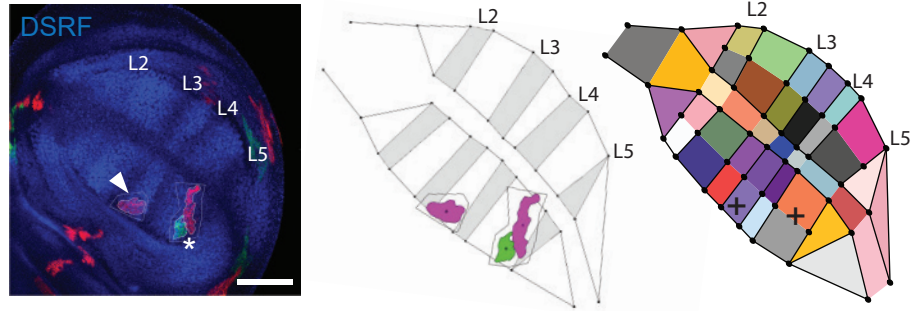


**Figure 2**

**A**



**B**



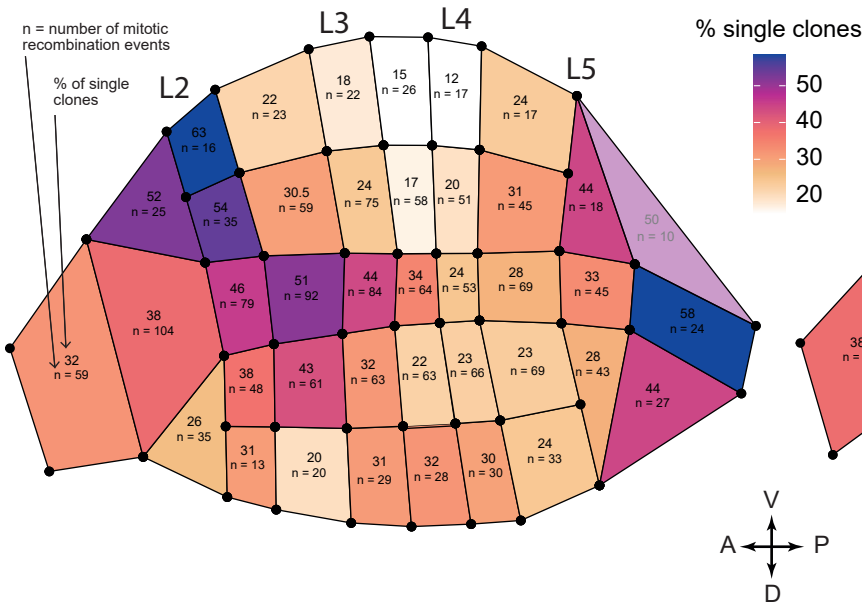
**C**

QMARCM - Control

96 discs  
546 mitotic recombination events

368 twin clones (67%)  
152 single RFP clones (28%)  
26 single GFP clones (5%)

Tub-GAL4 > UAS-GFP  
Tub-QF > QUAS-RFP



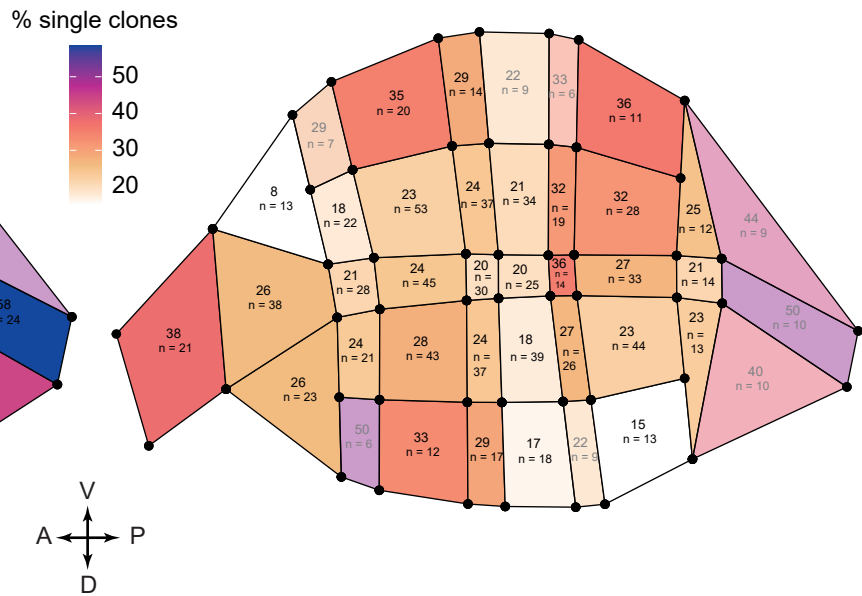
**C'**

QMARCM - Dronc<sup>DN</sup>

76 discs  
243 mitotic recombination events

180 twin clones (74%)  
58 single RFP clones (24%)  
5 single GFP clones (2%)

Tub-GAL4 > UAS-GFP, UAS-Dronc<sup>DN</sup>  
Tub-QF > QUAS-RFP

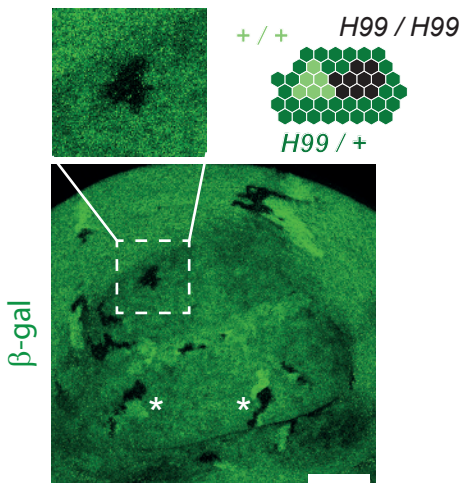


**D**

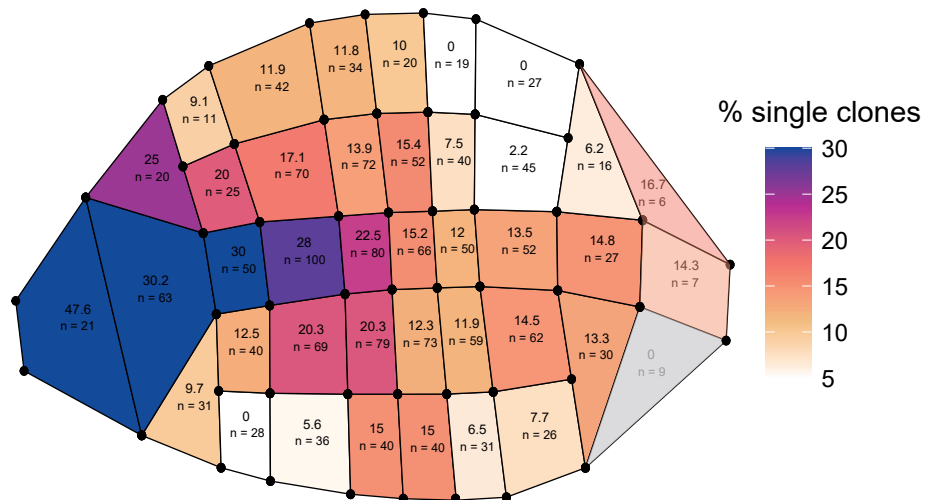
H99 - FRT80/ arm-lacZ FRT80

66 discs  
1242 mitotic recombination events

1140 twin clones (91.8%)  
102 single H99/H99 clones (8.2%)  
0 single +/- clones (0%)



