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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  
4 Alexis Matamoro-Vidal<sup>1</sup>, Tom Cumming<sup>1,2</sup>, Anđela Davidović<sup>3</sup> and Romain Levayer<sup>1\*§</sup>

5 1. Department of Developmental and Stem Cell Biology, Institut Pasteur, CNRS UMR  
6 3738, Université Paris Cité, Cell Death and Epithelial Homeostasis Unit, F-75015  
7 Paris, France

8 2. PPU program Institut Pasteur, Sorbonne Université, Collège Doctoral, F75005 Paris,  
9 France

10 3. Institut Pasteur, Université Paris Cité, Bioinformatics and Biostatistics Hub, F-75015  
11 Paris, France

12 \* Correspondance to: [romain.levayer@pasteur.fr](mailto:romain.levayer@pasteur.fr)

13 § Lead contact

## 14 **Abstract**

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

32 **roadmap for accurate assessment of the contribution of cell death to tissue**  
33 **shape, and outlines for the first time an important instructive role of cell death**  
34 **in modulating quantitatively local growth and the morphogenesis of a fast-**  
35 **growing tissue.**

36 **Keywords:** apoptosis, morphogenesis, growth, clone dynamics, quantitative mapping,  
37 *Drosophila*, wing imaginal disc

## 38 **Introduction**

39 The search of cellular mechanisms underlying variation in organ's size and shape is  
40 essential to our understanding of health and evolution. These mechanisms include  
41 changes in cell shape, cell proliferation, oriented cell division, oriented cell intercalation  
42 and also cell death [1]. Programmed cell death and apoptosis are indeed essential  
43 regulators of development and morphogenesis [2]. For instance, cell death is required  
44 to eliminate scaffolding tissues which are not present in the adult body. Apoptosis in  
45 epithelia is also a driving force of morphogenesis which can help to fuse tissues by  
46 generating pulling forces [3] or trigger fold formations by generating apico-basal  
47 traction forces [4, 5]. Finally, apoptosis has been associated with the buffering of  
48 developmental fluctuations which can eliminate miss-specified/miss-patterned cells  
49 through cell competition [6], or more recently, with the buffering of mismatch between  
50 the size of the tissue and the shape of morphogen gradients [7]. Interestingly, while  
51 the functions of apoptosis are numerous, its contribution to the regulation of organ size  
52 and growth, especially in tissues undergoing fast expansion, has been poorly studied.  
53 Accordingly, emphasis has mostly been given to cellular growth regulation including  
54 the increase of cell volume and cell proliferation [8-10]. Moreover, systematic and  
55 quantitative evaluations of the pattern and number of cell deaths remain relatively rare  
56 and tedious, especially in tissues which are not appropriate for long term live imaging  
57 [11]. As such, the exact pattern of cell death and its real contribution to the regulation  
58 of organ size and shape remain poorly documented in many situations. This also  
59 applies to the *Drosophila* wing imaginal disc, one of the best studied systems for  
60 understanding patterning and growth regulation [10, 12].

61 *Drosophila* wing imaginal discs are epithelial sacs composed of two epithelia: the  
62 peripodial cells (squamous epithelium) and a pseudostratified epithelium that will form  
63 the adult structures [13]. The disc is separated into domains that will give rise to the

64 adult wing (the wing pouch, or wing proper) and a distal region called the hinge which  
65 will connect the wing to the thorax. This wing pouch is patterned in different domains  
66 prefiguring adult wing structures: the pro-vein domains L2 to L5 and the dorsal-ventral  
67 boundary that will give rise respectively to the adult wing veins and the adult wing  
68 boundaries (**Figure 1A,B**). Wing imaginal discs undergo massive growth during larval  
69 development through several rounds of cell division (9 to 11 cycles) [14]. This growth  
70 phase is followed by profound remodeling and morphogenesis including tissue folding,  
71 elongation, and apposition of the two epithelial layers leading to the formation of the  
72 final adult wing shape (**Figure 1A,B**) [15], all this performed with a high degree of  
73 precision and reproducibility [16]. Decades of work sought to dissect the mechanisms  
74 controlling growth and size of the *Drosophila* wing, and have mostly focused on the  
75 distribution and regulation of cell proliferation while neglecting cell death [10]. Indeed,  
76 seminal works more than 20 years ago found that cell death is relatively minor in the  
77 wing disc and that it occurs sporadically without any noticeable pattern [17]. Yet, this  
78 did not exclude more subtle functions of cell death.

79 Accordingly, apoptosis was proposed to buffer developmental fluctuations to ensure  
80 reproducible wing size [18]. This was assumed to be based on cell competition: the  
81 context dependent elimination of suboptimal cells from growing tissues[19].  
82 Interestingly, while the studies of cell competition are mostly based on the analysis of  
83 mutant clone disappearance in the wing disc, we know virtually nothing about  
84 spontaneous clone disappearance during normal wing development and the  
85 prevalence, localisation and functions of physiological cell competition.

86 Here, we performed systematic mapping of apoptosis using a live marker of caspases  
87 and spatial registration in the larval wing imaginal disc. Unexpectedly, we found striking  
88 reproducible biases in the distribution of cell death which outlined hot-spots of  
89 apoptosis. These hot-spots correlate with a local increase of clone disappearance  
90 probability and significantly reduce local net-growth in the wing disc. Using  
91 morphometric analysis, we further demonstrate that these hot-spots can tune adult  
92 wing size and shape. Altogether, we reveal that apoptosis cannot be neglected for the  
93 study of growth/size and is also an essential modulator of local shape and growth even  
94 in a fast-expanding tissue. Our study also proposes a roadmap for a more systematic  
95 characterisation of the contribution of cell death to clone dynamics and tissue growth.

## 96 Results

### 97 Caspase activity is spatially biased in the larval wing pouch

98 We first systematically evaluated the distribution of apoptotic cells in the *Drosophila*  
99 wing disc at the larval wandering stage (96 h After Egg Laying - AEL). We used the  
100 live effector caspase reporter GC3Ai [20] driven by a wing specific driver (GMR11F02-  
101 GAL4, **Figure 1C**). The brightest GFP spots revealed by the reporter correspond to  
102 cell debris located on the basal sides, thus staining cells which already extruded ([20],  
103 **Figure 1D**). We confirmed the accuracy of the GC3Ai marker as we obtained a similar  
104 pattern with cleaved caspase3 staining (cleaved DCP1), although this was less  
105 sensitive and fails to mark the cells with faint GC3Ai signal (**Figure 1suppE-G**). To  
106 obtain an averaged map of the spatial distribution of cell death, we used spatial  
107 landmarks, whose positions were defined by the intervein marker *Drosophila* Serum  
108 Response Factor (DSRF, **Figure 1D,E**). The landmarks positions were used to divide  
109 every dissected wing disc into 40 sub-compartments (**Figure 1suppA-D**). In addition,  
110 we applied procrustes transformation (translation, rotation and scaling) on landmarks  
111 positions to align the discs and superimpose GC3Ai spatial data from many individuals  
112 on a single image (see **Methods**). The averaged distribution of apoptotic cells revealed  
113 a striking non-homogenous distribution of apoptosis with the strongest “hot spot”  
114 located in the anterior - dorsal quadrant, near the dorsal-ventral boundary (**Figure 1**  
115 **F,G**). This bias is not driven by the driver or any side effect of GC3Ai as a similar pattern  
116 was obtained in the *w* background with cleaved caspase3 staining (**Figure 1suppH-**  
117 **J**), and with GC3Ai driven by another GAL4 (*nubbin-gal4*, **Figure 1suppK**). To check  
118 whether the spatial bias of GC3Ai signal persists during wing disc development, we  
119 also monitored GC3Ai-positive cells in the early L3 wing disc (72h AEL). As we could  
120 not use the same spatial landmarks (the intervein regions are not yet defined at this  
121 stage) we instead used the location of the 4 compartments defined by AP and DV  
122 compartment boundaries (Anterior, Posterior, Dorsal and Ventral) as a spatial  
123 reference. The number of apoptotic cells was more than two times higher in the  
124 anterior-dorsal compartment than in the others (**Figure 1J-L**). This suggested that the  
125 spatial bias emerges at least during early L3 stage and persists for more than 24 hours.  
126 We then checked whether this pattern was indeed dependent on core regulators of  
127 apoptosis/caspases. Co-expression of GC3Ai with a dominant negative allele of Dronc  
128 (*Drosophila* caspase9) significantly reduced the amount of GC3Ai signal and reduced

129 the spatial bias (**Figure 1H, Figure 1supplL-N, R**). Similarly, depletion of the pro-  
130 apoptotic gene *hid* by RNAi in the wing pouch almost completely abolished the GC3Ai  
131 signal (**Figure 1I, Figure 1supplO-Q, R**). This suggests that the spatial bias is indeed  
132 caspase-dependent and most likely relies on the expression of the pro-apoptotic gene  
133 *hid*. Accordingly, we found a slight but systematic and reproducible increase of *hid*  
134 expression near the GC3Ai hot spot region using a GFP insertion at the *hid* locus  
135 (**Figure 1M,N**). Note however that other factors may also contribute to the spatial bias  
136 as we still observed some heterogeneity in cell death distribution upon Dronc inhibition  
137 or *hid* depletion (**Figure 1supplN, Q, S**).

138 Altogether, we conclude that contrary to what was previously observed [17], apoptosis  
139 is not spatially homogeneous in the wing imaginal disc and that some regions (anterior  
140 and close to the DV boundary, dorsal compartment) undergo higher rates of apoptosis.  
141 These hot-spots are in part explained by a spatial bias in the expression of the pro-  
142 apoptotic gene *hid*.

### 143 **The spatial bias in caspase activity influences clonal disappearance and local** 144 **net-growth.**

145 We next checked whether this spatial bias in apoptosis could have any consequence  
146 on clonal dynamics and local growth. Since ex-vivo live imaging of the wing disc does  
147 not allow long-term tracking of cell fate and local growth, we used an alternative  
148 approach based on twin-clone labelling. We first used the QMARCM technique [21] to  
149 stain the two daughter cells generated after mitotic recombination and their progeny  
150 with different fluorescent markers using alternate sets of transcription factors (Gal4 or  
151 the Qsystem, **Figure 2A,B**). Since cell movements and cell-cell intercalations are  
152 relatively neglectable in the wing disc [22, 23], the spatial proximity of patches of cells  
153 marked with GFP and RFP can be unambiguously attributed to a twin-clone in  
154 conditions of low frequency of clone induction. We assessed the probability of clone  
155 disappearance by scoring the number of single-coloured clones which are not in the  
156 vicinity of the other sibling clone 48h after clone induction. Such an observation can  
157 only be explained by the early disappearance of the lineage of the other daughter cell,  
158 and thus identifies a clone disappearance event (**Figure 2A**). We first noted that the  
159 QMARCM system has an intrinsic bias towards smaller clones and higher probability  
160 of clone disappearance for GFP/Gal4 clones relative to RFP/QF ones (**Figure**  
161 **2suppA**). This however should not preclude the analysis of potential spatial biases. By

