Int. J. Dev. Biol. 63: 17-27 (2019)
https://doi.org/10.1387/ijdb.180292am

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Genital disc growth in *Drosophila*

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ABSTRACT Prior to completion, apoptosis causes the secretion of different signals, including proliferative signals. Signaling associated with death was discovered in *Drosophila* and mostly characterized by the induction of experimental death. Thus, less is known about physiological death. Here, we analyzed physiological death in the genital disc, a structure with bilateral symmetry, in different growth scenarios. To this end, we prevented or promoted death in regions or in genetic mosaics. We observed that physiological death in the genital disc was associated with proliferative signals and that both processes were JNK-dependent. The proliferative signals promoted growth in the genitalia primordia but not in the analia. Due to the proliferative signaling, the prevention of death that produced undead cells provoked asymmetric growth, high variability in proliferation, and size reduction. Death can occur in the absence of JNK but without signaling. JNK is fundamental for growth and death associated with signaling.

KEY WORDS: *developmental apoptosis, apoptosis, growth, JNK, Drosophila*

Introduction

Apoptosis is a conserved process through which cells die. The decision to die can be autonomous or non-autonomous. Death factors are available in all cells; consequently, cells have anti-apoptotic molecules and mechanisms to antagonize this situation (review in Ayra and White, 2015). The degree of death prevention may depend on the genetic context. In this sense, one cause of physiological death is the withdrawal of growth-surviving factors. Indeed, sharp discontinuities or limited amounts of growth-surviving factors lead to death (review in Igaki, 2009) and have been proposed to occur by cell competition (Adachi-Yamada and O'Connor, 2002). The competition mechanism eliminates cells that differ in growth rate (Ripoll and Morata, 1975). Growth has three components: biosynthesis of products, proliferation and survival, the alterations of which produce cell competition. Competitive death occurs in those cells with reduced growth, called losers, in contraposition to those with enhanced growth, called winners leading to death of losers (Moreno and Basler, 2002).

In *Drosophila*, it has recently been shown that signaling from apoptotic cells can induce proliferation, death and survival of neighboring cells (review in Pérez-Garijo and Steller, 2015). We have focused on proliferation signals. The most studied are Decapentaplegic (Dpp), Wingless (Wg) (Ryoo *et al.*, 2004) and Hedgehog (Hh) (Fan and Bergmann, 2008). These molecules not only influence growth but also patterning, and thus, their secretions in physiological death are considered to be insignificant to induce proliferation (Morata *et al.*, 2011). Experimental induction of death and its simultaneous prevention allow cells to survive via the secretion of signals. These cells are called "undead" and induce extensive abnormal overgrowth in the wing disc (Pérez-Garijo *et al.*, 2004). The only death signal recognized is Eiger (Egr) (review in Pérez-Garijo and Steller, 2015), the *Drosophila* orthologue of mammalian TNF (Igaki *et al.*, 2002). Curiously, Egr an activator of the Jun-NH_2-Terminal-Kinase (JNK) pathway which is a potent inducer of death (Moreno *et al.,* 2004) with viable mutations (Igaki *et al.*, 2002), is also connected through JNK in the activation of the Hippo mechanism of size-growth control (Willsey *et al.,* 2016).

Abbreviations used in this paper: A/P, antero-posterior; Bsk, Basket, *Drosophila's* JNK protein; DfH99, deficiency 3 L H99; Drice, *Drosophila* ICE caspase protein; Dronc, *Drosophila* caspase 9; DP, death prevented; Dpp-Z, Dpp-βgal reporter gene; Dpp, decapentaplegic protein; EGFP, enhanced green fluorescent protein; Egr, Eiger protein; A8, A9, A10, eighth, ninth and tenth abdominal segments and primordia; hid, *head involution defective* gene; JNK, Jun-NH_2-terminal-kinase; L2, L3, second and third larval stages; Moe-GFP, moesina protein fused to GFP; PH3, phosphorylated histone 3; puc^{E69}= *puc-Z*, mutation in *puckered* due to a lac-Z insertion; allele *E69*, reporter gene for puckered and indirectly for JNK; p35, p35 baculovirus anti-apoptotic protein; RNAi, ribonucleic acid interference; RHG, Rpr, Hid and Grim proteins; *rpr*, *reaper* gene; TNF, tumor necrosis factor; Wg, wingless protein.