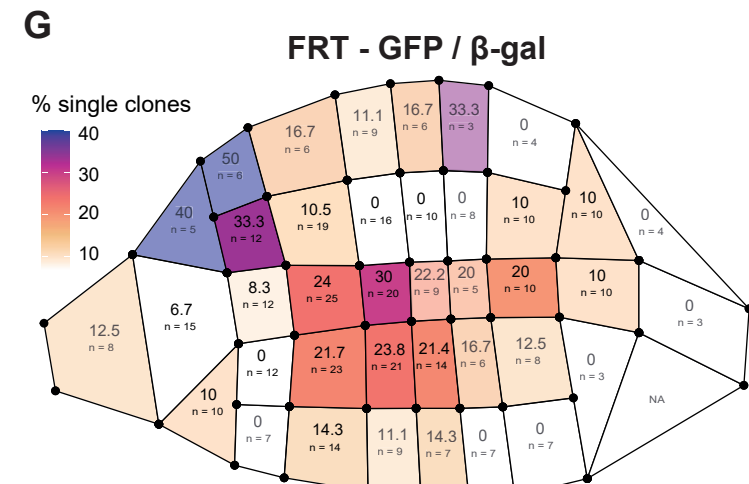
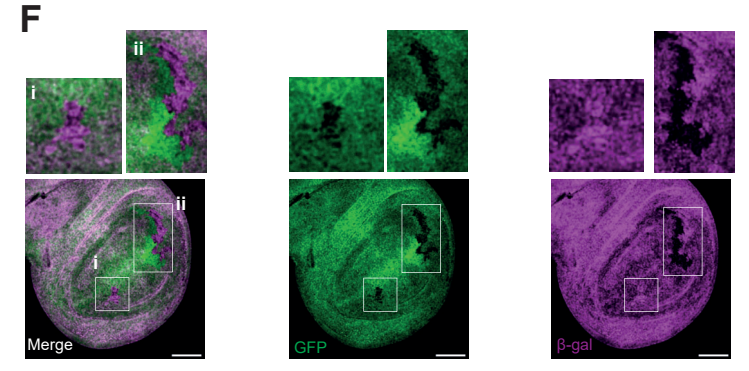
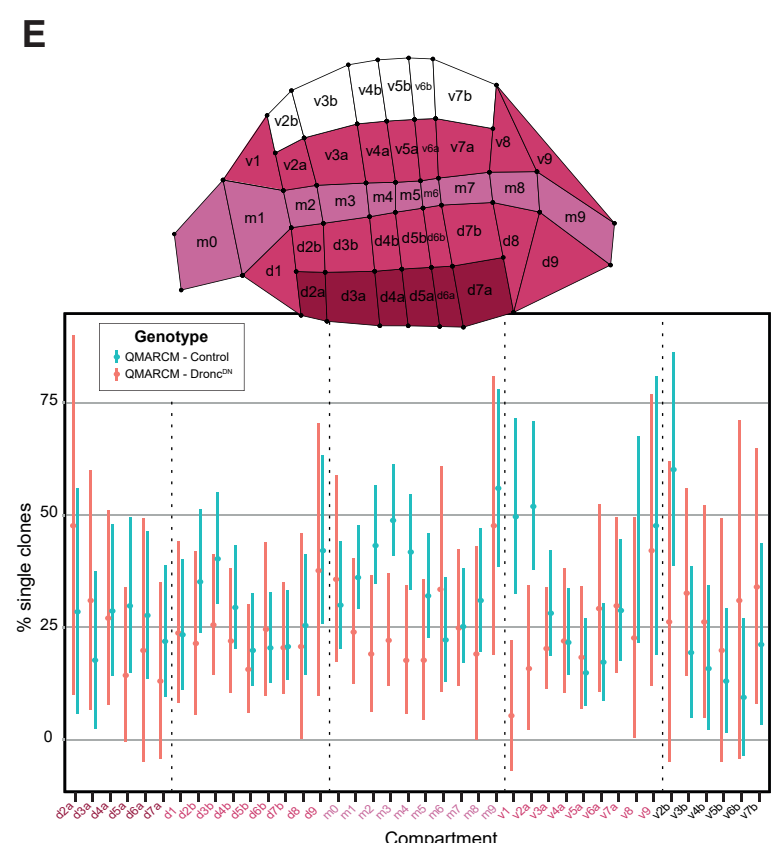
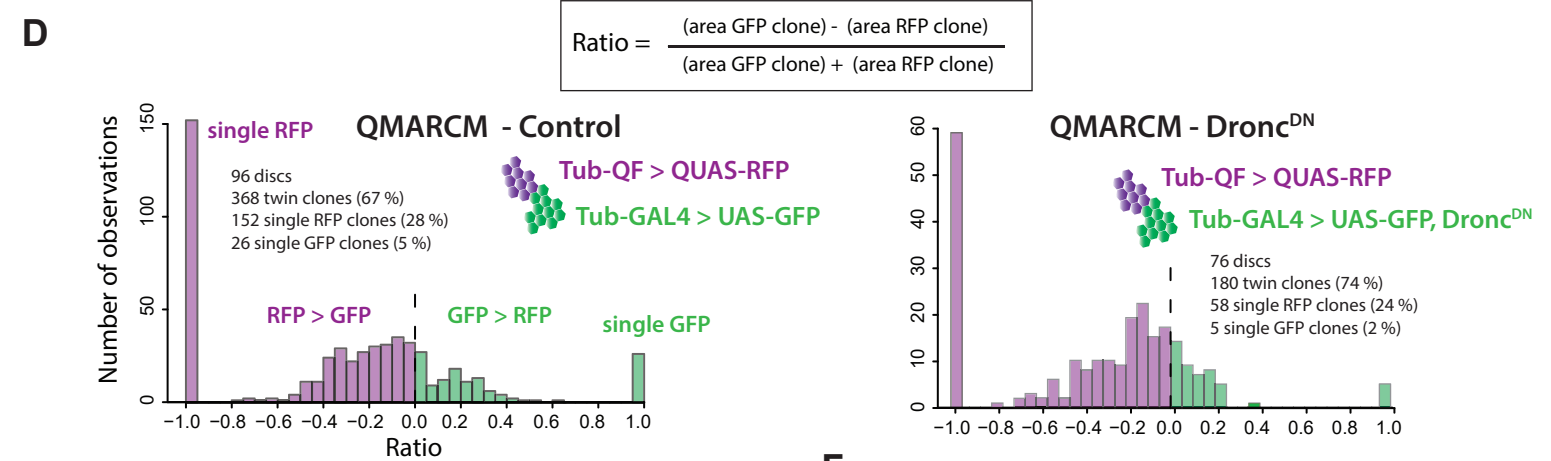
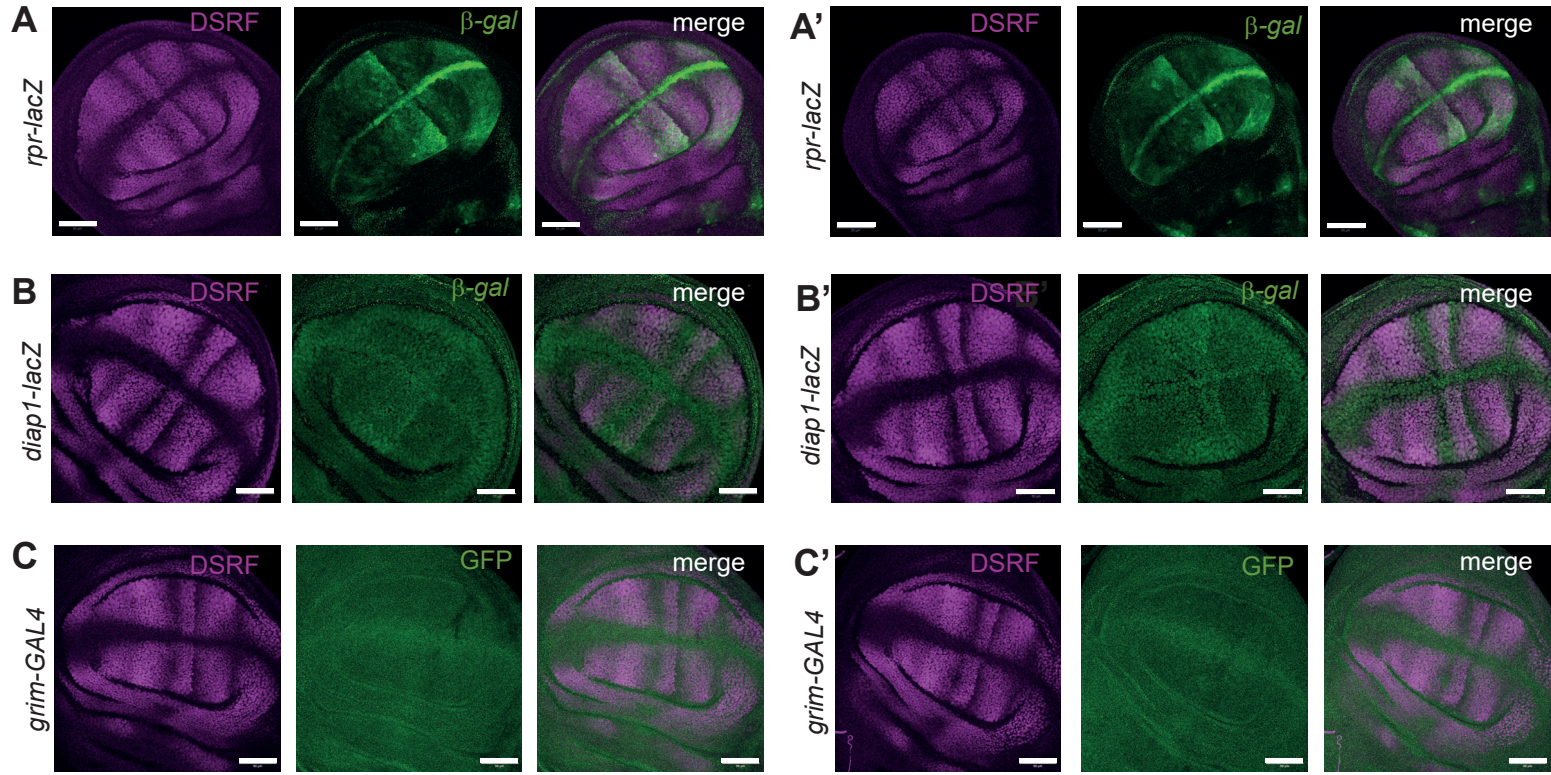
H99 and WT twin clones