162 multiplexing a large number of discs and using the same spatial landmarks as those  
163 used in **Figure 1suppC (Figure 2B)**, we obtained a coarse-grained spatial map of the  
164 probability of clone disappearance (**Figure 2C**). Strikingly, we observed that similar to  
165 caspase activation in the tissue, the probability of clone disappearance is not spatially  
166 homogenous. We identified clear hot-spots of clone death, including the anterior-dorsal  
167 region near the DV boundary (~two-fold increase compared to posterior compartments  
168 at similar DV positions, **Figure 2C, 2suppB**), as already outlined by the caspase  
169 positive cells mapping (**Figure 1G**). Note that this clone strategy reflects the pattern of  
170 apoptosis at earlier stages of development compared to the one revealed with GC3Ai.  
171 A similar pattern was observed using an alternative twin-clone method (twin-spot  
172 MARCM [24], using RNAi depletion of RFP and GFP via a GAL4 expressed in the wing  
173 pouch, **Figure 2D, 2suppB**) suggesting that the pattern of clone disappearance is not  
174 specific to the clone induction system. Importantly, clone size was also on average  
175 smaller (estimated through total apical surface and number of cells) in regions showing  
176 high rates of apoptosis and clone disappearance (**Figure 3A, Figure 3suppC,E**). This  
177 suggested that spatial biases in caspase activity not only modulate the probability of  
178 clone survival, but also the cumulative local growth in the wing disc. Indeed, these local  
179 differences in clone size match the differences numerically estimated from an  
180 exponential growth model with no spatial difference in proliferation rate and including  
181 the spatial differences in apoptotic rates calculated from our measurements of clone  
182 disappearance (see **Methods, Figure 3B-D**). This indicates that local differences in  
183 clone size could be solely explained by spatial differences in apoptotic rates. To check  
184 whether these spatial differences are indeed driven by caspases, we repeated the  
185 QMARCM clonal assay upon inhibition of caspase in the Gal4 sibling clones (using  
186 UAS-Dronc<sup>DN</sup>). While this did not totally abolish clone disappearance, the spatial  
187 pattern was flattened with no visible hot-spot of clone disappearance in the anterior  
188 side (**Figure 2C', 2suppB**). The spatial pattern of clone disappearance was also  
189 affected upon global inhibition of *hid* by RNAi with the twin-spot MARCM assay (**Figure**  
190 **2D', 2suppB**). These two approaches also lead to more homogenous clone size  
191 between anterior and posterior compartments in the wing disc (**Figure 3suppD,F**).

192 Altogether, we conclude that the spatial bias in *hid* expression and caspase activity  
193 generates a hot-spot of clone disappearance which also significantly reduces local  
194 growth rate mostly in the anterior-dorsal region near the DV boundary. This suggests

195 that local increases of caspase activity could play an instructive role in significantly  
196 modulating local growth, which therefore may affect the final shape and size of the  
197 tissue.

### 198 **Patterned caspase activity modulates adult wing shape and size**

199 We therefore checked whether local biases in caspase activity could significantly  
200 impact adult wing shape and size. To obtain a precise and quantitative description of  
201 adult wing shape and size, we used a quantitative assay based on semi-automatic  
202 wing segmentation, landmark positioning and procrustes alignment of wings (which  
203 includes translation, rotation and global rescaling) [25, 26] (**Figure 4suppA**).  
204 Interestingly, inhibition of cell death in the wing tissue using *hid* RNAi led to a significant  
205 increase in wing size (**Figure 4B**, 5.5%, a similar range to what was obtained upon  
206 ectopic overexpression of Dp110/PI3K [27]), suggesting that apoptosis has a net  
207 negative effect on adult tissue size. We also observed significant changes to adult wing  
208 shape, including a global increase in the size of the most anterior and posterior  
209 domains, as well as a global wing rounding (**Figure 4C,E,F**). To check whether local  
210 increases in apoptosis were indeed responsible for the local modulation of shape and  
211 size, we used various drivers to inhibit apoptosis in different wing subdomains (**Figure**  
212 **4A**). Accordingly, inhibition of *hid* in the patched domain (*ptc-gal4*, expressed in a band  
213 near the AP compartment boundary, **Figure 4A**) led to a global increase of wing size  
214 dominated by the expansion of the region overlapping the *ptc* domain (**Figure 4B,C**).  
215 This suggested that apoptosis has a local impact on growth even in regions other than  
216 the apoptosis hot-spots identified at larval stage. We also used drivers restricted to the  
217 DV boundary either in the anterior (encompassing the caspase hot-spot, **Figure 1G**)  
218 or posterior part of the wing disc (**Figure 4A**). Interestingly, while *hid* depletion in the  
219 posterior-DV boundary domain had a mild effect on wing size (similar to inhibition of  
220 *hid* in the *ptc* domain), this effect was enhanced upon inhibition in the anterior-DV  
221 boundary domain reaching differences close to the one observed upon inhibition of *hid*  
222 in the full wing pouch (4.1% versus 5.5% increase, **Figure 4B**). This suggests that  
223 apoptosis around the anterior DV margin (the strongest hot-spot of apoptosis) can  
224 account for a significant proportion of the effect on adult wing size. Importantly, the  
225 expansion of the most anterior domain of the wing was only observed upon inhibition  
226 of *hid* in the anterior-DV boundary domain (**Figure 4C**), suggesting again that  
227 apoptosis has a local effect on size. The observed effects on adult size might also be

228 due to changes during the pupal stage. Therefore we also performed experiments with  
229 conditional *hid* depletion to assess the relative contribution of *hid* expression at larval  
230 stage versus pupal stage to the final wing shape (**Figure 4suppB,C**). While depletion  
231 of *hid* starting at early-mid pupal stage only had a minor effect on wing shape (**Figure**  
232 **4D, 4suppE**), *hid* depletion from early larval stage to early pupal stage was sufficient  
233 to recapitulate the rounding of the wing and the main shape modulation observed upon  
234 *hid* depletion throughout development (**Figure 4D-F, 4suppE**). Note that we could not  
235 use this context to analyze the effect on absolute size, as it was completely dominated  
236 by the well-documented effect of temperature on wing size [28, 29] (**Figure 4suppD**).

237 To have a more quantitative description of shape changes induced by these genetic  
238 backgrounds, we used Principal Components Analysis (PCA) applied to the 48  
239 landmarks defined by the wing segmentation (**Figure 4suppA**). The combinatorial  
240 effect of *hid* depletion during larval and pupal stages is well reflected by this PCA  
241 analysis (**Figure 4D**), which can decompose the total effect of *hid* depletion throughout  
242 development (“full expression”, purple arrow) into a strong effect coming from the larval  
243 stage along Principal Component 1 (“early expression”, blue arrow, along PC1) and a  
244 mild effect during pupal stage (“late expression”, green arrow, mild effect along PC2).  
245 Accordingly, PC1 mostly reflects the rounding of the wing (**Figure 4E**, accounting for  
246 61% of the variation in the PCA analysis) while PC2 mostly relies on the shift of the  
247 posterior cross-vein (**Figure 4E**, accounting for 13% of the variation of the PCA  
248 analysis). The effect of this temporal depletion, combined with the wing pouch  
249 specificity of the other drivers strongly argue for the important role of *hid* expression  
250 pattern in the pouch during larval development on adult wing shape.

251 Altogether, we conclude that spatial biases in apoptosis distribution in the growing wing  
252 disc can significantly modify the shape of the adult wing by locally reducing net growth.  
253 This suggests that the fine spatial tuning of apoptosis in a fast-growing tissue plays an  
254 instructive role for organ shape and size regulation.

## 255 **Discussion**

256 In this study, we outlined an unexpected pattern of apoptosis in the growing wing  
257 imaginal disc with a significant upregulation in the anterior and dorsal compartment of  
258 the wing. To our knowledge this is the first demonstration that apoptosis is spatially  
259 biased in the wing imaginal disc. Moreover, our quantitative assessments of clonal

260 growth and adult wing shape clearly show that this local upregulation of apoptosis has  
261 a significant impact on local net growth and final adult shape. So far, the  
262 characterisation of cell death has never been performed in a systematic manner using  
263 spatial landmarks allowing superimposition of data from many individuals, which may  
264 explain why such biases have been missed [17]. Similarly, the impact of apoptosis on  
265 tissue shape and size has never been studied with such quantitative readouts. More  
266 generally, the assessment of apoptosis levels and distributions is usually based on  
267 cleaved caspase3 and TUNEL staining [11]. However, in conditions where the lifetime  
268 of apoptotic debris is relatively short, these assays may easily miss potential patterns  
269 of interest and may underestimate the real contribution of apoptosis. Our work  
270 emphasises the need for more systematic quantitative assessments of death  
271 distribution in order to reach solid conclusions about the contribution of apoptosis to  
272 organ shape/size regulation. The pipeline used in this study may easily be applied in  
273 other developmental contexts which are not amenable for long term live imaging, as  
274 long as markers can be used for spatial registration and tools are available for clone  
275 generation.

276 Compensatory proliferation is one of the best studied processes that relates apoptosis  
277 to the induction of cell proliferation [30]. So far, this process was mostly characterised  
278 either in conditions of massive death induction through irradiation and genetic induction  
279 of apoptosis in large domains [31, 32], or through the perturbation of the core apoptotic  
280 pathway (e.g.: by blocking some of the essential caspases, caspase3 in  
281 *Drosophila*[33], or caspase9 in mammalian epidermis[34]). However, to our knowledge  
282 there is no study that has clearly identified biases in cell proliferation distribution in the  
283 vicinity of physiological apoptosis *in vivo*. Surprisingly, we observed a net negative  
284 effect of the local increase of cell death on local growth and the size of the final adult  
285 compartment, and also outlined a significant effect of physiological death on the final  
286 size of the adult wing (**Figure 2** and **Figure 4**). This suggests that compensatory  
287 proliferation is unlikely to occur in the context of physiological apoptosis, or at least that  
288 its contribution is relatively minor and not sufficient to compensate for cell loss by  
289 apoptosis. Further quantitative studies of the coupling between apoptosis and cell  
290 proliferation will be essential to assess its real contribution to physiological growth  
291 regulation. Interestingly, a recent study outlined a significant positive upregulation of  
292 proliferation near dying MDCK cells, however this effect is strongly context-dependent

293 and is not visible at low stiffness or high density values[35], conditions that may apply  
294 to the wing imaginal disc.

295 Cell competition is the process describing the context-dependent elimination of viable  
296 but suboptimal cells [6]. Studies of clonal elimination in the wing imaginal disc have  
297 thoroughly contributed to our understanding of this process. Interestingly, most of  
298 these studies assumed that clone disappearance in physiological conditions is largely  
299 neglectable. Our results suggest that a significant proportion of WT clones may  
300 undergo early elimination in the wing imaginal disc, and that this elimination is spatially  
301 biased. On the one hand, this opens the possibility for spontaneous competition  
302 occurring in the wing imaginal disc, which so far has not been thoroughly explored  
303 (although its existence has been suggested by indirect genetic evidence [7, 18, 36]).  
304 On the other hand, it also suggests that cell elimination during competition may be  
305 influenced by this spatial pattern of caspase activity and apoptosis. Further quantitative  
306 description of clonal elimination during competition may reveal such spatial bias and  
307 would help to study the influence of pre-existing patterns of caspases/apoptosis on cell  
308 competition and tissue plasticity.

309 In this study, we showed that local modulation of *hid* expression can fine-tune the local  
310 level of apoptosis which will impact local growth and adult tissue shape in a subtle and  
311 quantitative way, similarly to the phenotypic changes observed at the  
312 macroevolutionary scale [37]. As such, the evolution of the cis-regulatory elements of  
313 pro-apoptotic genes may constitute an additional lever for shape evolution that could  
314 be used to fine tune adult appendage shape. Interestingly, these pro-apoptotic genes  
315 are most likely less pleiotropic than pro-proliferative pathways such as morphogens,  
316 RTK signaling pathways, or Hippo pathways that are classically studied for the  
317 regulation of size and growth [10]. As such, modulating pro-apoptotic genes levels and  
318 pattern of expression may constitute a relatively parsimonious way to evolve wing and  
319 appendage shape.