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Submitted: 30 August, 2018; *Accepted:* 7 January, 2019.

ISSN: Online 1696-3547, Print 0214-6282
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Printed in Spain

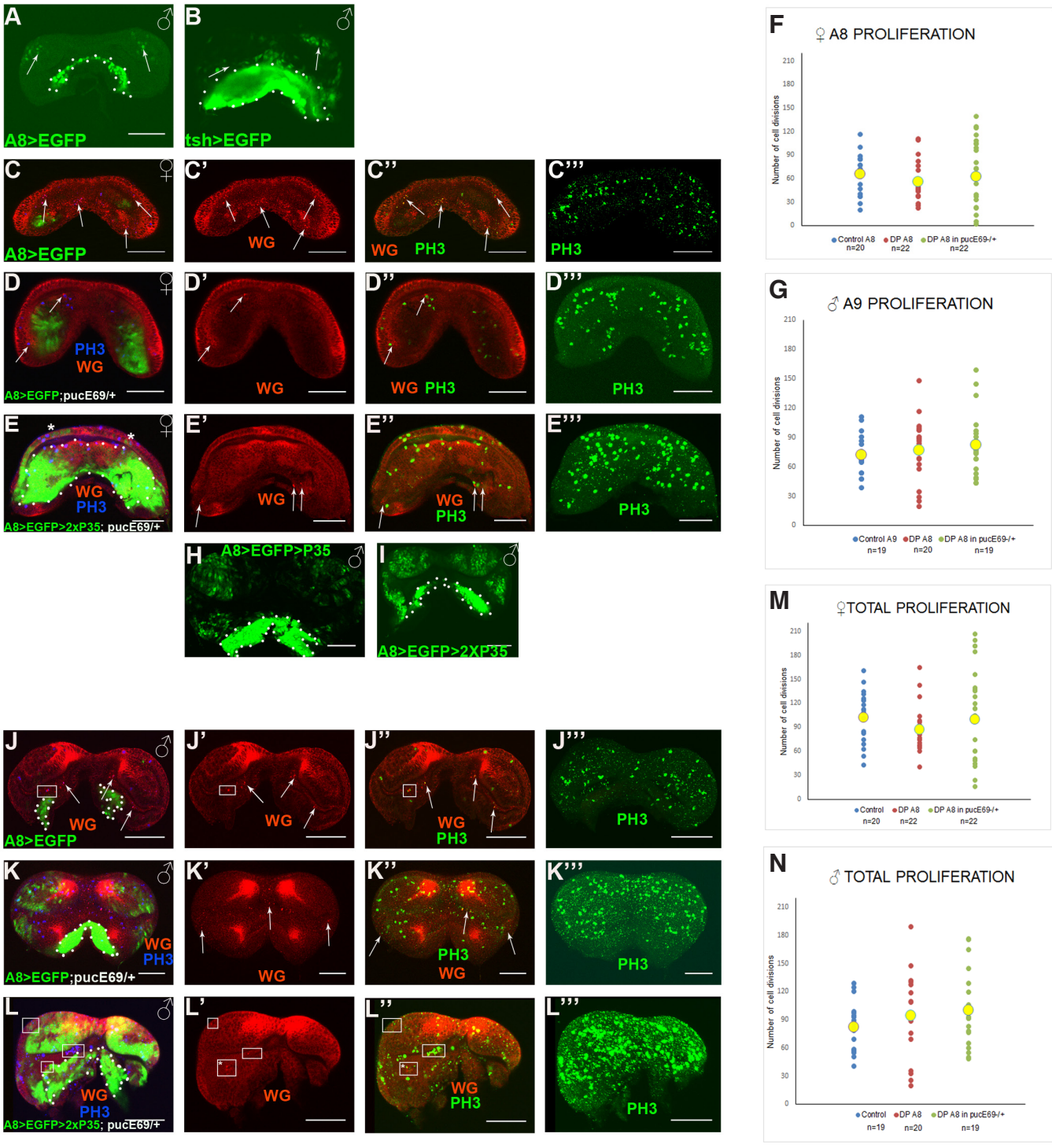

Fig. 1. Death prevention in the A8 primordia and in A8 male migratory cells. *In all figures, white bars correspond to 50 μm; sex symbols are indicated in the upper right angle. In this figure, white dots highlight A8 primordium.* **(A-B)** *A8 migratory cells (arrows) shown by different Gal4 drivers.* **(C)** *Control.* **(D)** *Control.* **(E)** *Experimental. Note that the prevention of death caused the female A8 cells to migrate to A9 and the cells neighboring them to be dividing (asterisks). Ectopic Wg expression co-expressed with PH3 (white arrows)* **(C"- D"-E").** **(C'"-E'")** *Z projections for PH3 staining. The wt total proliferation* **(C'")** *increased in* puc^{E69}*/+* **(D'")** *and with prevention of death* **(E'")**. **(F)** *Proliferation did not change with only prevention of death, but it did in a* puc^{E69}*-/+ background, however, the differences were not significative (p>0.01).* **(G)** *The proliferation in A9 was highly variable due to death prevention in A8 migrating cells and with the addition of* puc^{E69}*-/+ background.* **(H-I)** *The A8 cells in A9 increased in direct relation to the stringency of death prevention.* **(J)** *Control.* **(K)** *Control.* **(L)** *Experimental. Notice the* puc^{E69}*/+ condition alone or with death preventing conditions increased the A8 cells in the A9 respect to* **(J).** *Proliferation in A9 occurred in the neighborhood of A8 cells or among them* **(K-L).** **(J'-L')** *Ectopic Wg expression co-expressed with PH3, arrows and rectangular highlighted areas* **(J"-K"- L").** *The asterisk points to co expression of Wg- PH3 in the A8 primordium.* **(J'"- L'")** *Z projections for PH3 staining. The total proliferation increased from* **(J'")** *to* **(L'")**. **(M-N)** *The total proliferation showed the same tendencies than in the primordia and also the differences were not significative (p>0.01).*