**Figure 2: clone disappearance is spatially patterned in the developing wing.**

**A:** Rationale for inferring cell death events from twin-clone induction experiments. Upon twin-clone induction and 48 h of growth, the observation of twin clones composed of one GFP and one RFP clone is expected. However, the occurrence of a cell death event soon after clone induction will result in the occurrence of a single GFP or RFP clone without any twin counterpart in its vicinity. **B:** Spatial mapping for twin and single clone occurrences in the wing disc. Left, image of a wing disc at 96 h AEL stained with anti-DSRF and showing a single RFP clone (arrowhead) and a twin clone GFP-RFP (asterisk). Middle, manual segmentation of the tissue using the DSRF signal, and automated segmentation of the clones. Spatial map of the wing disc divided in 40 compartments (following the DSRF-based segmentation) into which clones positions are mapped. **C-D:** Spatial maps showing the pattern of single clone occurrences under different genetic conditions. In each case, the genotypes of the clones, the number of discs studied, the total number of mitotic recombination events, of twin clones, and of single clones observed are given. Colour scale of the heat maps shows the % of single clones observed in each compartment, as a proportion of the total number of mitotic recombination events assigned to this compartment ( $n$ ). For clarity, the compartments for which  $n < 10$  were shaded, since the inferred proportion from such low sample size are very unreliable. A: Anterior, P: Posterior, V: Ventral, D: Dorsal. **C-C':** Spatial map of single clone occurrences obtained by inducing twin clones using the twin spot QMARCM system, in control conditions (**C**) and by additionally expressing UAS-Dronc<sup>DN</sup> in the GFP clone (**C'**). **C** and **C'** share the same colour scale. **D:** Spatial map of single clone occurrences (% for each compartment) obtained by inducing twin clones using mitotic recombination and the H99 deletion (covering the pro-apoptotic genes *hid*, *grim* and *reaper*) with *arm-LacZ* allele to mark the sister WT chromosome, left scheme shows the  $\beta$ -gal signal associated with each genotype (dark: homozygous mutant, 2  $\beta$ -gal copies: WT, 1 copy: heterozygous). A representative wing disc is shown on the bottom left (green:  $\beta$ -gal). White stars show twin clones, dotted square and close up view an example of single *H99* mutant clone. Scale bar=50 $\mu$ m.

# Supplementary figure 2

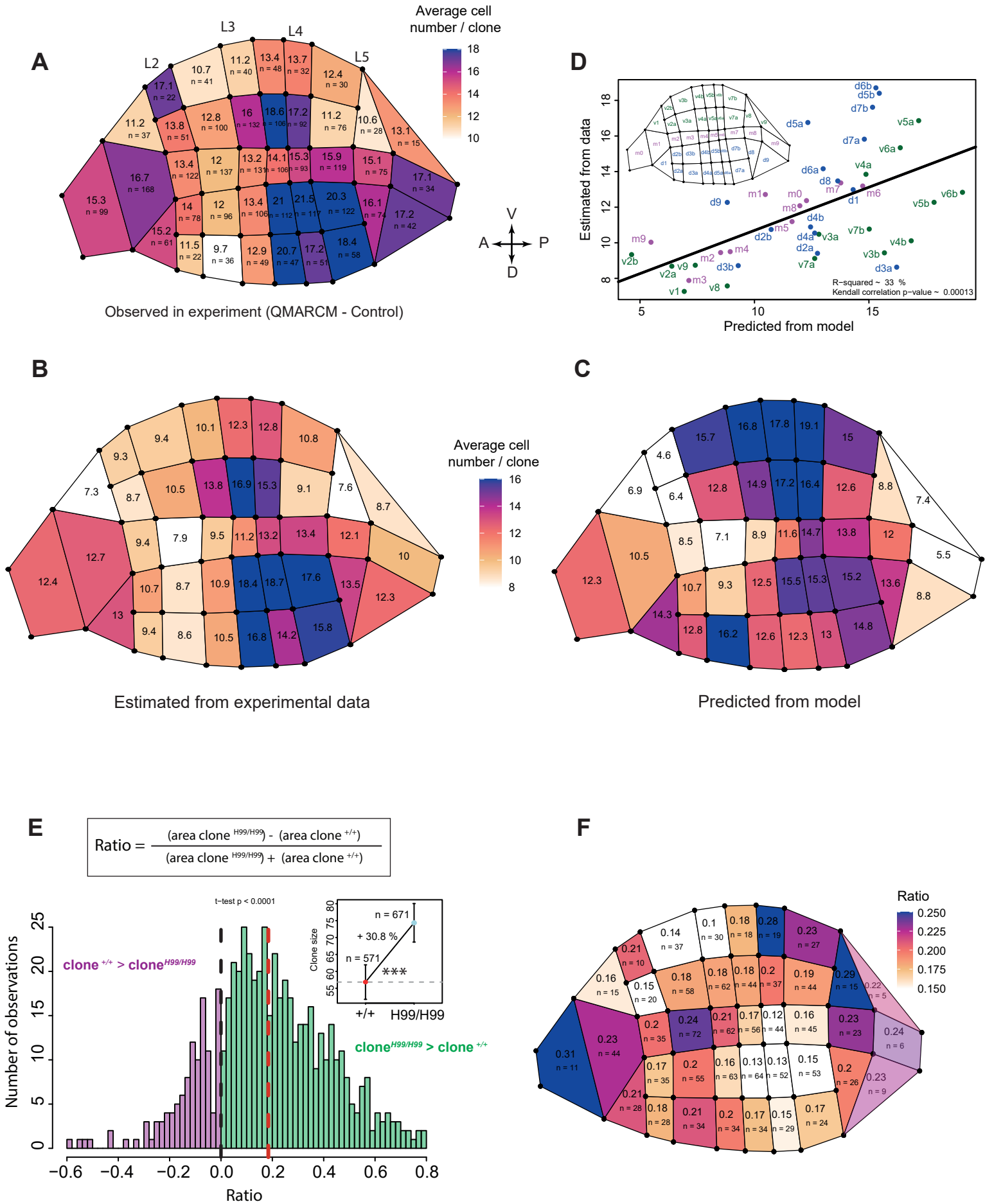




## Supplementary figure 2 (related to Figure 2)

**A, A'**: z-projection of two wing discs expressing *rpr-LacZ* reporter (green,  $\beta$ -gal staining) and stained with DSRF (magenta). **B, B'**: z-projection of two wing discs expressing *diap1-LacZ* reporter (green,  $\beta$ -gal staining) and stained with DSRF (magenta). **C, C'**: z-projection of two wing discs expressing *grim-GAL4 UAS-GFP* reporter (green, GFP signal) and stained with DSRF (magenta). Scale bars from **A** to **C'**=50 $\mu$ m. **D**: Distribution of twin-clone Ratio values for each of the two genetic conditions studied to establish the spatial map of single clone occurrences with QMARCM. For each mitotic recombination event, the computed ratio is the difference between areas of the green and red clones over the sum of these areas. Negative ratios indicate twin clones for which the red clone is bigger than the green one whereas twin clones where the green clone is bigger than the red one have positive values. Ratios values of -1 and +1 indicate mitotic recombination events where the green or the red clone were lost and have thus a null area. **E**: Frequency of the single colour clones for the 40 compartments in four genotypes (QMARCM blue, QMARCM Dronc<sup>DN</sup> red). A wing disc diagram shows the delimitation and names of the 40 compartments on which the quantifications were made. Error bars are s.e.m. **F**: Example of a wing disc obtained by inducing twin clones using mitotic recombination between *nls-GFP* and *arm- $\beta$ -gal*, stained for anti  $\beta$ -gal. A single plane is shown, processed with a gaussian blur filter 3D ( $x = 2, y = 2, z = 1$ ) and illustrating an example of a single clone  $\beta$ -gal 2x (inset i) and of a twin clone  $\beta$ -gal 2x/GFP 2x (inset ii). Scale bars: 50  $\mu$ m. **G**: Spatial map of single clone occurrences (% for each compartment) obtained by inducing twin clones using mitotic recombination between *nls-GFP* and *arm- $\beta$ -gal*. Colour scale shows the % of single clones observed in each compartment, as a proportion of the total number of mitotic recombination events assigned to this compartment (n).