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### 333 **Authors contribution**

334 RL and AMV discussed and designed the project and wrote the manuscript. TC  
335 performed part of the experiments on adult wing shape and twin clones analysis as  
336 well as the wing disc segmentation. AD provided the theoretical calculation of the  
337 expected distribution of clone size based on differences in apoptotic rates. AMV  
338 performed all the other experiments and analysis. Every author has commented and  
339 edited the manuscript.

### 340 **Declaration of interests**

341 The authors declare no competing interest

### 342 **Methods**

#### 343 **Resource availability**

##### 344 *Lead contact*

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

##### 347 *Material availability*

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

##### 350 *Data and Code availability*

351 All code generated in this study and the raw data corresponding to each figure panel  
 352 (including images) can be shared upon request and will be uploaded soon to a  
 353 repository.

### 354 **Experimental model and subject details**

#### 355 *Drosophila melanogaster* husbandry

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

#### 366 *Drosophila melanogaster* strains

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

Fly line	Chromosome location	Origin (citation)	RRID
<i>UAS-GC3Ai/TM6b</i>	III	[20], Magali Suzanne	none
<i>w; UAS-GC3Ai/Cyo ; MKRS/TM6b</i>	II	Bloomington and [20]	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	[38], 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

368 **Table 1** : description and origin of the *Drosophila melanogaster* strains used in this  
369 study.

370

371 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>ΔGFP</sup>/TM3</i>	-	96h AEL
1supp A-G, R,S	<i>w; + ; GMR11F02-gal4/UAS-GC3Ai</i>	-	96h AEL
1supp H-J	<i>w<sup>118</sup></i>	-	96h AEL
1supp K	<i>UAS-dicer2; nubbin-gal4/+ ; UAS-GC3Ai/+</i>	-	96h AEL
1supp L-N,R,S	<i>w; UAS-dronc<sup>DN</sup>/+ ; GMR11F02-gal4/UAS-GC3Ai</i>	-	96h AEL
1supp O-Q, R,S	<i>w; UAS-GC3Ai/+ ; GMR11F02-gal4/UAS-hid-dsRNA</i>	-	96h AEL
2B, 2C, 2supp A,B	<i>yw hs-flp22, UAS-GFP/+; ET40-QF, QUAS-tdTomato/Cyo-If; FRT82B, tub-QS/FRT82B, tub-gal80</i>	8 min hs, 48h ACI	96h AEL
2C', 2supp A,B	<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
2D, 2supp A,B	<i>yw hs-flp22; UAS-mCd2RFP , UAS-dsRNACD8 FRT40A/UAS-mcd8GFP, UAS-dsRNACD2, FRT40A; GMR11F02-gal4/+</i>	10 min hs, 48h ACI	96h AEL
2D', 2supp A,B	<i>yw hs-flp22; UAS-mCd2RFP , UAS-dsRNACD8 FRT40A/UAS-mcd8GFP, UAS-dsRNACD2, FRT40A; UAS-hid dsRNA/GMR11F02-gal4</i>	10 min hs, 48h ACI	96h AEL
3A-D	<i>yw hs-flp22, UAS-GFP/+; ET40-QF, QUAS-tdTomato/Cyo-If; FRT82B, tub-QS/FRT82B, tub-gal80</i>	8 min hs, 48h ACI	96h AEL
3supp A-C	<i>yw hs-flp22, UAS-GFP/+; ET40-QF, QUAS-tdTomato/Cyo-If; 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	<i>yw hs-flp22; UAS-mCd2RFP, UAS-dsRNACD8 FRT40A/UAS-mcd8GFP, UAS-dsRNACD2, FRT40A; GMR11F02-gal4/+</i>	10 min hs, 48h ACI	96h AEL
3supp F	<i>yw hs-flp22; UAS-mCd2RFP, UAS-dsRNACD8 FRT40A/UAS-mcd8GFP, UAS-dsRNACD2, FRT40A; UAS-hid dsRNA/GMR11F02-gal4</i>	10 min hs, 48h ACI	96h AEL
4A	<i>yw; UAS-mCd2RFP, UAS-dsRNACd8 FRT40A ; GMR1102-gal4/TM3</i>	-	96h AEL
4A	<i>w; UAS-lacZ/aristaless-gal4</i>	-	96h AEL
4A	<i>w; UAS-lacZ/trithorax-gal4</i>	-	96h AEL
4A	<i>w; UAS-lacZ/ptc-gal4</i>	-	96h AEL
4B-C	<i>w; UAS-lacZ/+; GMR11F02-gal4/+</i>	-	adult
4B-C	<i>w; +; GMR11F02-gal4/UAS-hid dsRNA</i>	-	adult
4B-C	<i>w; UAS-lacZ/aristaless-gal4; +</i>	-	adult
4B-C	<i>w; aristaless-gal4/+; UAS-hid dsRNA/+</i>	-	adult
4B-C	<i>w; UAS-lacZ/trithorax-gal4; +</i>	-	adult
4B-C	<i>w; trithorax-gal4/+; UAS-hid dsRNA/+</i>	-	adult
4B-C	<i>w; UAS-lacZ/ptc-gal4 ; +</i>	-	adult
4B-C	<i>w; ptc-gal4/+ ; UAs-hid dsRNA/+</i>	-	adult
4D-F	<i>w; UAS-lacZ/tub-gal80<sup>ts</sup>; GMR11F02-gal4/+</i>	-	adult
4D-F	<i>w; +/tub-gal80<sup>ts</sup>; GMR11F02-gal4/UAS-hid dsRNA</i>	-	adult
4supp A	<i>w<sup>118</sup></i>	-	adult
4supp D-E	<i>w; UAS-lacZ/tub-gal80<sup>ts</sup>; GMR11F02-gal4/+</i>	-	adult
4supp D-E	<i>w; +/tub-gal80<sup>ts</sup>; GMR11F02-gal4/UAS-hid dsRNA</i>	-	adult

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

374

### 375 Immunostaining

376 Dissections of larval wing imaginal discs were performed on PBS in ice. Dissected  
377 discs were fixed for 20 min in 4 % formaldehyde (SIGMA F8775), rinsed 3 times in PBT  
378 (PBS 0.4 % Triton), followed by 10 min permeabilisation in PBT. Primary and  
379 secondary antibodies were incubated for 2 h at room temperature (or 12 h at 4 °C)  
380 under rocking agitation. After each antibody incubation, discs were rinsed 3 times in  
381 PBT, followed by 3 washes of 30 min. Discs were mounted in Vectashield® (EUROBIO  
382 SCIENTIFIC / H-1000) and imaged using a confocal spinning disc microscope (Gataca  
383 systems) with a 40X oil objective or a LSM880 equipped with a fast Airyscan using a  
384 40X oil objective. The following primary antibodies were used: rat anti E-cadherin  
385 (1/100, DCAD2 concentrated DSHB), mouse anti DSRF (1/500, gift of Seth Blair), rat  
386 anti Delta (1/1000, gift of François Schweisguth), chicken anti GFP (1/1000, abcam  
387 ab13970), rabbit anti RFP (1/500,), rabbit anti DCP-1 (1/100, Cell Signaling 9578S),  
388 chicken anti Beta-gal (1/1000, Abcam ab 9361), mouse anti Wingless (1/250, 4D4

389 concentrated DSHB), mouse anti Patched (1/250, Apa-1 concentrated, DSHB),  
390 phalloidin alexa 647 (1/50, Invitrogen). Secondary antibodies were: anti rabbit alexa  
391 555 (1/500, Invitrogen), anti chicken alexa 488 (1/500, Invitrogen), ultrapurified anti  
392 mouse 405 (1/500, Jackson ImmunoResearch / 715-476-151), ultrapurified anti mouse  
393 CY3 (1/500, Jackson ImmunoResearch / 715-165-151), ultrapurified anti rat 647  
394 (1/500, Jackson ImmunoResearch / 712-605-153).

### 395 **Image processing**

396 All images were processed using FIJI[39] . For the twin clones analyses, Z projections  
397 of z-stacks were done using the Fiji LocalZProjector plugin using E-cad or phalloidin  
398 staining as a reference plane[40], allowing to project a limited number of planes around  
399 the apical junction plane while following the local disc curvature. *hid*<sup>AGFP</sup> pattern  
400 measurements were done after fixation and GFP immunostaining and maximum z  
401 projection. The mean GFP intensity was measured in each compartment (defined with  
402 DSRF staining) and normalised by the mean GFP intensity of the full wing pouch.

### 403 **QMARCM and twin spot MARCM experiments**

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

$$415 \quad \textit{Centroid} (x, y)_{\textit{mitotic figure}} = \sum_{\textit{clone } i} (\textit{Centroid} (x, y)_{\textit{patch } i}) \cdot \frac{\textit{Area}_{\textit{patch } i}}{\textit{Total Area twin clone}}$$

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

419 analyser[41]) 3 wing discs stained with E-cad (Figure 3suppA') and DSRF to position  
420 landmarks, and by estimating the averaged apical cell area for each compartment. The  
421 3 wing discs segmented for apical cell area shown a very congruent pattern (Figure  
422 3suppA), thus allowing to pool the data from the 3 discs (Figure supp3 B). The average  
423 surface of clone for each compartment was then divided by the local averaged cell size  
424 to obtain an estimation of cell number per clone and to correct for effects driven by  
425 inhomogeneity of cell apical size throughout the disc.

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

427 Positions of 36 landmarks were manually set on Z projections of wing discs using FIJI.  
428 Landmarks were positioned according to the pattern of DSRF revealed by  
429 immunostaining, along the veins, margin and folds of the wing pouch (**Figure 1supp**  
430 **A,B**). Two methods were used to characterise a spatial map of the tissue based on  
431 landmarks position. For the first one, landmarks were geometrically aligned within each  
432 genotype using the General Procrustes Analysis (GPA) [42] as implemented in R  
433 geomorph package [43]. GPA translates the set of landmarks of each wing disc to the  
434 same origin, scales them to size, and rotates them until the coordinates of  
435 corresponding points align as closely as possible. GPA allows to superimpose as  
436 closely as possible several wing discs, thus allowing superimposition of the signal from  
437 many individuals (**Figure 1F; Figure 1supp,M,P**). For the second mapping method,  
438 14 additional landmarks were added (landmarks 37 to 50) at the centre of segments  
439 defined by other landmarks (for example landmark 37 was set as the centre of the  
440 segment defined by landmarks 21 and 22, **Figure 1suppC**). The resulting 50  
441 landmarks were thus used to define polygons dividing the wing disc tissue into 40  
442 compartments (**Figure 1suppC**). With this later approach, clones and apoptotic bodies  
443 (defined by GC3Ai or cleaved DCP-1 figures) were assigned to a compartment  
444 according to the coordinates of the centre of mass of the clone/apoptotic body. In order  
445 to account for uncertainty in clone/apoptotic body localisation (e.g., because of clone  
446 movement between the time of induction and imaging), as well as for uncertainty in  
447 landmark positioning (e.g., because of immunostaining variability or user error), clone  
448 assignment included consideration of an error margin. We used the *st\_buffer* function  
449 implemented in the *sf* package of R software to add an error margin at a distance of  
450 11.8  $\mu\text{m}$  (40 pixels) around compartment margins (**Fig. 1suppD**). As a result, buffered  
451 boundaries of neighbour compartments overlap within each other and a given position

452 in the tissue could be assigned to belong to more than one compartment. This results  
453 in a smoothing of the spatial map allowing to account for uncertainty in cells and  
454 landmarks positions.