The death pathway is well known in *Drosophila* (review in Ayra and White, 2015). The products of the pro-apoptotic genes *reaper (rpr), head involution defective (hid)* and *grim* (RHG), *sickle* and *jafrac2* promote activation of the enzymes caspases, the effectors of apoptosis. In most cases of developmental death analyzed, mediation of JNK pathway is required (review in Igaki, 2009). JNK is an activator of pro-apoptotic genes (Moreno *et al.,* 2004; Benitez *et al.,* 2010) and establishes with their products positive feedback loops (review in Igaki 2009). However, not all death may be mediated by JNK, and RHG have other activators (review in Igaki, 2009; Ayra and White, 2015). Death and its associated signaling proceed through independent pathways, although both share factors. Probably these factors summoned for the death process display the signaling nonspecifically (Morata *et al.*, 2011). The death factors involved in the secretion of proliferative signals are as follows: the stress activated protein p53 (Well *et al.*, 2006), the caspase Dronc (Huh *et al.*, 2004; Well *et al.*, 2006),) in certain contexts Drice (Fan and Bergmann, 2008), the JNK pathway (Ryoo *et al.*, 2004) and the pro-apoptotic genes (Well *et al.*, 2006). For secretion of the death/growth signal Egr, JNK is required (review in Pérez-Garijo and Steller, 2015). It is remarkable the pivotal action of JNK in apoptosis, the signaling associated and in the promotion of growth.

In general, death signaling has been studied by the experimental induction of death, so it is interesting to analyze physiological death with respect to this subject. One way to study this phenomenon is to select a structure in which death normally occurs and impose experimental preventions in regions (Benitez *et al.*, 2010) or genetic mosaics (Arias *et al.*, 2015) with the objective of preventing or promoting this process and examining the effects. In practice, under these experimental conditions, two types of cells can be generated: undead cells and cells with reinforced death prevention. It is interesting to note the effects of undead cells derived from the prevention of physiological death. The cells with increased survival have a growth advantage. In this sense, when confronted with neighbors less protected, cells of this type induce death via cell competition (Arias *et al.*, 2015). It would be interesting to assess whether competitive death is or not associated with proliferative signals and in any case which are the effects.