**Figure 3**

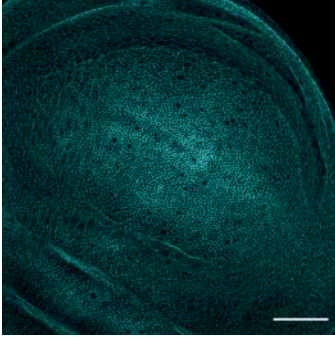


### Figure 3: Spatial variation of apoptosis modulates local growth and clone size

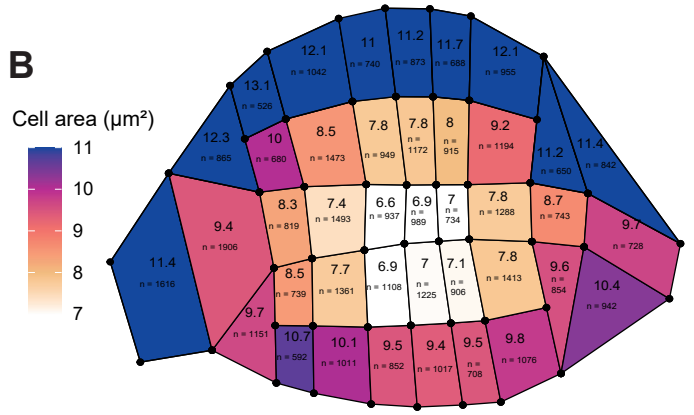
**A:** Spatial map for average cell number per clone in QMARCM – control experiment. Clones were considered irrespective of their colour. For each compartment, the average clone surface was calculated and divided a posteriori by the average cell apical size obtained in the same compartment to obtain an estimation of the averaged cell number per clone (see **Methods** and **Figure 3supp**). **B:** Spatial map of the mean value of clone size ( $\mu(t)$ , see **Methods**) which integrates the experimental average clone size as well as the 0 values (estimated from the proportion of single colour clone, see **Methods**). **C:** Spatial map of the averaged predicted number of cells per clone assuming a constant and homogenous growth rate throughout the pouch and heterogeneous rate of apoptosis estimated from the local proportion of single coloured clone (see **Methods**). **B** and **C** share the same colour scale bar. **D:** Correlation between the average cell number per clone predicted from the model and estimated from experimental data for each compartment. The black line is the linear regression. The R-squared of the regression and the p-value of a correlation test (Kendall) are shown. Each dot is the data for one compartment, coloured and named according to the map shown on the left side of the plot (blue: dorsal compartments d1 to d9; magenta: margin compartments m0 to m9; green: ventral compartments v1 to v9). **E:** Distribution of twin-clone area ratio values for each H99 mitotic clones (H99 homozygous mutant versus WT sibling clone). For each mitotic recombination event, the computed ratio is the difference between areas of the H99 and WT clones over the sum of these areas (top inset). Negative ratios: WT clone bigger than H99 clone, Positive ratios: H99 clone bigger than WT clone. Black dotted line shows 0, red dotted line the median of the distribution. Top right inset shows the bulk averaged clone size (30.8% bigger for H99 compared to WT,  $p < 0.0001$ ), dots averages, error bars 95% confidence intervals, n, number of clones. Note that single clones were not used for this comparison (which would have a value of +1 or -1). **F:** Spatial map for averaged clone size ratio for H99 and WT sibling clones (see in **E**) for each of the 40 compartments of 66 wing discs. Colour scale is shown on the right.

# Supplementary figure 3

**A**



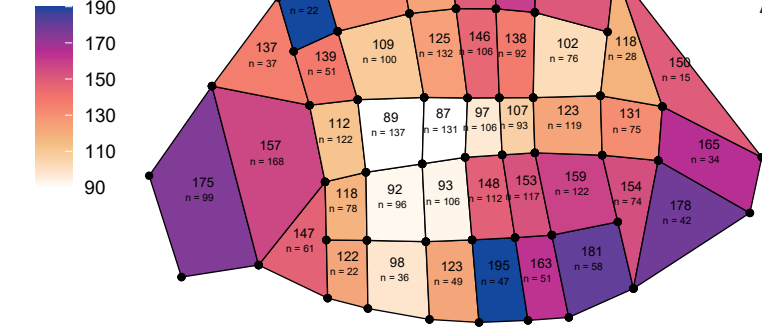
**B**



**C**

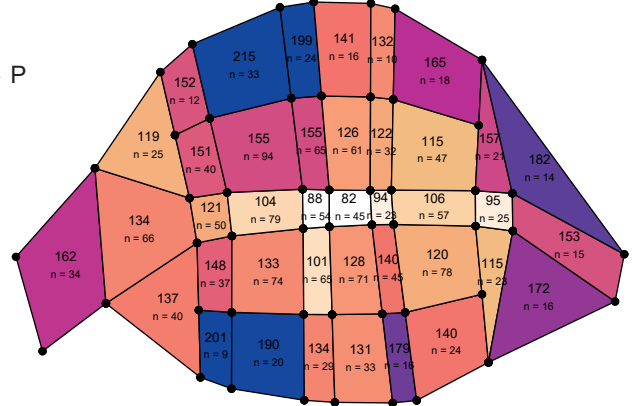
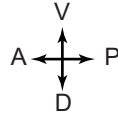
QMARCM - control (n = 96 discs)

Mean clone size ( $\mu\text{m}^2$ )



**D**

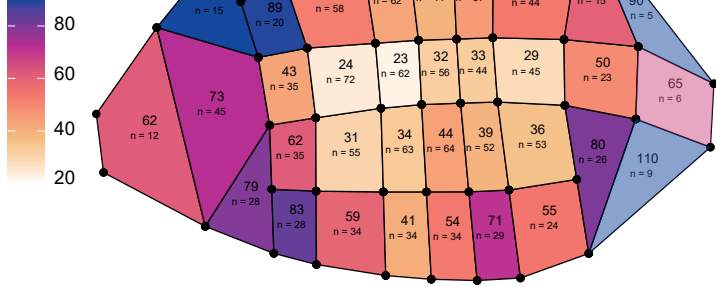
QMARCM - Dronc<sup>DN</sup> (n = 76 discs)



**E**

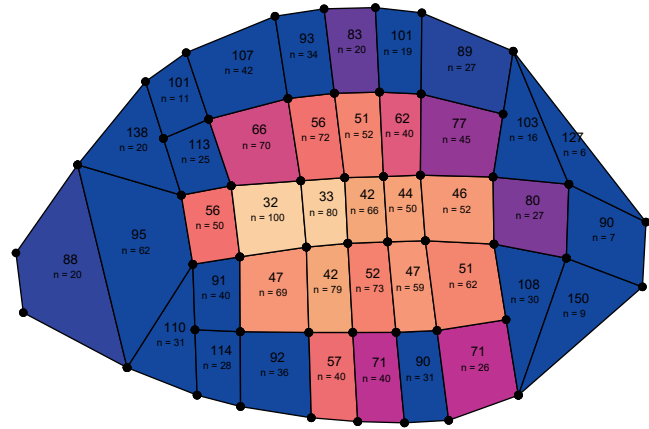
FRT -  $\beta$ -gal clones (n = 65 discs)

Mean clone size ( $\mu\text{m}^2$ )