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

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

461 Crosses were done by placing 50-100 couples in egg laying cages with juice-agar  
462 plates kept at 25 °C. Freshly hatched larva from the agar plates were transferred to  
463 *Drosophila* vials with fly food as described above at a density of 30 individuals / vial,  
464 and grown at 18 °C, 25 °C or 29 °C until emergence of adults. Flies were collected and  
465 stored in ethanol 70 % at room temperature until dissection. Only left wings from  
466 females were taken, and mounted dorsal side up on a glass slide in a solution of 1:1  
467 ethanol 80% and lactic acid 90%. Imaging was carried out on a ZEISS Discovery V8  
468 stereomicroscope using a ZEISS Axiocam ICc 5 camera. All wings were imaged in the  
469 same orientation and with the same imaging parameters. For the experiments using  
470 Gal80ts (**Figures 4** and **4supp**), flies were crossed in cages supplied with agar petri  
471 dishes and allowed to lay eggs for ~ 6 h. Freshly hatched larva were transferred to  
472 regular vials at a density of 30 individuals / tube, and placed at 18 °C or 29 °C. Once  
473 arrived at the fluid pupal stage, individuals where switched temperature (from 18 °C to  
474 29 °C and vice-versa). Upon hatching, flies were collected for wing dissection.

475 Wing measurements were done using a semi-automated procedure for estimating the  
476 positions of 12 landmarks and 37 semi-landmarks along the wing outline and veins  
477 (**Figure 4suppA**). This was done using Wings4 software [25, 26] which fits a B-spline  
478 model to the wing from which the coordinates of landmarks and semi-landmarks are  
479 extracted. Wings4 outputs were examined using CPR software[26] which allows to  
480 screen for outliers and to generate a consolidated dataset of landmarks and wing  
481 areas.

482 Landmarks data were geometrically aligned within each experiment using the General  
483 Procrustes Analysis (GPA)[42] as implemented in R geomorph package[43]. GPA  
484 translates all wing images to the same origin, scales them to unit-centroid size (centroid  
485 size is a measure of specimen size computed as the square root of the sum of squared  
486 distances of all the landmarks from the specimen's centroid) and rotates them until  
487 the coordinates of corresponding points align as closely as possible. Differences in  
488 landmarks coordinates resulting from the GPA represent shape differences between  
489 wings. Wing shape of each individual is characterised by the value of 96 variables,  
490 coming from the Procrustes transformation of x and y coordinates of the 48 landmarks  
491 and semi-landmarks. To reduce dimensionality of the data, wing shape variation was  
492 analysed by Principal Components Analysis as implemented in the function  
493 *plotTangentSpace* (now deprecated and replaced by *gm.prcomp*) of the R *geomorph*  
494 package. Visualisation of the wing shape variation among the principal component  
495 axes was done by comparing wing shape of the individual presenting the lowest value  
496 along the axis, with the individual presenting the highest value. To enable visualisation  
497 of local growth differences in the adult wing upon apoptosis inhibition, we used the  
498 program Lory[44] to show one pattern of relative expansion or contraction that can  
499 transform mean shape of control genotype into mean shape of genotypes where  
500 apoptosis was inhibited.

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

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

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

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

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

518 
$$p_0(t) = P(n(t) = 0).$$

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

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

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

524 which gives the estimator of  $p_0$ ,

525 
$$(1) \hat{p}_0 = \frac{q}{2-q}$$

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

527

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

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

530 
$$\mu(t) = p_0(t) \cdot 0 + p_1(t) \cdot 1 + \dots + p_i(t) \cdot i + \dots,$$

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

534 
$$\hat{\mu} = \frac{k_0}{N} \cdot 0 + \frac{k_1}{N} \cdot 1 + \dots + \frac{k_i}{N} \cdot i + \dots,$$

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

537 However, since we cannot observe clones of size 0 we cannot measure  $k_0$  nor  $N$ . We  
 538 therefore use  $p_0 = \frac{k_0}{N}$  (the probability of clone extinction, see above), and the total

539 number of observed clones  $N_{obs} = N - k_0$ . From these two we can estimate  $N = \frac{N_{obs}}{1-p_0}$ .

540 Hence, we estimate  $\mu$  with

541 (2) 
$$\hat{\mu} = (1 - \hat{p}_0) \left( \frac{k_1}{N_{obs}} \cdot 1 + \dots + \frac{k_i}{N_{obs}} \cdot i + \dots \right).$$

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

548 (3) 
$$\mu(t) = e^{(b-a)t}$$

549 Furthermore, the probability to be extinct at the time  $t$  ( $n(t) = 0$ ) can be expressed as  
550 follows [45]:

551 (4) 
$$p_0(t) = \frac{a - ae^{-(b-a)t}}{b - ae^{-(b-a)t}}$$

552

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

554 From the twin clone experimental data, we retrieve for each compartment the  
555 proportion of single clone occurrence (**Figure 2C**) and the averaged clone size (**Figure**  
556 **3A**) which, together with (1), allows us to estimate  $p_0$ , and  $\mu$  for each of the 40  
557 compartments of the wing disc. These estimates can then be used to estimate  
558 parameters  $a$  and  $b$  (apoptosis and division rate). For the fixed  $t$ , based on (3) and (4)  
559 we have:

560 (5) 
$$b = \frac{\ln(\mu)}{t} + a$$

561 and

562 (6) 
$$p_0 = \frac{a - \frac{a}{\mu}}{b - \frac{a}{\mu}}$$

563 Hence

564 (7) 
$$a = \frac{\ln(\mu)}{t \left[ \frac{1}{\mu} + \frac{\mu-1}{\mu p_0} - 1 \right]}$$

565 *Estimating the expected differences of clone size between compartments assuming*  
 566 *constant proliferation rate*

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

## 579 **Statistics**

580 Data were not analysed blindly. No specific method was used to predetermine the  
 581 number of samples. The definition of  $n$  and the number of samples is given in each  
 582 figure and associated legend. Error bars are standard error of the mean (s.e.m.) or  
 583 confidence interval 95%.

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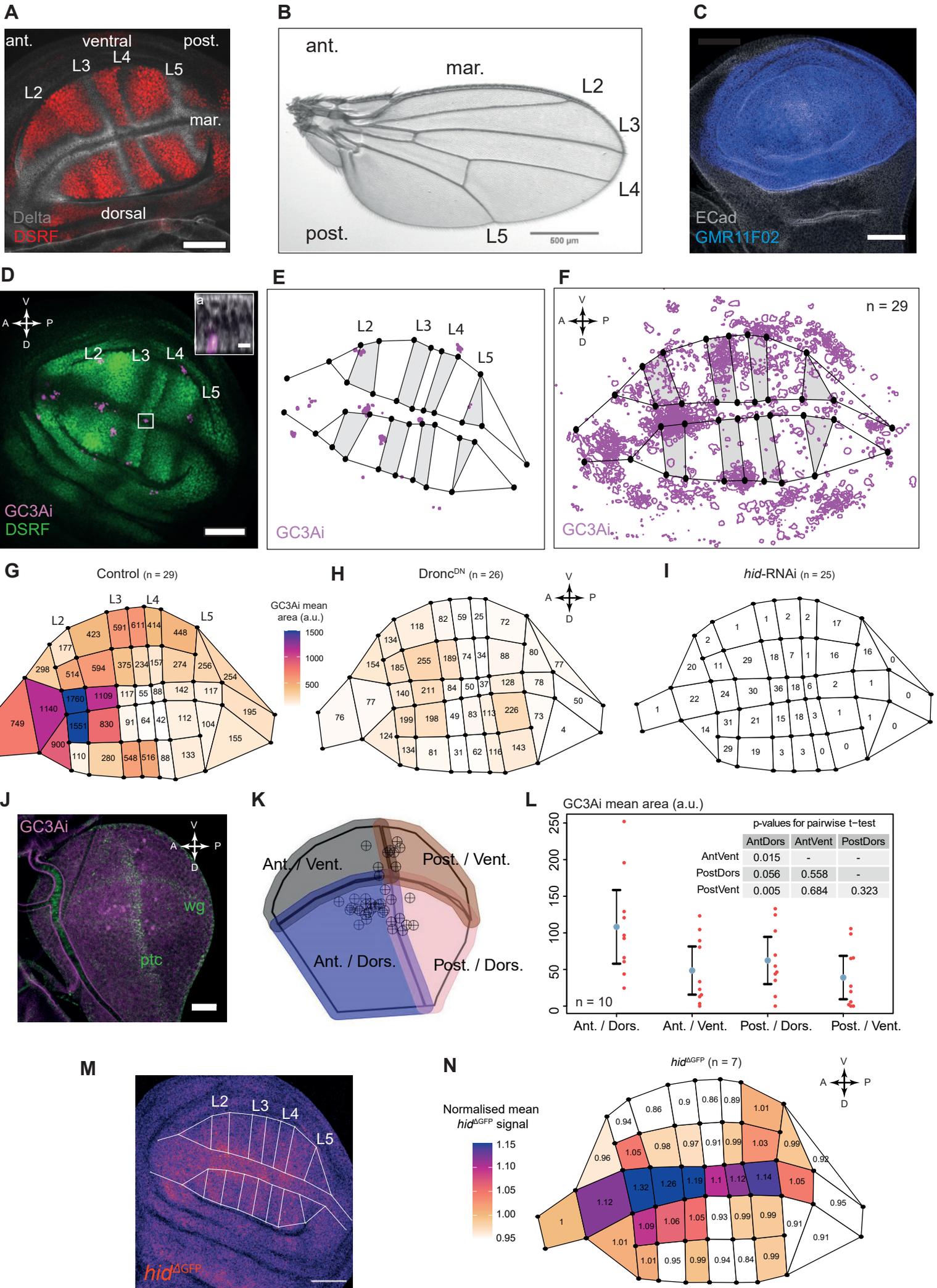
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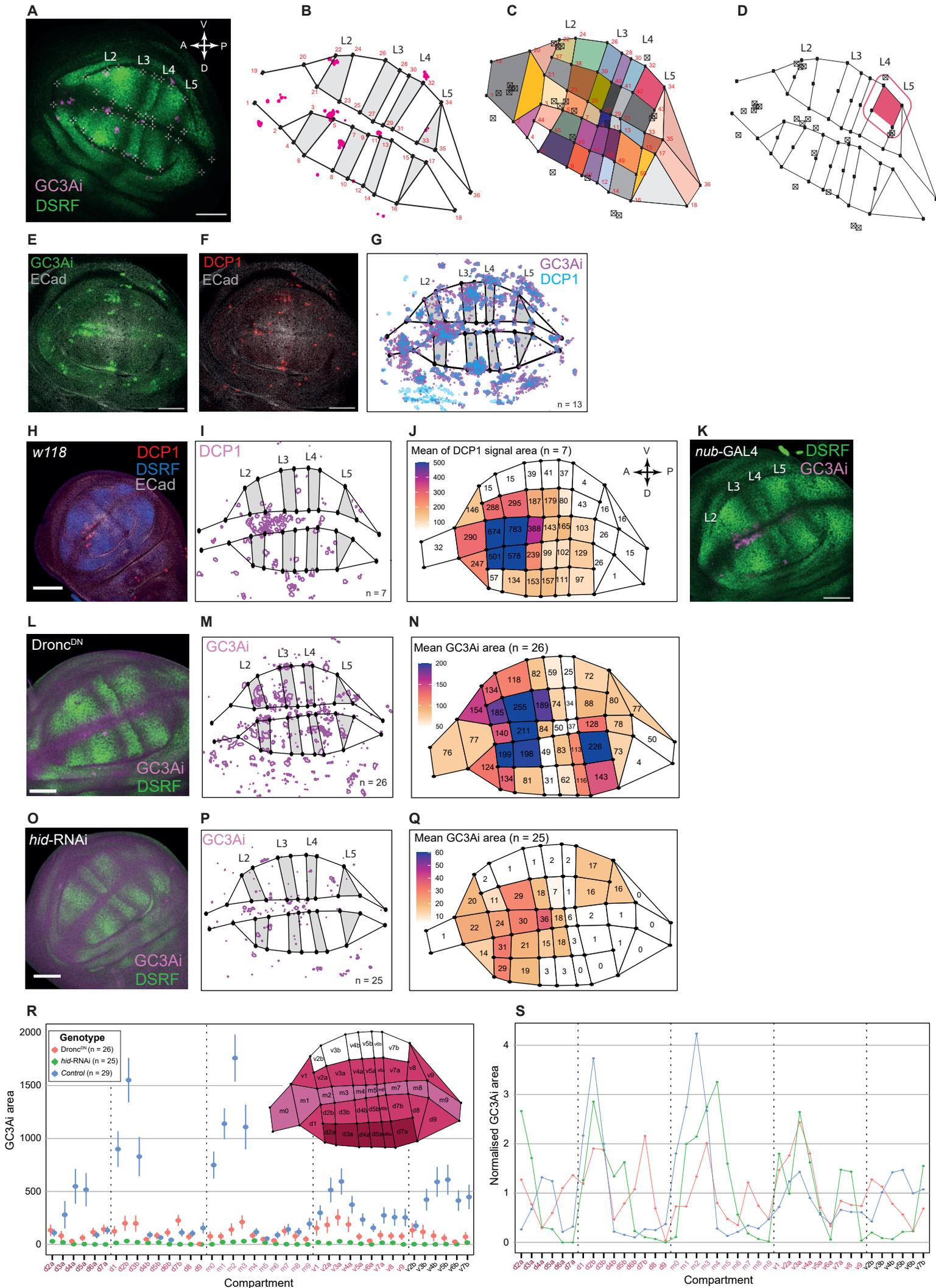
**Figure 1**