The genital disc meets the characteristics to test the relevance of physiological death because it occurs during development (Benitez *et al.*, 2010). It is a bilateral structure a useful characteristic to follow growth. Male and female discs were formed by the same precursor cells (abdominal cells of the eighth, ninth and tenth segments, A8, A9 and A10), but the sex genes impose sexual differences in terms of growth/patterning (review in Estrada *et al.*, 2003). Individualization of sex growth differences broadens the study of growth. One such difference is the regulation of *hid*; that is down-regulated in females' genitalia in contrast to males' genitalia (Benitez *et al.*, 2010). We need to make a correction to that previous work specifying that *hid* in the analia does not exhibit sex regulation. Another difference in males is that A8 cell death does not occur during the larval period; these cells rarely divide, scarcely growth and are migratory. A complete opposite behavior occurs in the same cells of the female (Benitez *et al.*, 2010). Independently of sex, the disc size is the product of the coordinated growth of the three primordia (Gorfinkiel *et al.*, 2003). Death occurs in the larval and pupal stages (Benitez *et al.*, 2010; Arias *et al.*, 2015); however, its detection as apoptotic nuclei is quite rare. The causes seemed to be the rapid speed of the process and the engulfment of apoptotic corpses (review in Ayra and White, 2015). However, since cells target to death can produce and secrete mitogens signals, which should necessarily be augmented if the execution of death is interrupted, their detection and effects are other ways to infer the occurrence of death.

We manipulated the death pathway in primordia, in the domain of Dpp and in genetic mosaics. In the first two regions, we interfered with the death process at the level of JNK, *hid,* the caspases Dronc and/or Drice and with the anti-apoptotic baculovirus' protein p35. In some of these experiments, we added a genetic background of reduced repression of JNK activity *(pucE69*/+) (Martín-Blanco *et al.*, 1998). More JNK activity, depending on the genetic context and experimental conditions, will promote growth, death, death or undead signaling. We analyzed null genetic mosaics for the RHG genes (homozygous DfH99 cells) (Grether *et al.*, 1995) and for the unique *Drosophila* JNK, Basket (Bsk) (Riesgo-Escovar *et al.*, 1996). In the first case, cells targeted to die could not do the death pathway but could signal; in the second case, the opposite behavior might be observed. Finally, to weigh the growth role of JNK, we induced clones DfH99 in a null background for Egr and Bsk^- clones in a *pucE69*/+ background. Since death and undead events can be accompanied by proliferation, we centered our analysis on the measurement of proliferation, symmetry/asymmetry and size.

Results

Death prevention in A8 primordia and A8 male migratory cells

The A8 driver in males allows the visualization of A8 cells in the A9 primordium, suggesting that they are migratory (Fig. 1A, arrows). Their existence is supported by another A8 driver, teashirt-Gal4, which displays the same pattern (Fig 1B, arrows). The migratory characteristic arises from the prevention of apoptosis in these cells (Benitez *et al.*, 2010).

Despite preventing death in the females A8 primordium produced overgrowth in discs (Fig. 1E; compare with C,D) and adults (Benitez *et al.*, 2010) this was not reflected in the quantification of the proliferation (Fig. 1F). To solve the contradiction and with the object to favor the undead cells secretions we did the same experiments but in a background with reduced repression of JNK, *pucE69*/+. In accordance, the proliferation became more variable (Fig. 1F). The lack of a pronounced increase in proliferation could be due to two circumstances: first, delay in development that prevention of death produces (Wells *et al.*, 2016) which probably made the L3 discs sample were not homogeneous at the growth achieved and second to a growth detrimental effect due to undead cells (analyzed in next section). In support with the formation of undead cells in which JNK des-repression favors their signaling we detected ectopic Wg expression which corresponded to dividing cells (Fig. 1E). Interestingly, in the controls (Fig. 1 C,D) there were also ectopic Wg overlapping with PH3, but remarkably in these cases must correspond to death events. Therefore, female A8 death is associated with the secretion of proliferative signals. Importantly and corroborating the experimental death prevention, these cells became migratory, as occurred in males (Fig. 1E, asterisks).