**F**

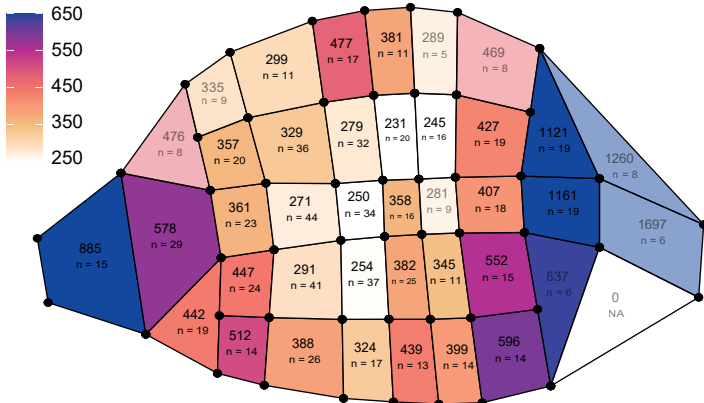
FRT - H99 clones (n = 65 discs)



**G**

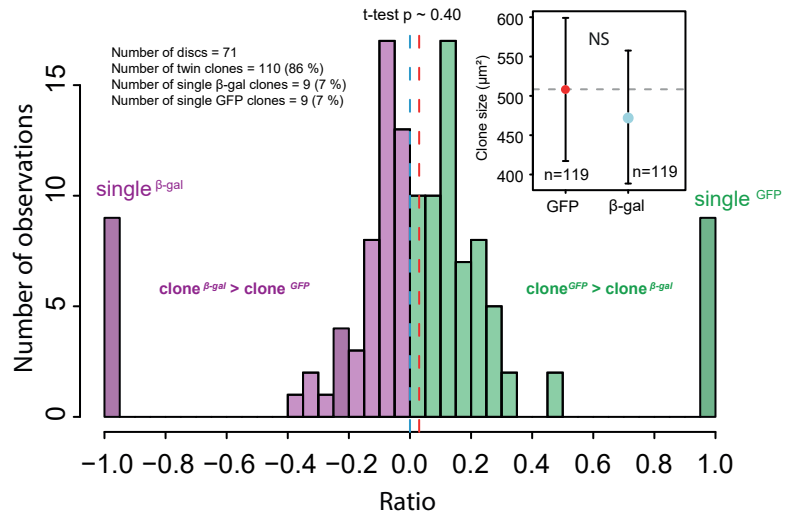
FRT - pooled GFP /  $\beta$ -gal clones (n = 71 discs)

Mean clone size ( $\mu\text{m}^2$ )



**H**

$$\text{Ratio} = \frac{(\text{area clone } GFP) - (\text{area clone } \beta\text{-gal})}{(\text{area clone } GFP) + (\text{area clone } \beta\text{-gal})}$$

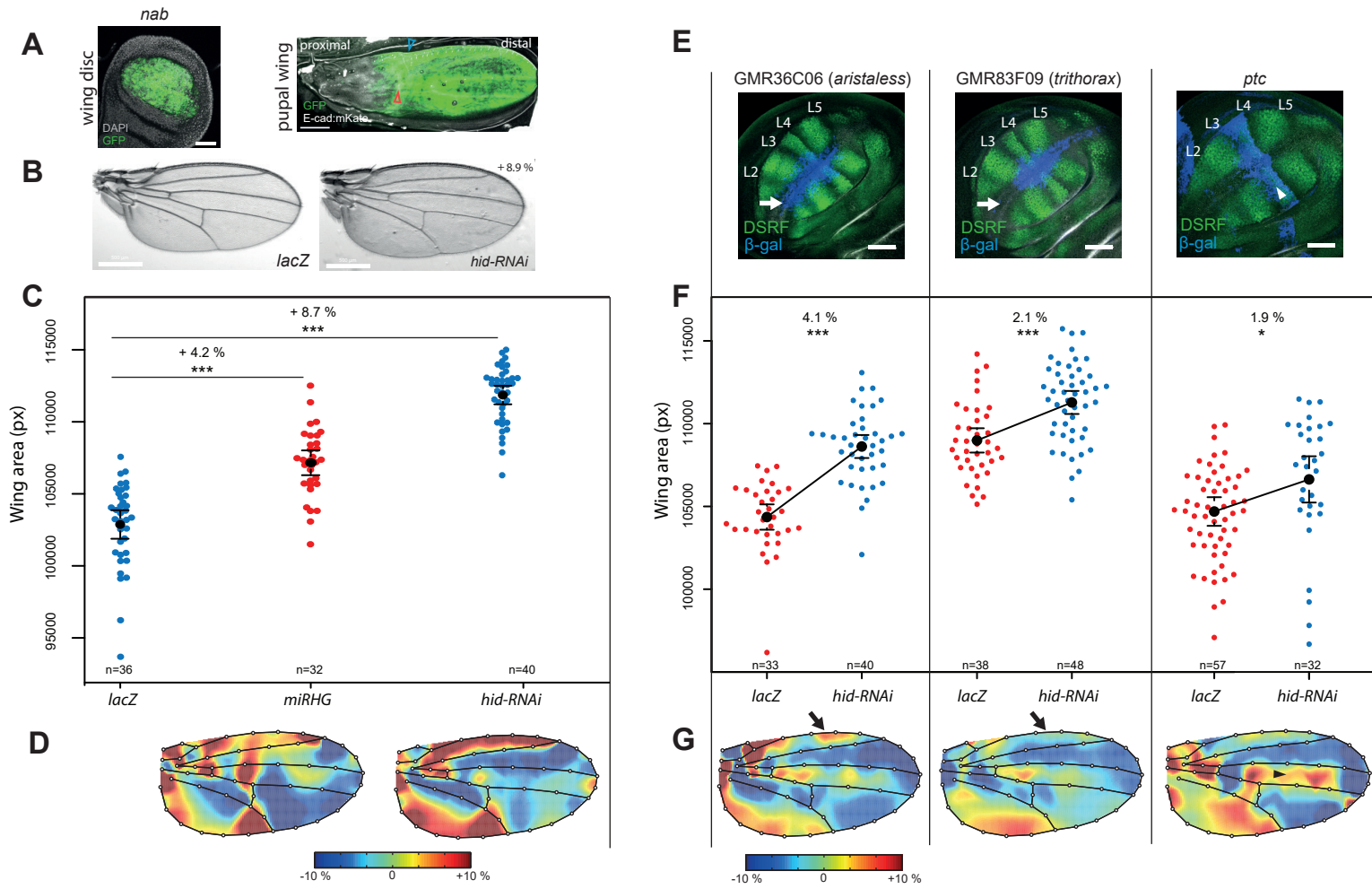


### Supplementary figure 3 (associated with Figure 3)

**A:** Example of a local projection of a wing disc stained with E-cad used for cell apical area measurements. Scale bar=50  $\mu\text{m}$ . **B:** Heat map showing the average spatial pattern for cell apical area from three discs. Within each compartment, the value for average cell size ( $\mu\text{m}^2$ ) and the number of cells measured (over the three discs) are given. Overall, the three discs had very similar patterns of spatial variation of cell apical area (not shown). **C-G:** Heat maps showing the average spatial pattern for clone surface area ( $\mu\text{m}^2$ ). For each compartment, the average surface area of the clones and the number of clones examined (n) are given. The data are shown for five conditions: QMARCM system, in control conditions (tub-QF, QUAS-RFP and tub-Gal4, UAS-GFP) (**C**) and upon expression of UAS-Dronc<sup>DN</sup> in the GFP clone (**D**); twin FRT clones H99/ $\beta$ -gal for  $\beta$ -gal clones (**E**) and H99 homozygous clones (**F**); neutral twin FRT clones GFPnls/ $\beta$ -gal for GFP and  $\beta$ -gal clones pooled together (**G**) Note that the colour scale is different between **C,D**; **E,F**; and **G**. Compartments for lower sample sizes ( $n < 10$ ) are shaded. Note that colour scales of heat maps were saturated to better display relevant local variations. **H:** Distribution of twin-clone area ratio values for neutral FRT GFPnls/ $\beta$ -gal mitotic clones (homozygous GFP versus homozygous  $\beta$ -gal sibling clone). For each mitotic recombination event, the computed ratio is the difference between areas of the GFP and  $\beta$ -gal clones over the sum of these areas (top inset). Negative ratios:  $\beta$ -gal clone bigger than GFP clone. Positive ratios:  $\beta$ -gal clone smaller than GFP clone. Blue dotted line shows 0, red dotted line the median of the distribution (not different from zero, t-test p-value  $\sim 0.40$ ). Top right inset shows the bulk averaged clone size (no difference between GFP and  $\beta$ -gal t-test p-value  $\sim 0.58$ ), dots averages, error bars 95% confidence intervals, n, number of clones. Note that values of +1 and -1 denote the presence of single clones (single GFP and single  $\beta$ -gal respectively).