## Figure 1: caspase-3 activity 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 boxed area, 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. Black dots are landmarks. **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) and *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, green) and anti Patched (ptc, green) 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. To account for uncertainty in centroid location of the GC3Ai spots and of 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 except in **B**, 500  $\mu$ m, and in **J**, 25  $\mu$ m.

# Figure 1 - supplementary

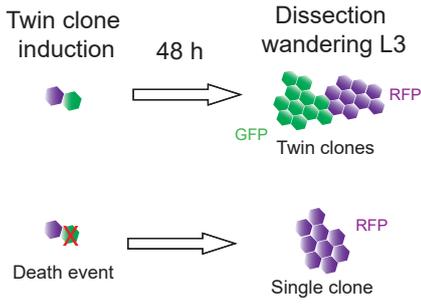


## Supplementary Figure 1 (associated with Figure1)

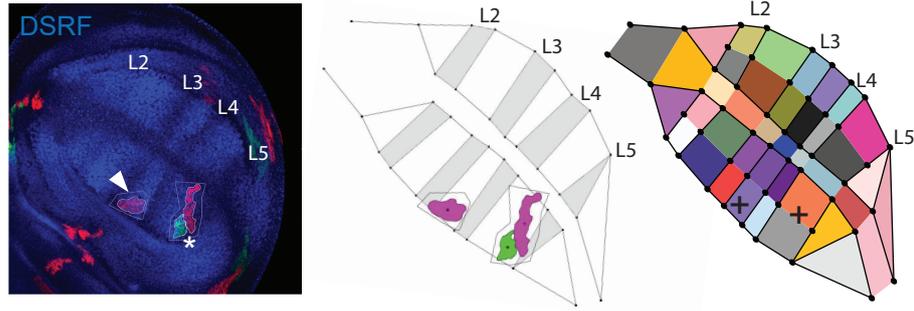
**A:** Wing disc from **Figure1 A** showing the 36 landmarks (white crosses) that were manually positioned at the intersections between veins (L2 to L5), folds and margin of the tissue, visible thanks to DSRF (green). **B:** Map of the wing disc shown in **A**, drawn from the landmark positions (black 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 by adding 14 additional 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  $\mu\text{m}$  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 and cleaved DCP-1 (*Drosophila* caspase3). **E** and **F** show the same wing disc with GC3Ai signal (**E**) or anti cleaved DCP-1 immunostaining (**F**). **G:** Superimposition of GC3Ai (magenta) and cleaved DCP-1 (blue) signals from 13 discs. The 13 discs were superimposed using the General Procrustes Alignment (GPA) based on the 36 landmarks positions. **H:** Wings disc from *w118* genotype stained for anti cleaved DCP-1, anti DSRF and anti E-cad. **I:** Superimposition of the segmentation data from 7 discs scaled, rotated and aligned using the GPA approach, showing a pattern for cleaved DCP-1 (magenta) similar than the one for GC3Ai (**Fig. 1F**). **J:** Heat-map showing the average cleaved DCP-1 positive area on each of the 40 compartments of the wing disc in *w118* genetic background. Numbers within each compartment show the average value for cleaved DCP-1 positive area. These values are also shown as a colour coded heat-map. The pattern is similar to the one of GC3Ai (**Figure 1G**). **K:** Wing disc stained with anti DSRF expressing GC3Ai under the control of *nubbin-GAL4*. **(L-N):** Pattern of GC3Ai in *UAS-Dronc<sup>DN</sup>* background driven by *GMR11F02-GAL4*, single representative wing disc (**L**), superimposition of 26 discs using GPA (**M**), and with a heat map showing the average GC3Ai signal area over 26 discs (**N**). **(O-Q):** Pattern of GC3Ai in *hid-RNAi* background driven by *GMR11F02-GAL4*, single representative wing disc (**O**), superimposition of 25 discs using GPA (**P**), and heat map showing the average GC3Ai signal area over 25 discs (**Q**). **R:** Mean GC3Ai signal area for the 40 compartments in three genotypes (*Control*, *hid-RNAi*, *Dronc<sup>DN</sup>*). The inset shows a wing disc diagram with delimitation and names of the 40 compartments on which the quantifications were made. Error bars show s.e.m. **S:** This plot shows the same data than in **R**, but that were normalised to show within each genotype how much the GC3Ai signal varies in a given compartment relatively to the average amount over all the disc. The plot was obtained by dividing the mean value of each compartment (shown in **R**) by the mean over all the compartment means within each genotype.

**Figure 2**

**A**



**B**



**C**

QMARCM - Control

96 discs

546 mitotic recombination events

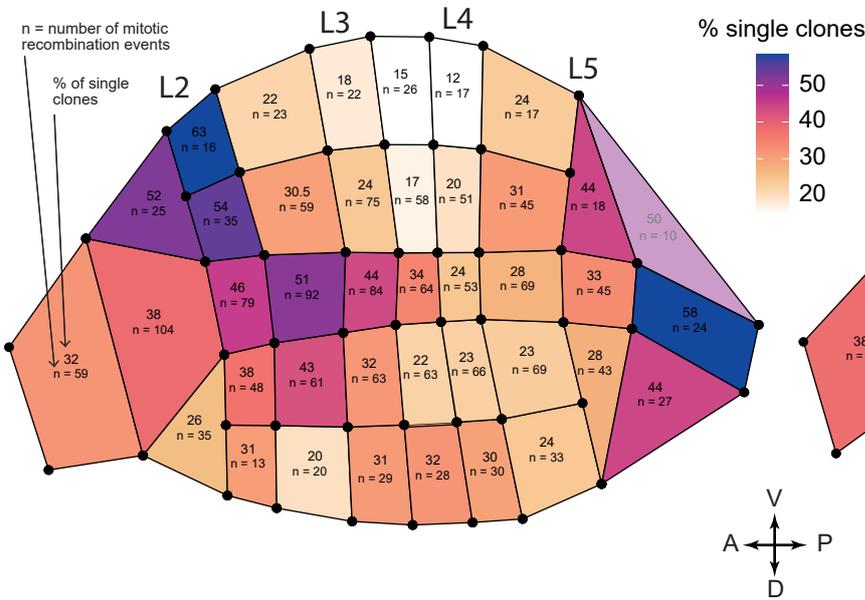
368 twin clones (67 %)

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

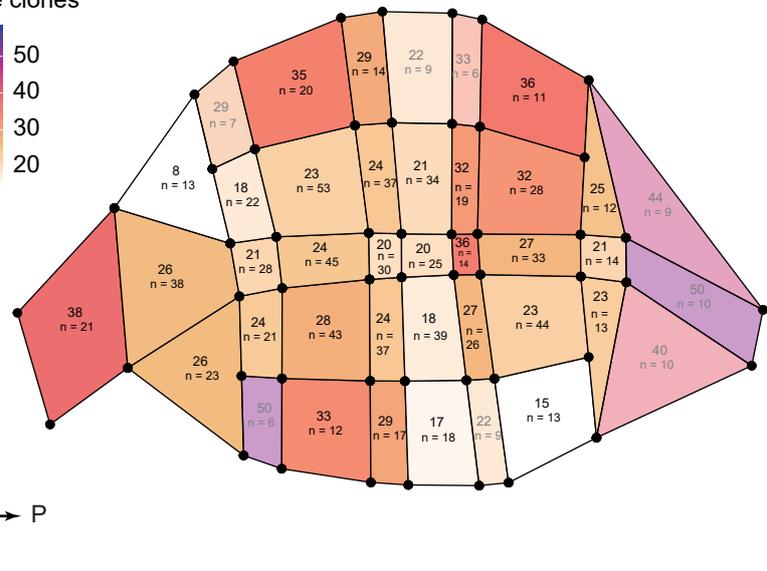
180 twin clones (74 %)

58 single RFP clones (24 %)

5 single GFP clones (2 %)

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

Tub-QF > QUAS-RFP



**D**

MARCM - Control

67 discs

293 mitotic recombination events

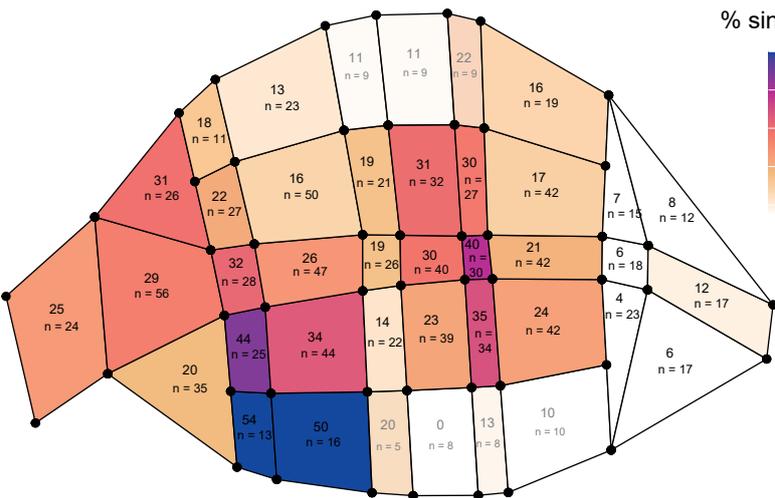
229 twin clones (78 %)

47 single RFP clones (16 %)

17 single GFP clones (6 %)

GMR-GAL4 > UAS-GFP

GMR-GAL > UAS-RFP



**D'**

MARCM - Hid-RNAi

67 discs

446 mitotic recombination events

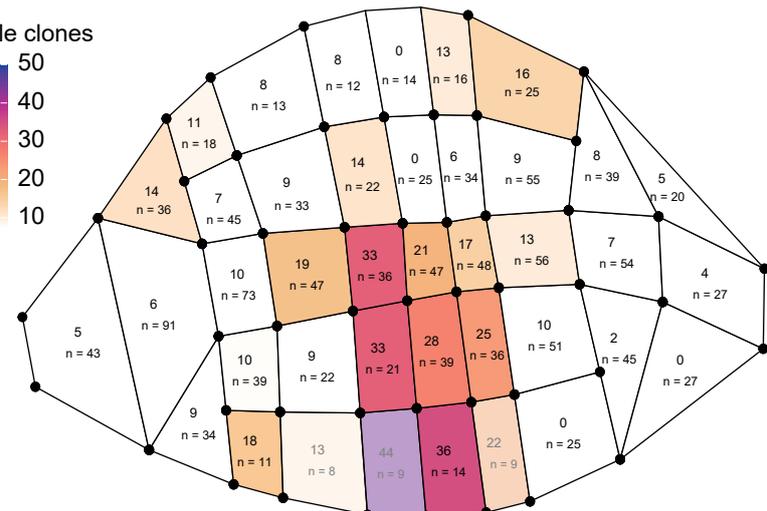
396 twin clones (89 %)