In males, the prevention of death in A8 cells increased the variability in proliferation in the A9 primordium (Fig. 1G). The proliferation in the A8 primordium did not change (data not shown), in consonance, death was already prevented therein (Benitez *et al.*, 2010). Thus, in males, it is necessary to explain the proliferation in

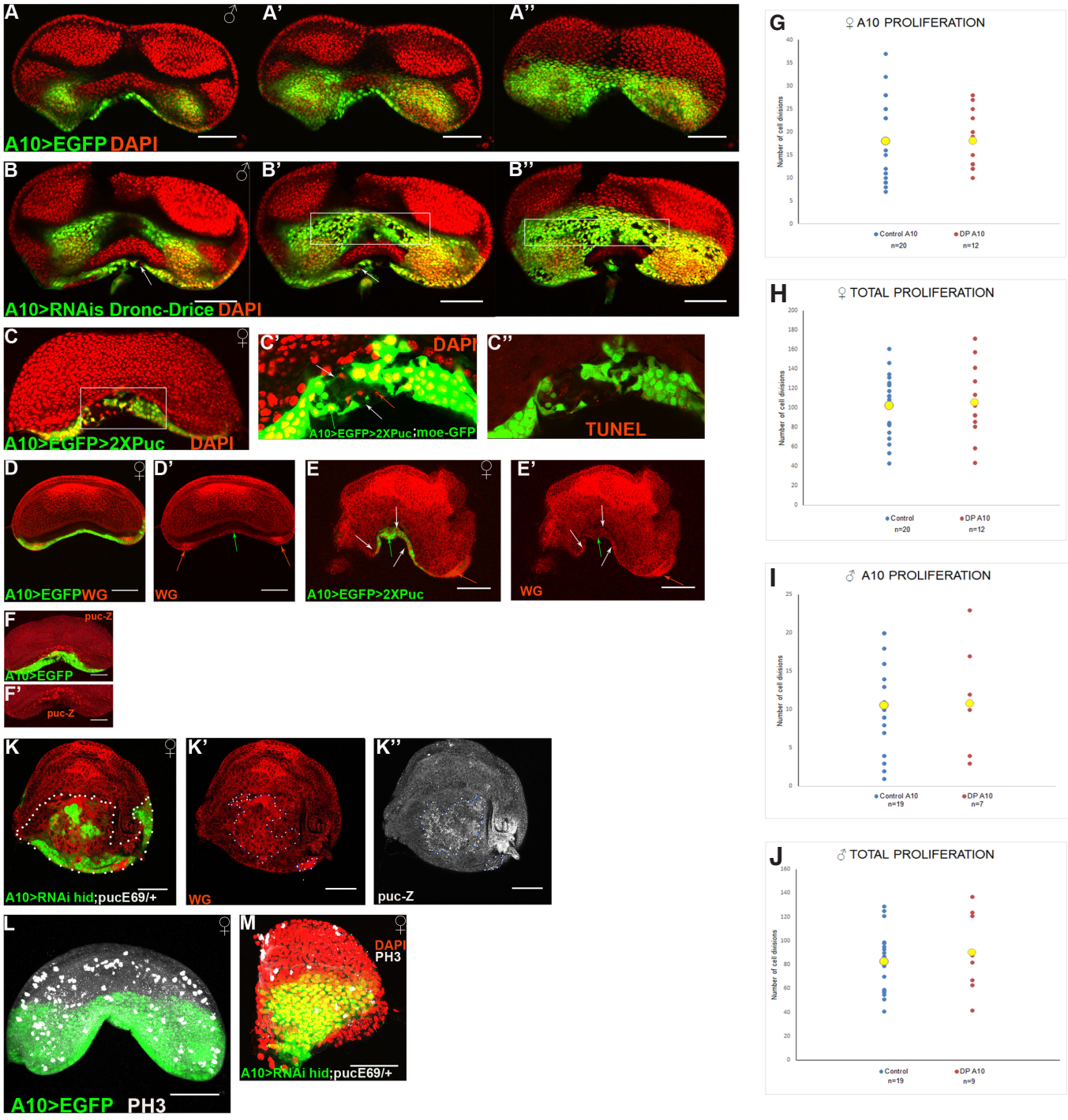

Fig. 2. Death prevention in the A10 primordium. *The A10 primordium is shown by the expression of EGFP.* **(A,B)** *Confocal Z sections in similar planes.* **(A)** *Control.* **(B)** *Experimental. Undead cells in the medial region (rectangular highlighted area) and in the A10 part of the stalk (arrows).* **(C-C″)** *Undead cells.* **(C)** *The rectangular highlighted area corresponds to the A10 part of the stalk.* **(C′)** *GFP is in the A10 nuclei (EGFP) and in the plasma membrane (Moe-GFP, white arrows). The undead cells do not have GFP in the nuclei neither in the cytoplasm. They are surrounded by plasma membrane (white arrows). The nuclei are smaller than those exhibiting GFP (red and green arrows, respectively).* **(C″)** *Undead neighbor cells showing cytoplasmic engulfed TUNEL particles.* **(D-D′)** *Wt expression of Wg in A10 (red arrows), notice in* **(D′)** *the stalk cells (green arrow) there is ectopic Wg expression.* **(E-E′)** *A10 wt Wg expression (red arrow). Undead cells secrete Wg (white arrows), but also wt neighbors cells do (green arrows). Notice that two doses of Puc could not stop the JNK's transcription of Wg.* **(F)** *Stalk cells normally express high levels of JNK pathway activity (puc-Z).* **(F′)** *Only puc-Z.* **(G-J)** *In both sexes, prevention of death in A10 did not change the proliferation in the primordium* **(G-I)** *or in the disc* **(H-J)**. **(K-K″)** *Undead cells in a* pucE69/+ *background highly impaired growth. A10 is highlighted by white dots. Notice that undead cells that do not express GFP show upregulated expressions of Wg and JNK pathway activity (highlighted by white and blue dots).* **(L)** *Control disc showing cell divisions in a plane that includes A10.* **(M)** *Tiny abnormal disc with very few cell divisions due to undead cells in A10 in a* puc^{E69}*/+ background.*

the A9 preventing death in the A8 migrating cells. The A8 cells have extensive prevention of death as a consequence of their inherent genetic conditions plus the ones we introduced. We thought that death with its proliferative signaling was mainly produced in A9 and less in A8 migrating cells for various reasons. First, the migrating cells greatly proliferated in direct relation to the stringency of the death-preventing conditions (Fig. 1 H,I), suggesting that they could be the beneficiaries of death/proliferative signaling rather than been the target of death. Second, A9 cells did not show