**Figure 4**

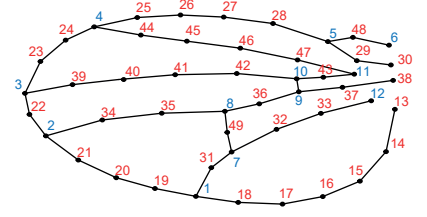
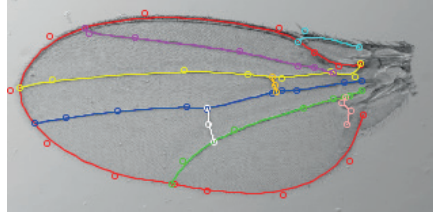
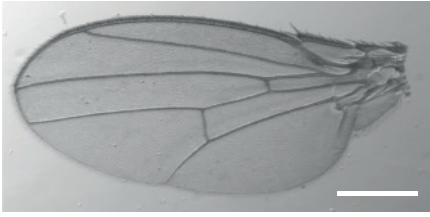


#### Figure 4: Apoptosis affects locally and globally wing size and shape.

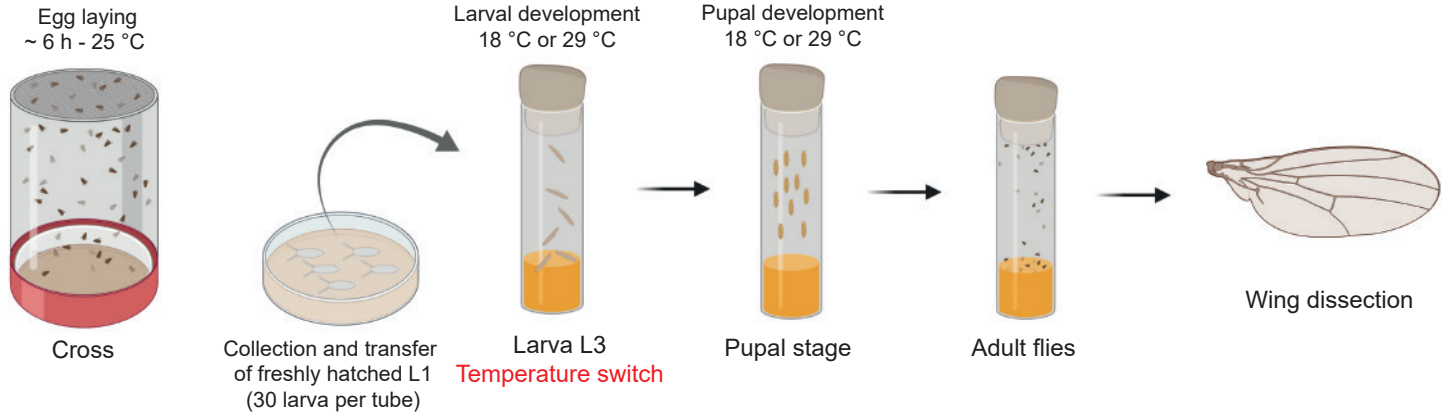
**A:** Pattern of expression at ~ 96 h AEL of the Nab-GAL4 driver used to express *hid-RNAi* at larval stage (left, GFP, green) and pupal stage (right, 30h APF, GFP, green, E-cad::mKate, magenta, red and blue arrowhead point at the hinge/blade boundary recognised by the accumulation of E-cad along antero-posterior axis (red arrowhead) and the infold on the anterior side (blue arrowhead). Scale bars, 50µm top, 200 µm bottom. **B:** Images of adult wings in the control (*Nab-GAL4, UAS-LacZ, Area=103661 px*) and upon downregulation of apoptosis by *hid* depletion (*Nab-GAL4, hid-RNAi, Area =112857px*). Wings are bigger and slightly rounder. The images shown are representative of the average size observed in control and *hid* downregulation groups and differ by 8.9 % in area. Scale bars, 500 µm. **C:** Variation of adult wing size in control (*UAS-LacZ*), upon expression of the microRNA targeting *hid*, *grim* and *reaper* (*miRHG*) and depletion of Hid (*hid-RNAi*) all with the Nab-GAL4 driver. Each dot is a wing. Black dots are means and error bars 95% confidence interval. n=number of wings. \*\*\*:  $p < 0.0001$ . **D:** Local variation of tissue shape caused by expressing *miRHG* or *hid-RNAi* relative to the control wing (*lacZ*). Colours represent changes in relative area necessary to transform the average wing from the control group to the perturbed group after alignment, rotation and size normalisation, red expansion, blue shrinkage (number of wings shown in **C**). Note that this comparison is done after rescaling the wings in order to compare variations of shape. Colour bar shows the upper and lower limits in deformation. Highest colour intensity is reached at 10% increase and decrease in local area. Left: proximal, right: distal, top: anterior, bottom: posterior. **E:** Pattern of expression in the wing disc at ~ 96 h AEL of various GAL4 drivers used to express *hid-RNAi*. Green, DSRF, blue, UAS-β-gal. L2,L3,L4,L5 show the different prospective veins. White arrows show the most anterior region of the disc, where GAL4 is expressed in the case of *aristaless-GAL4*, but not in the case of *trithorax-GAL4*. Scale bars, 50µm. Left: anterior, right: posterior, top: ventral, bottom: dorsal. **F:** Variation of adult wing size for the different genotypes. Each dot is a wing. Black dots are means and error bars 95% confidence interval. n=number of wings. \*\*\*:  $p < 0.0001$ , \*:  $p = 0.02$ . **G:** Local variations of tissue shape caused by expressing *hid-RNAi* relative to the control wing (*lacZ*) for each GAL4 driver. Colours represent changes in relative area necessary to transform the average wing from the control group to the perturbed group, red expansion, blue shrinkage (number of wings shown in **F**). Left: proximal, right: distal, top: anterior, bottom: posterior.

# Supplementary figure 4

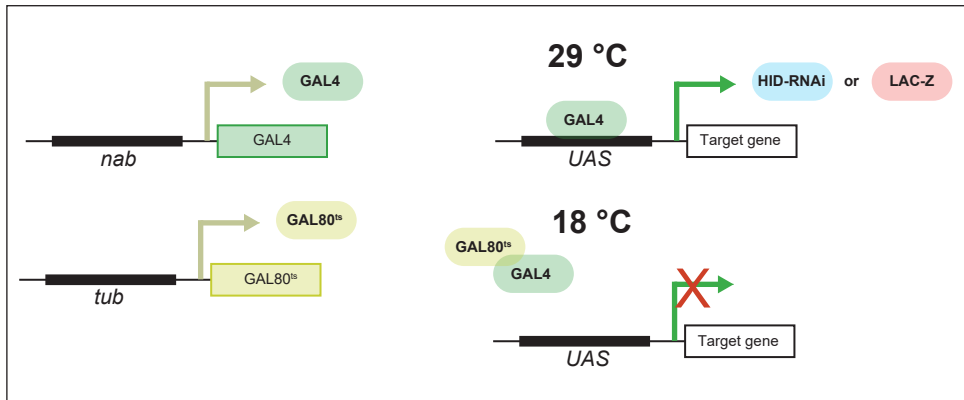
**A**



**B**



**C**



4 conditions

Condition	Larva	Pupa
No expression	18 °C	18 °C
Larval expression	29 °C	18 °C
Pupal expression	18 °C	29 °C
Larval + Pupal expression	29 °C	29 °C