42 single RFP clones (9 %)

8 single GFP clones (2 %)

GMR-GAL4 > UAS-GFP, UAS-HID-RNAi

GMR-GAL > UAS-RFP, UAS-HID-RNAi



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

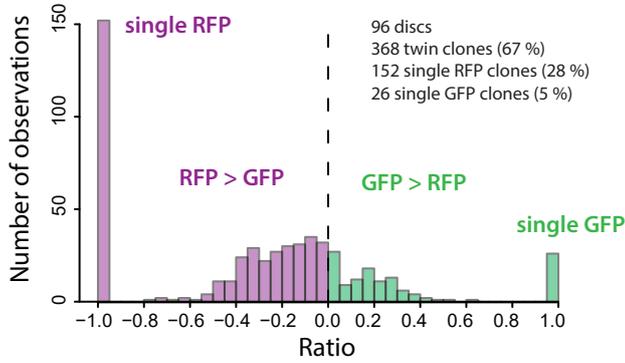
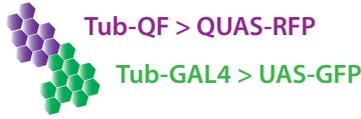
**A:** Rationale for inferring cell death events from twin-clone induction experiments. Upon twin-clone induction and 48 h of growth, the observation of twin clones composed of one GFP and one RFP clone is expected. However, early occurrence of cell death events after clone induction may eliminate one sibling resulting 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. Right, spatial map of the wing disc divided in 40 compartments (following the DSRF-based segmentation) into which clones positions are mapped (black crosses show location of clones centroids). **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 percentage of single clones observed in each compartment, relative to the total number of mitotic recombination events assigned to this compartment ( $n$ ). For clarity, the compartments for which  $n \leq 10$  were shaded, since the inferred proportions from such low sample size are very unreliable. **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-D':** Spatial map of single clone occurrences obtained by inducing twin clones using the twin spot MARCM system driven by the *GMR11F02-GAL4*, in control conditions (**D**) and by additionally expressing *UAS-hid-dsRNA* in all the pouch (**D'**). **D** and **D'** share the same colour scale.

# Figure 2 - supplementary

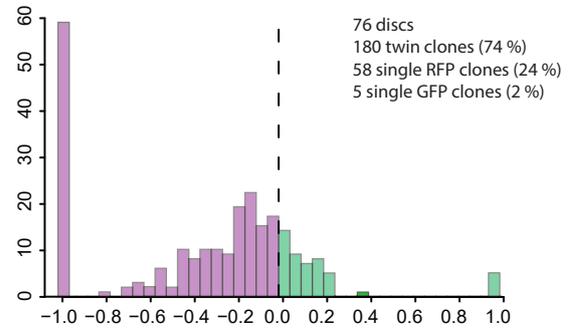
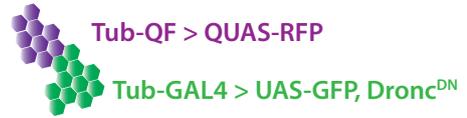
**A**

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

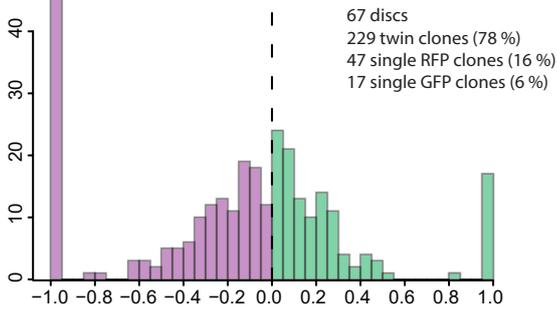
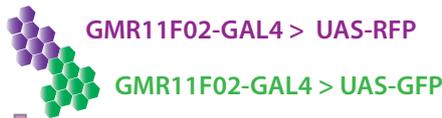
## Case 1 : QMARCM - Control



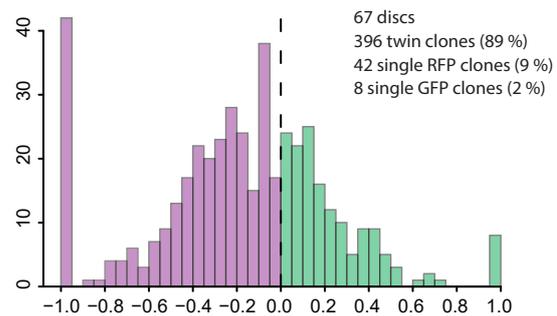
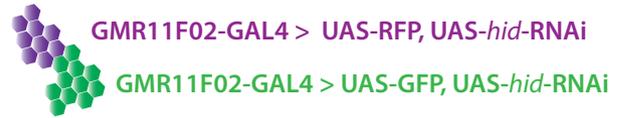
## Case 2 : QMARCM - Dronc<sup>DN</sup>



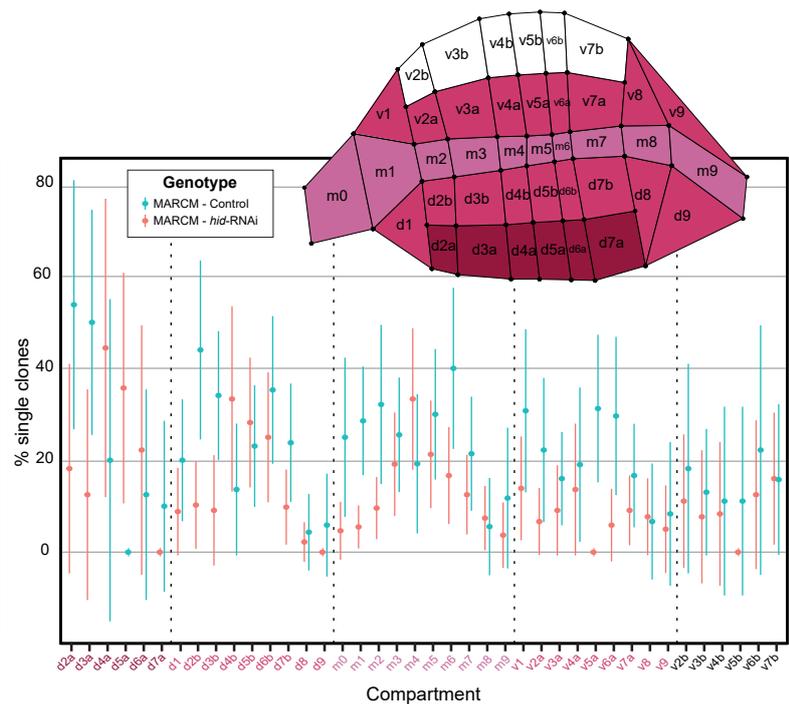
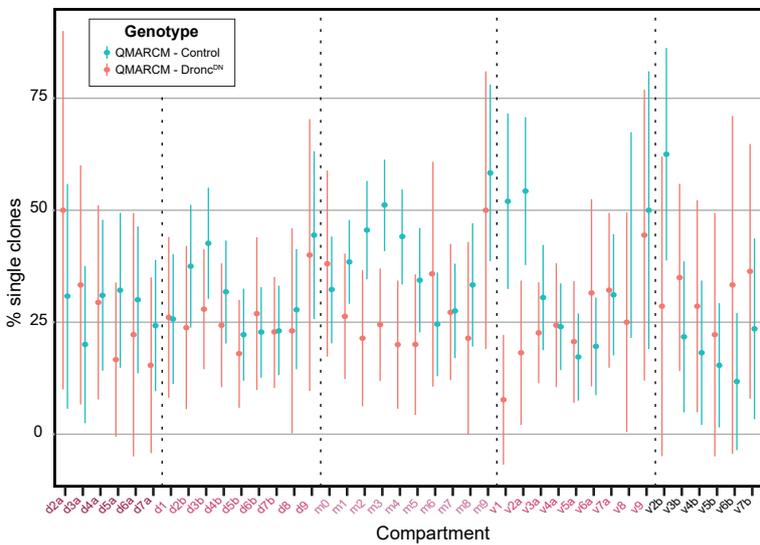
## Case 3 : MARCM - Control