death prevention because, in such a case, the primordium would be homogeneous for this characteristic and, thus, A9 would be equivalent to the A8 primordium, which is not the case. Third, the proliferation observed was in the A8 cells or in their vicinity suggesting A9 death took place in the neighborhood with A8 cells (Fig. 1 K,L). Consistently, the same locations were observed for cells divisions when the females A8 cells became migratory (Fig. 1E, asterisks).

Finally, and consistently the A8 cells could eventually be targeted for death, but they formed undead cells we detected ectopic Wg expression associated with PH3 (Fig. 1L, arrows and rectangular highlighted areas). As occurred in females, the detected co- expressions were seen even in the controls (Fig. 1 J,K), but in males there is the uncertainty if they came for a death or an undead cell events. In particular, we wanted to note the detection of ectopic Wg with PH3 at the edges of the A8 primordium, where cell division is normally located (Benitez *et al*., 2010), confirming that male A8 death induces proliferation (Fig. 1L, asterisk).

Since apoptosis can have non-autonomous long-range effects (Milán *et al*., 1997; Pérez-Garijo and Steller, 2015), we compared the total proliferation. Consistently with the primordia, total proliferation varied (Fig. 1 M,N; compare with F,G).

Death prevention in the A10 primordium

In the A10 primordium in both sexes, death occurs at the larval stage at a precise site in the medial region, including cells that belong to the A10 part of the stalk (Fig. 2B; compare with A). This death may be related to the development of the hindgut/anus and/or the eversion process. The stringency and time at which A10 cell death was prevented determined the size, abnormal morphology and number of undead cells produced. The A10 site-specific concentration of undead cells permitted us to describe them (Fig. 2C). The undead cells became rounded (Fig. 2C). Their nuclei were smaller (Fig. 2C'). They stopped expressing the marker EGFP, indicating low levels of translation (Fig. 2C'). The A10 undead cells did not divide, and their neighbors rarely did. Instead, their size increased (Fig. 2C'); the neighbors showed engulfed TUNEL-positive particles (Fig. 2C''), suggesting undead cells might be inducing the neighbors' death who avoided the prevention. Undead cells expressed Wg (Fig. 2 E,E', white arrows), but interestingly also did neighbors that were apparently normal (Fig. 2 E,E', green arrow). Even in wt discs these cells express Wg (Fig. 2D', green arrow) in coincidence with high JNK activity (Fig. 2 F,F'). The expression of Wg in stalk cells, which have not yet been targeted for death, admits that death signaling is a readout of JNK's activation and does not belong to the death process (Morata *et al*., 2011).