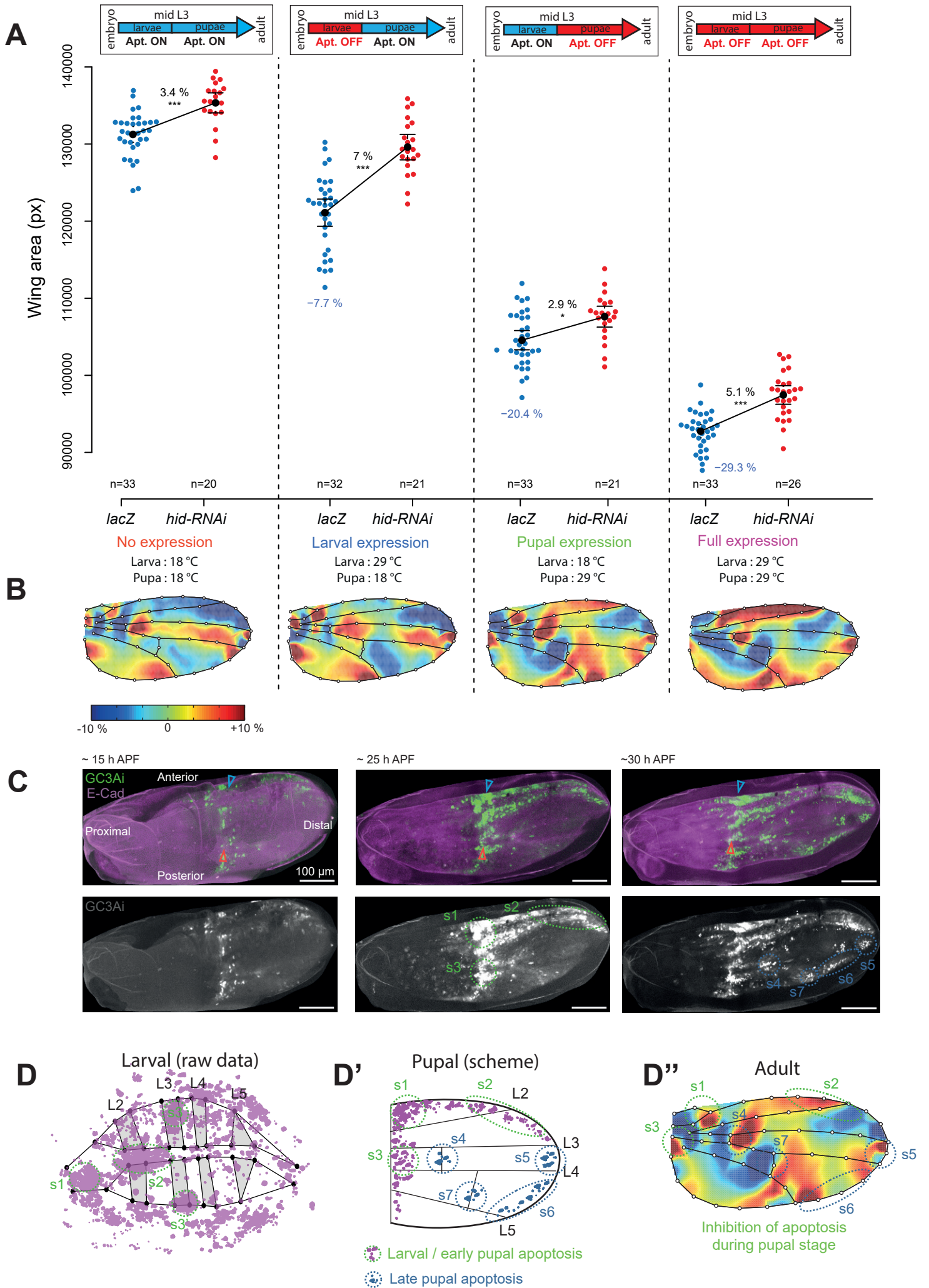
## Supplementary figure 4 (related to Figure 4 and Figure 5)

**A:** Adult wing segmentation pipeline. Left: wing example. Middle: the software Wings4 fits 9 spline curves (i.e., equations that give the location of the curve at any point between its end points) to veins and edges of the wing. Each spline is shown with a specific colour. Circles denote the positions of control points, which can be dragged manually by the user to maximise the overlap between the spline curves and veins and edges of the wing. Left: the program CPR extracts from the spline curves the positions of 12 landmarks (blue, numbered 1-12) and 36 semi-landmarks (red, numbered 13-48). Landmarks are strongly reliable points because they are located at the end of the curves. Semi-landmarks are less reliable because they are sampled along the curves by spacing them equally along each curve segment between each pair of landmarks.

**B:** Experimental design for conditional gene expression during early or late developmental stages. Flies were crossed in cages supplied with agar petri dishes and allowed to lay eggs for ~ 6 h. Freshly hatched larva were transferred to regular vials at a density of 30 individuals / tube, and placed at 18 °C or 29 °C. Once arrived at the L3 wandering stage, individuals were switched temperature (from 18 °C to 29 °C and vice-versa). Upon hatching flies were collected for wing dissection.

**C:** Rationale for conditional gene expression using tub-GAL80ts. GAL80ts has ubiquitous expression under the control of tubulin promoter whereas GAL4 expression is restricted to wing tissue under the control of GMR11F02-GAL4. At 18 °C, GAL80ts binds to the GAL4 and inhibits its activator function, precluding the expression of the target genes. At 29 °C Gal80ts is unable to bind GAL4, allowing GAL4 binding to the upstream activating sequence (UAS) and transcription of target genes (*lacZ* or *hid-RNAi*).

**Figure 5**



## Figure 5: Larval and pupal patterned apoptosis affects adult wing size and shape

**A:** Variation of adult wing size in control (*UAS-LacZ*) and upon depletion of Hid (*hid-RNAi*) with the Nab-GAL4 driver and conditional activation at different developmental stages using the thermosensitive GAL4 inhibitor GAL80<sup>TS</sup> and controlled temperature shift (29°C, Hid depletion, 18°C no Hid inhibition). See **Figure 4supp**. The % in black show that variation between the Hid RNAi and corresponding control. The % in blue show the variation of size for the control at a given temperature relative to the 18° control (no expression). The top schemes show the period of development during which apoptosis is inhibited. Each dot is a wing. Black dots are means and error bars 95% confidence interval. n=number of wings. \*\*\*:  $p < 0.0001$ , \*:  $p = 0.001$ .

**B:** Local variation of tissue shape caused by expressing *hid-RNAi* relative to the control wing (*lacZ*) for each temperature condition. Colours represent changes in relative area necessary to transform the average wing from the control group to the perturbed group, red expansion, blue shrinkage (number of wings shown in **A**). Highest colour intensity is reached at 10 % increase and decrease in local area. Left: proximal, right: distal, top: anterior, bottom: posterior.

**C:** Snapshots of z-local projections of a live pupal wing expressing UAS-GC3Ai (green) in the Nab-GAL4 domain (wing pouch) and E-cad::mKate between 16 and 32h APF (hours After Pupal Formation). See **Video S1**. Representative of 4 movies. s1 to s3 and green ellipses show early domains of apoptosis (already appearing at 16h APF), s4 to s7 and blue ellipses show late domains of apoptosis (appearing around 30h APF). **Blue and red arrowheads show the position of the margin between the blade and hinge, marked with a infold on the anterior side (blue arrowhead) and strong concentration of E-cad along AP axis (red arrowhead)**. Scale bars=100µm.

**D:** Averaged pattern of apoptosis in the wing disc (**Figure 1F**) and the corresponding early pupal apoptosis zones (s1 to s3, green ellipses). Anterior: left, posterior: right, ventral: top, dorsal: bottom.

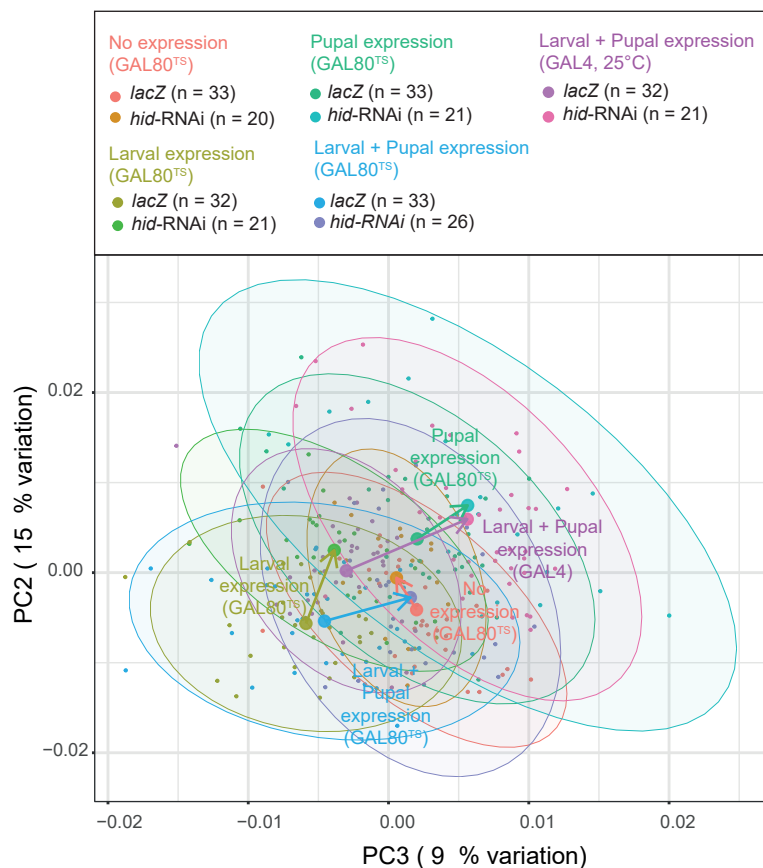
**D'**: Schematic of the apoptosis pattern observed in the pupal wing with early domains (s1 to s3, green ellipses) and late domains (s4 to s7, blue ellipses). Note the correspondence between early domains and the pattern of apoptosis observed in the wing disc (**D** magenta dots). Left: proximal, right: distal, top: anterior, bottom: posterior.