## Case 4 : MARCM - hid-RNAi



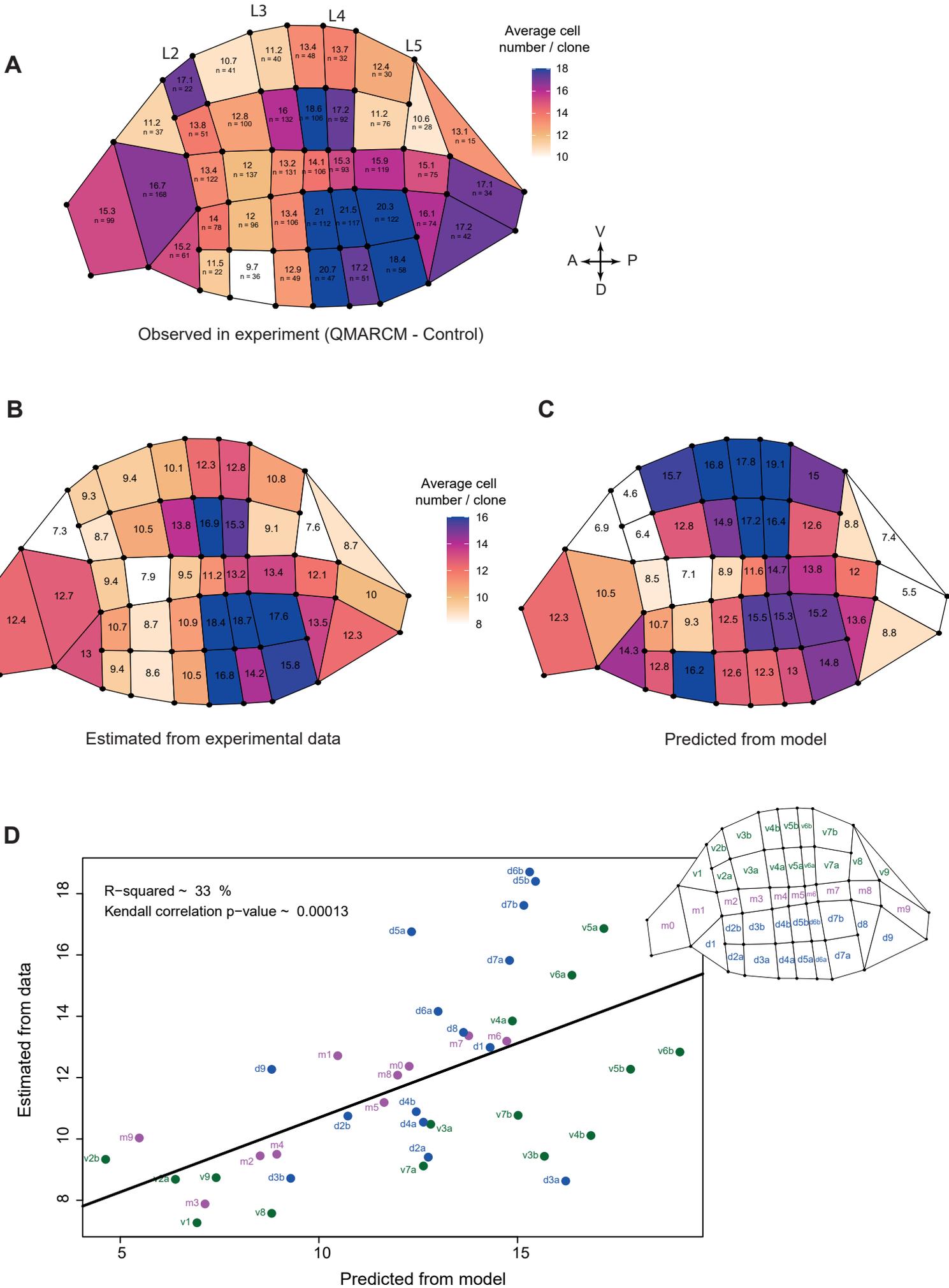
**B**



## Supplementary Figure 2 (associated with Figure 2)

**A:** Distribution of twin-clone Ratio values for each of the four genetic conditions studied to establish the spatial map of single clone occurrences. 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, respectively, the green or the red clone were lost and have thus a null area. **B:** Frequency of the single colour clones for the 40 compartments in four genotypes (left, QMARCM, QMARCM *Dronc*<sup>DN</sup>, right, twin spot MARCM, twin spot MARCM *hid*-RNAi). The inset shows a wing disc diagram with delimitation and names of the 40 compartments on which the quantifications were made. Error bars are 95% confidence interval (CI), with  $CI = \pm 1,96 \cdot \sqrt{\frac{p(1-p)}{n}}$ , with  $p$ , frequency of the single colour clones and  $n$ , number of mitotic recombination events observed.

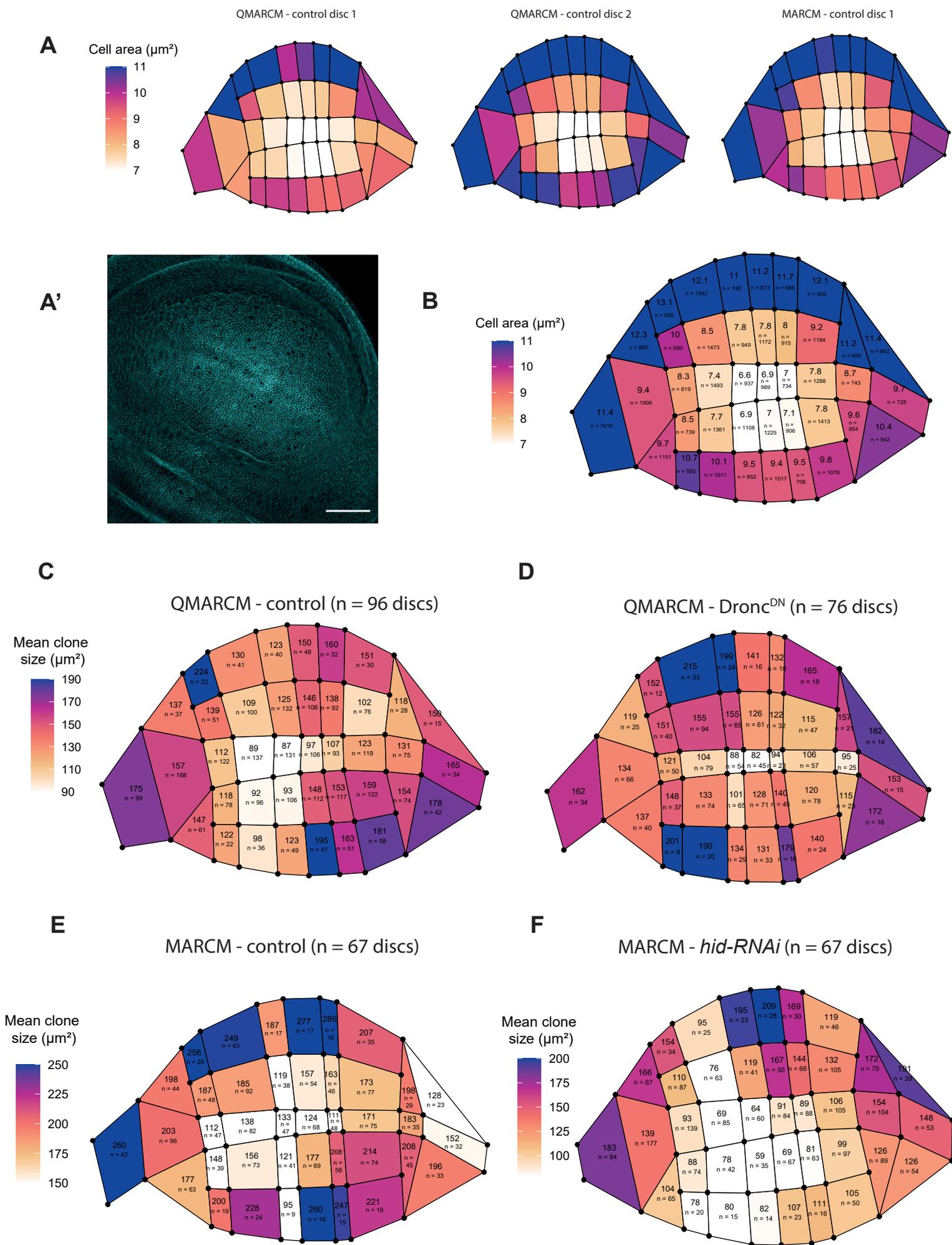
**Figure 3**



**Figure 3 – Local variation of clone size can be predicted by the local variation of apoptosis.**

**A:** Spatial map for clone size (expressed in mean number of cells/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**) estimated by integrating the experimental average clone size for each compartment 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 right side of the plot (blue: dorsal compartments d1 to d9; magenta: margin compartments m0 to m9; green: ventral compartments v1 to v9).

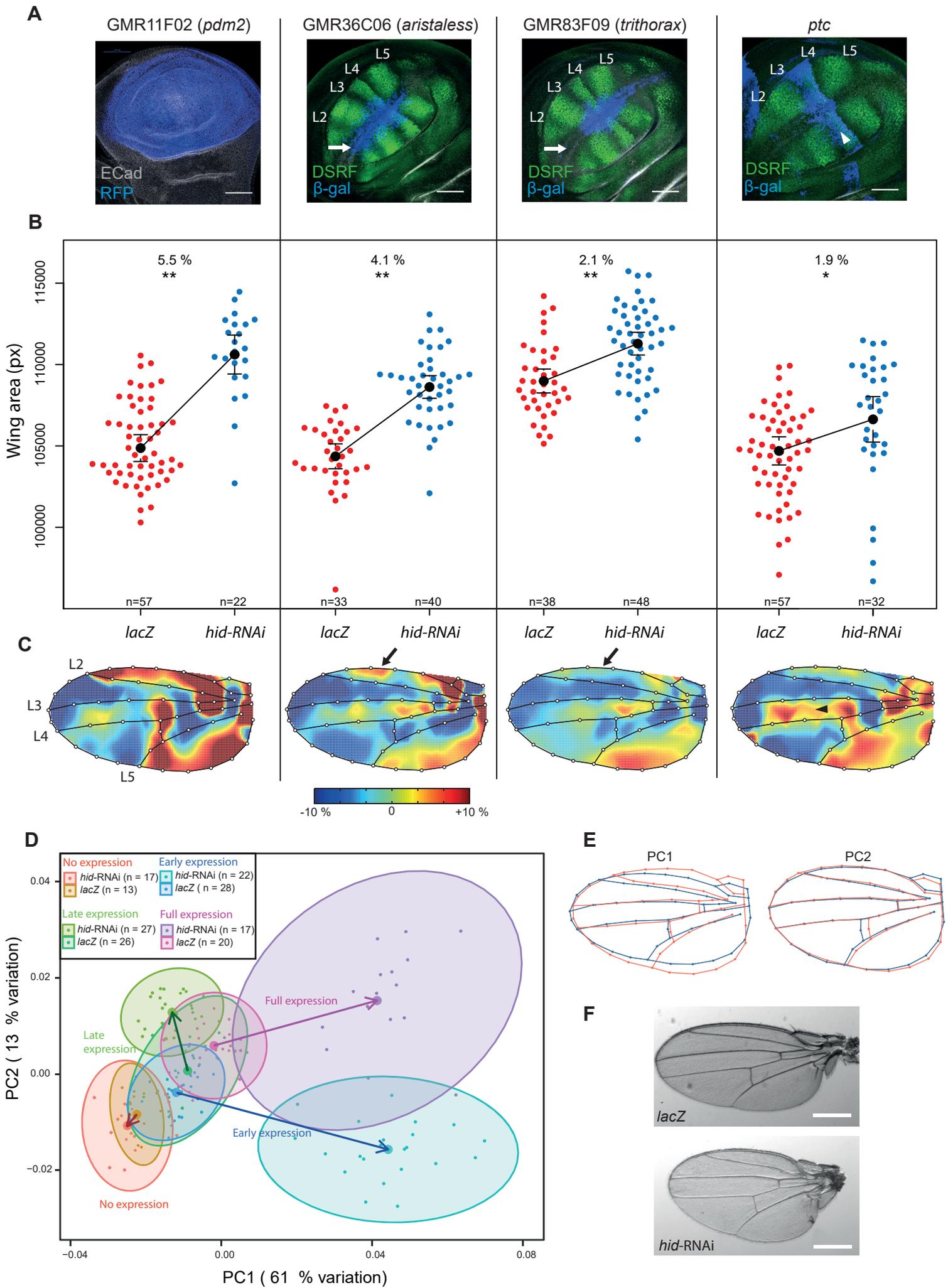
**Figure 3 - supplementary**



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

**A:** Heat maps showing the spatial pattern for cell apical area for three segmented discs estimated after local projection of E-cad staining. Two discs were of the *QMARCM – control* genotype and another one from the twin spot *MARCM – control* genotype. The colour code shows the averaged apical cell area for each compartment (same colour scale bar for the three discs). **A':** Example of a local projection of a wing disc stained with E-cad (the one shown in **A**), allowing the extract cell apical area. Scale bar: 50  $\mu\text{m}$ . **B:** Heat map showing the average spatial pattern of cell size from the three discs shown in A. Within each compartment, the value for mean cell size ( $\mu\text{m}^2$ ) and the number of cells measured (over the three discs) are given. **C-F:** Heat maps showing the average spatial pattern for clone surface area ( $\mu\text{m}^2$ , based on local projections performed at the level of adherens junctions). Each clone was considered irrespectively of its colour and its occurrence as single clone or in a twin spot. For each compartment, the average surface area of the clones and the number of clones examined (n) are given. The data are shown for the four conditions used in this study: *QMARCM* system, in control conditions (**C**) and upon expression of *UAS-Dronc<sup>DN</sup>* in the GFP clone (**D**); twin spot *MARCM* system driven by the *GMR11F02-GAL4*, in control condition (**E**) or by expressing *UAS-hid-RNAi* in all the pouch (**F**).

**Figure 4**

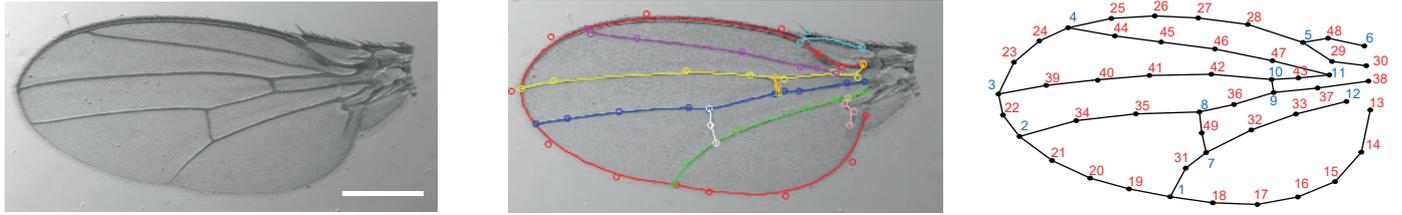


**Figure 4: Apoptosis affects locally and globally wing size and shape during early stages of development.**

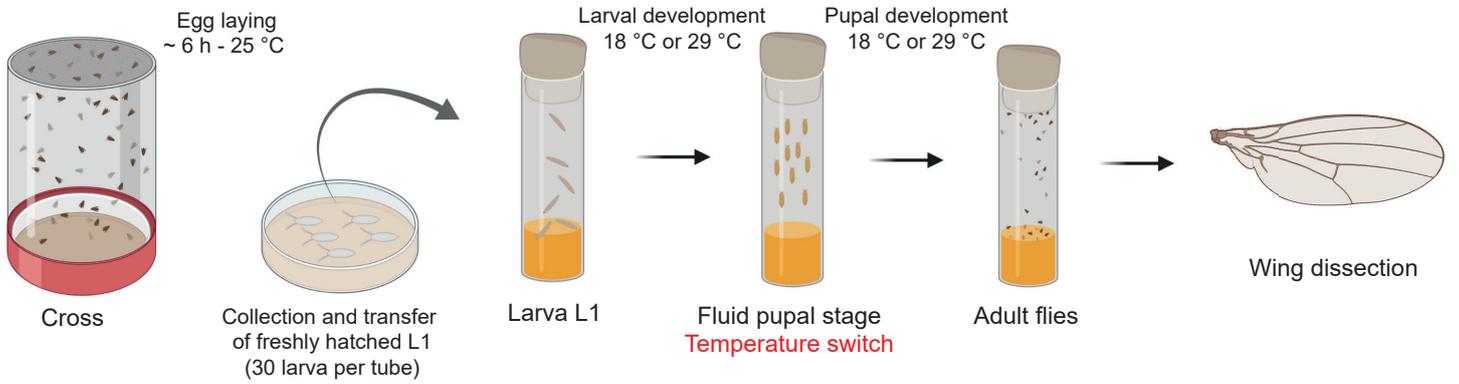
**A:** Pattern of expression at ~ 96 h AEL of the GAL4 drivers used to express *hid-RNAi*. White arrows show the most anterior region of the disc, where GAL4 is expressed in the case of *aristaless-GAL4*, but not in the case of *trithorax-GAL4*. White arrowhead highlights the *ptc* domain. **B:** Variation of adult wing size caused by expression of *hid-RNAi* using different GAL4 drivers. For each GAL4 driver shown in **A**, the effect of expressing *hid-RNAi* compared to *lacZ* is shown. Blue and red dots represent individual wing size values, black dots are means, and error bars are 95 % confidence interval of the mean. For each driver, the % of variation of the *hid-RNAi* mean relative to the *lacZ* mean, as well as the results of pairwise t-tests between *lacZ* and *hid-RNAi* groups are shown. \*\*:  $p < 0.001$  ; \* :  $p < 0.01$ . « n » denotes the number of wings for each group. **C:** Local variation of tissue shape driven by expression of *hid-RNAi* with the GAL4 drivers shown in **A**. Colours represent changes in relative area necessary to transform the average wing from the *lacZ* group into the average wing of *hid-RNAi* group (red: increase, blue: decrease, number of wings shown in **B**). Colour bar shows the upper and lower limits in deformation. Highest colour intensity is reached at 10 % increase and decrease in local area in *hid-RNAi* group with respect to *lacZ* group. Arrows / arrowhead show the regions corresponding to the regions pointed by arrows / arrowhead in **A**. **D:** Principal components analysis (PCA) showing variations of wing shape (according to the positions of 49 Procrustes-aligned landmarks and semi-landmarks) caused by expression of *hid-RNAi* at different developmental stages (using Gal80<sup>ts</sup> and switch between 18°C and 29°C). Each small dot represents one wing shape. 8 conditions are shown (no expression, early expression, late expression and full expression of *hid-RNAi* or *lacZ* with GMR11F02-GAL4 driver). Big dots represent the mean of each condition. Vectors connect the mean of the *lacZ* group to the one of *hid-RNAi* group of the corresponding condition, thus allowing to visualise magnitude and direction of the effect on wing shape due to *hid-RNAi* expression. Ellipses are 95 % confidence intervals of the means. **E:** Diagrams showing the variation of wing shape along the first and the second principal component (PC) axes of the PCA shown in **D**, from low PC values (blue) to high values (red). **F:** Pictures of mounted wings illustrating the shape rounding observed upon *hid-RNAi* expression during early stages as compared to expression of *lacZ*. Scale bars are 500  $\mu\text{m}$ .

# Figure 4 - supplementary

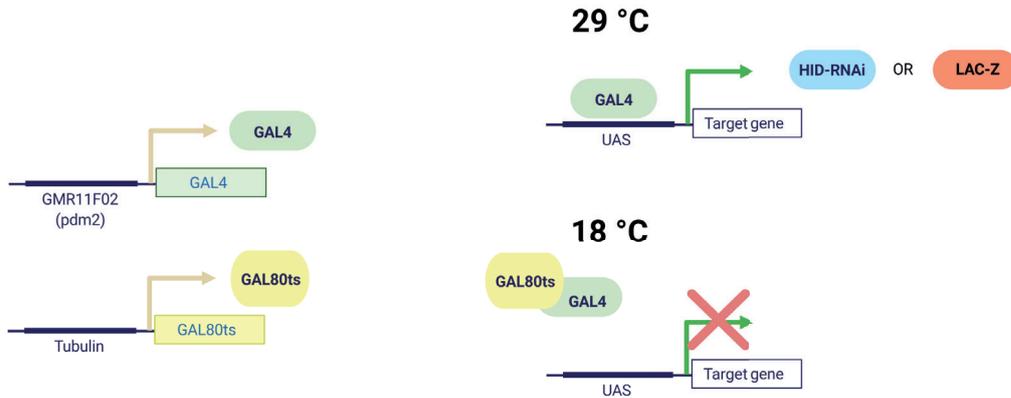
**A**



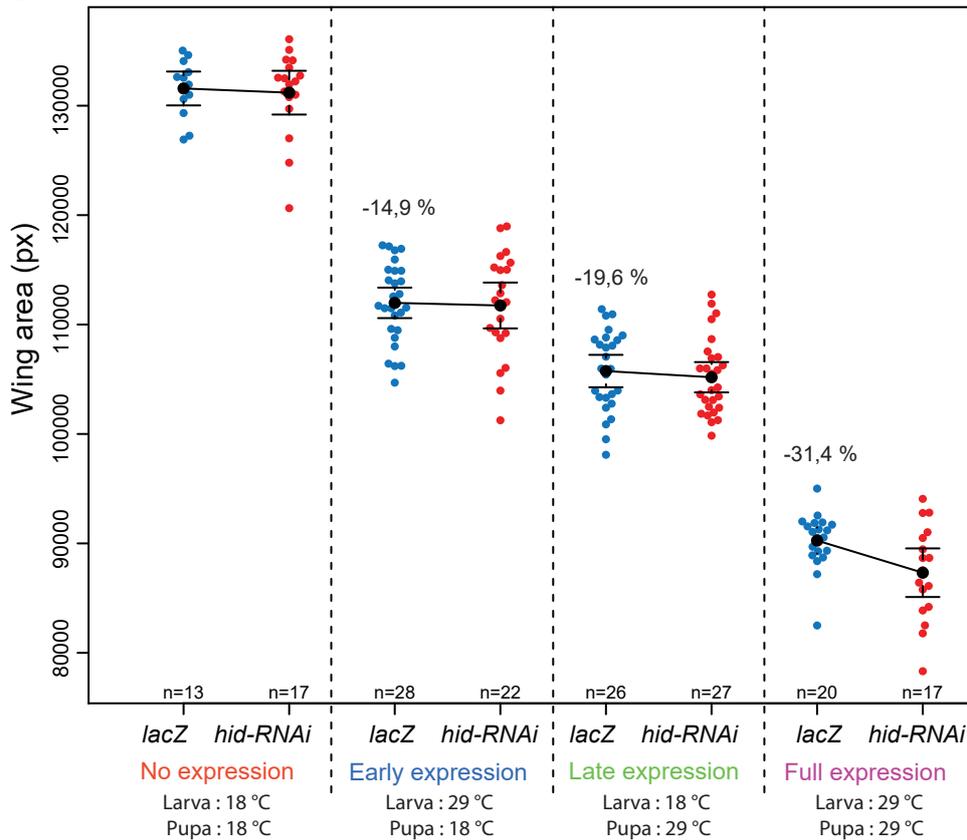
**B**



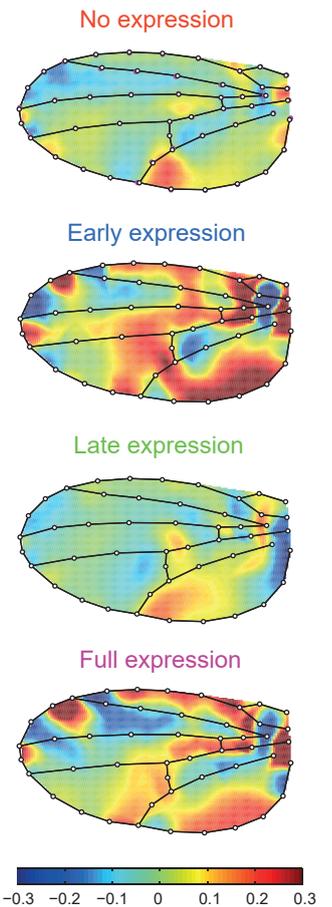
**C**



**D**



**E**



## Supplementary Figure 4 (associated with Figure 4)

**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. Right: 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. **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 at 25 °C. 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 fluid pupal 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-GAL80<sup>ts</sup>. GAL80<sup>ts</sup> has ubiquitous expression under the control of tubulin promoter whereas GMR11F02-GAL4 expression is restricted to wing tissue. At 18 °C, GAL80<sup>ts</sup> binds to the GAL4 and inhibits its activator function, precluding the expression of the target genes. At 29 °C Gal80<sup>ts</sup> is unable to bind GAL4, allowing GAL4 binding to the upstream activating sequence (UAS) and transcription of target genes (*lacZ* or *hid-RNAi*). **D:** Variation of adult wing size caused by expression of *hid-RNAi* under the control GMR11F02-GAL4 driver at different developmental stages. The effect of expressing *hid-RNAi* compared to *lacZ* is shown, for four conditions (No expression; Expression before fluid pupal stage (Early expression); Expression after fluid pupal stage (Late expression); Expression during all development (Full expression)). Each blue and red dot represents an individual wing size value, black dots are means, and error bars are 95 % confidence interval of the mean. For each condition, the percentage of variation of the *lacZ* mean relative to the “No expression” condition is shown. Pairwise t-test for *lacZ* compared to *hid-RNAi* within each condition are all non-significant. **E:** Local variations of shape caused by expressing *hid-RNAi* at different developmental stages. Colours represent changes in relative area necessary to transform the average wing for *lacZ* group into the average wing of *hid-RNAi* group (red: expansion, blue: contraction). Colour bar shows the upper and lower limits in deformation. Highest colour intensity is reached at 30 % increase and decrease in local area in *hid-RNAi* group with respect to *lacZ* group. Panels B and C were created with BioRender.com.