**D''**: Map of wing deformation associated with Hid inhibition at pupal stage (**5 B**, pupal expression), red expansion, blue constriction relative to the control discs, and the position of the pupal apoptosis zones (early, s1 to s3, green ellipses, and late, s4 to s7, blue ellipses). Left: proximal, right: distal, top: anterior, bottom: posterior.

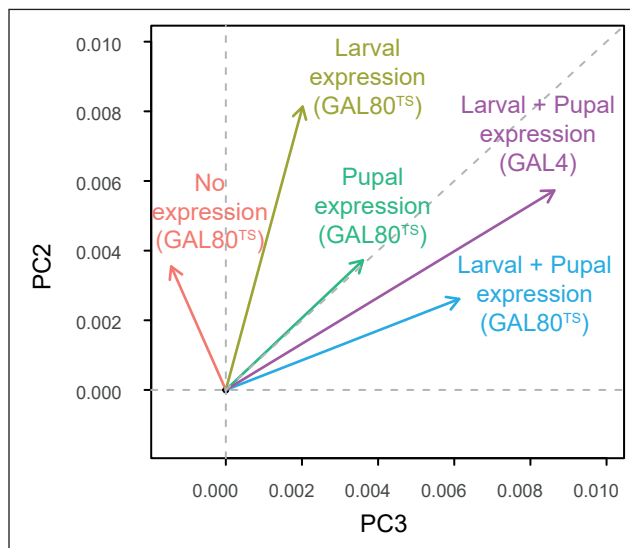


# Supplementary figure 5

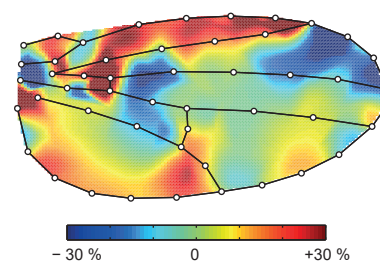
**A**



**B**

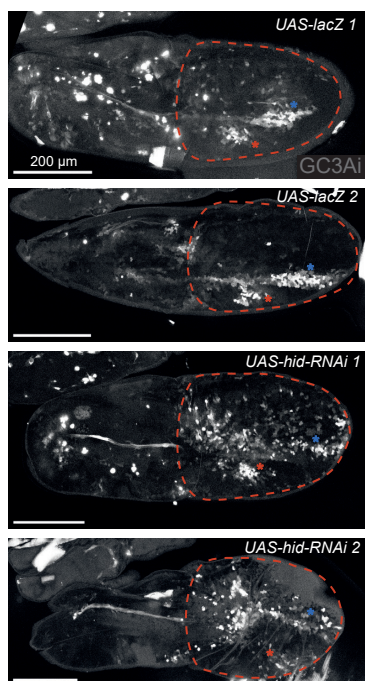


**C**



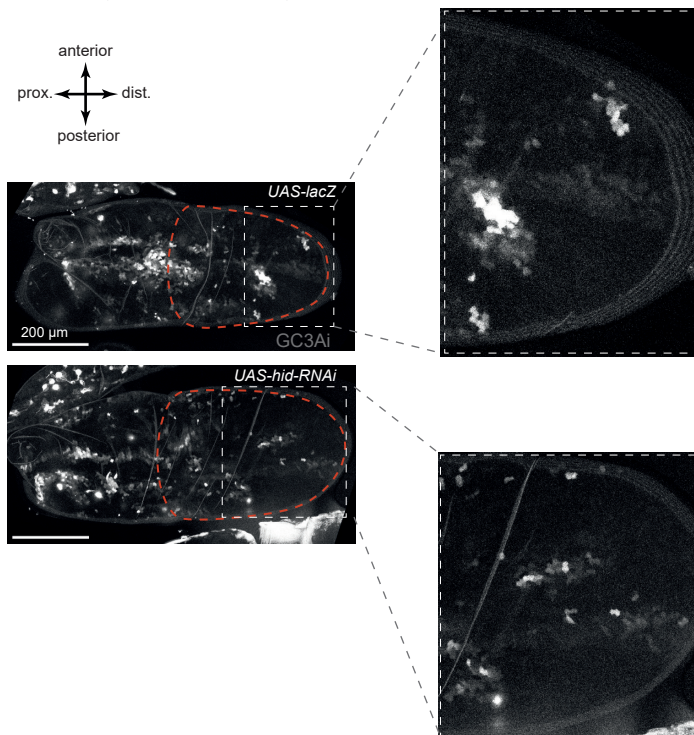
**D**

*act-lexA > lexAOP-GC3Ai, nab-GAL4, tub-GAL80<sup>TS</sup>*  
 Larval expression (larval 29 °C and pupal 18 °C)



**E**

*act-lexA > lexAOP-GC3Ai, nab-GAL4, tub-GAL80<sup>TS</sup>*  
 Pupal expression (larval 18 °C and pupal 29 °C)





## Supplementary figure 5 (related to Figure 5)

**A:** Principal Component Analysis of adult wing shape using the position of every landmark as inputs (see **Figure 4suppA**) comparing *Nab-GAL4, UAS-LacZ* and *Nab-GAL4, UAS-hid RNAi* with *Gal80<sup>TS</sup>* for different temperature switches (see **Figure 5**). The first principal component, which was mostly reflecting the variation driven by the temperature, was removed in order to focus on shape variation associated with the genotypes and *hid* depletion. Each dot represents one wing, the ellipses contain 95% of the data and bold dots are the median of each genotype (number of wings shown in the legend). Vectors represent the transformation in the morphospace to go from the control to the *Hid* depleted flies for a given temperature protocol. **B:** The same vectors represented separately. Variations along PC3 (x-axis) reflects the shape variation associated with *Hid* depletion, which is stronger upon full depletion (all developmental stages). Note that the sum of larval and pupal depletion variations along PC3 axis matches the amplitude of the full depletion along PC3 axis. **C:** Representation of the shape variation along PC3 showing the transformation required to go from the two extreme wings along PC3 (from low to high PC3) after scaling, rotation and translation. Red regions are expanding, blue regions are shrinking (max variations of +/-30%). Overall, movement toward the right on PC3 is associated with a rounding of the wing (expansion of the anterior and posterior sides, shrinkage of the most distal parts). Left: proximal, right: distal, top: anterior, bottom: posterior. **D:** Maximum z-projections of pupal wings expressing *lexAOP-GC3Ai* under the control of *act-lexA*. *UAS-lacZ* or *UAS-hid-RNAi* were conditionally expressed with *tub-GAL80<sup>TS</sup>* in the *nab-GAL4* domain during the larval stage, by growing the larva at 29 °C and shifting them at 18 °C mid-L3 stage. Acquisitions were done 48 h +/- 6 h after temperature shift. The presumptive blade domain is outlined with a dashed red line. Asterisks mark GC3Ai figures consistently observed in the two controls (*UAS-lacZ 1 and 2*). In *UAS hid-RNAi*, levels of GC3Ai were restored to levels qualitatively similar to the control. **E:** Maximum z-projections of pupal wings expressing *lexAOP-GC3Ai* under the control of *act-lexA*. *UAS-lacZ* or *UAS-hid-RNAi* were conditionally expressed with *tub-GAL80<sup>TS</sup>* in the *nab-GAL4* domain during the pupal stage, by growing the larva at 18 °C and shifting them at 29 °C at mid-L3 stage. Acquisitions were done 24 h +/- 6 h after temperature shift. The presumptive blade domain is outlined with a dashed red line. The most distal part of the *nab* domain (grey dashed rectangle) is enlarged to appreciate the overall reduction in GC3Ai levels (representative of 3 wings for each).

## Supplementary video legends

### **Video S1: Dynamics of apoptosis in the pupal wing (related to Figure 5)**

Local projection of a living pupal wing marked with E-cad::mKate (magenta) and expressing GC3Ai with the Nab-GAL4 driver (green and bottom) between 16h and 35h APF (After Pupal Formation). Anterior: top, Posterior: bottom, Proximal: left, Distal: right. Scale bar=100 $\mu$ m.