In accordance, undead cells did not divide and non-undead neighbors rarely did, the proliferation in the primordium and the disc did not change (Fig. 2 G-J). However, A10 death signaling was certainly demonstrated in a *pucE69*/+ background (Fig. 2 K,M), which provoked a great impair to normal growth, probably

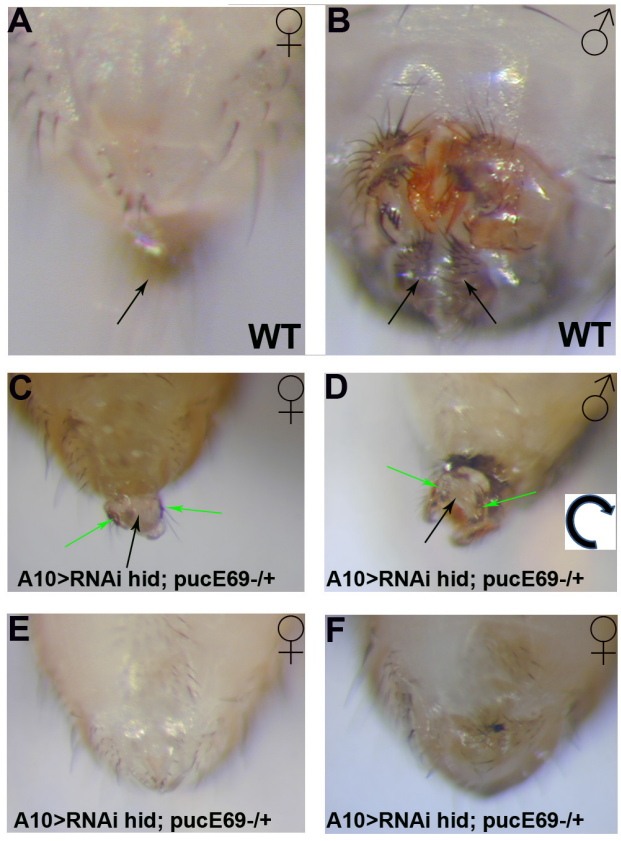

Fig. 3. Adult terminalias. (A,B) *Wt female and male analias (black arrows).* **(C-F)** *A10> RNAi* hid; puc^{E69}*/+ adults.* **(C,D)** *Notice the abnormal growth of undead cells (black arrows) and reduced growth of normal tissue (green arrows).* **(D)** *The prevention of death in A10 perturbs the 360° rotation of the male terminalia; the inset shows the rotation done.* **(E)** *The analia is lost.* **(F)** *The terminalia is lost.*

because the signaling disrupts the endogenous Wg-Dpp gradient (Fig. 2K') (Adachi-Yamada and O'Connor, 2002). In accordance, very few were the dividing cells in the A10 and outside (Fig. 2M; compare with L). Progressively, all cells could die, as shown by the adult phenotypes (Fig. 3 C-F; compare with A,B), remarkably including the undead cells. The impaired to normal growth shown by A10 undead cells signaling justified the delay in developmental prevention of death produces (Wells *et al*., 2003).

Competitive death mediated by JNK

We recently proposed that growth proceeds in the genital disc by intercalation at the Dpp discontinuity through competitive death (Arias *et al*., 2015). In support of this proposition, the prevention of death in the Dpp domain increased proliferation (Fig. 4B; compare with A). However, in the analyzed sample, the proliferation was highly variable with not statistical significant differences (Fig. 4 C,D). The variability was consistent with the formation of random undead cells whose signaling induces proliferation, but in turn delays development and ultimately reduces growth. In agreement, the corresponding adults were smaller (♀-11% and ♂-17% in the length of A/P axis) (Fig. 4E).

Another way to see the proliferative effect of death is to measure

proliferation in the discs where we induced null genetic mosaics for the RHG genes. Since clones that were null for the RHG genes outgrew their twins (Arias *et al.,* 2015) and competitive death was associated with proliferation (Fig. 4 B-D). The imposed cell competition should increase proliferation, which was what occurred in both sexes. Remarkably in this case the differences were statistical significant (Fig. 4 F,G). In accordance, proliferation is link to competitive death it was commonly detected at the boundaries of clones, involving clones, twins and background cells (Fig. 4H). However also appeared clearly 'undeads' among null RHG cells detected by ectopic Dpp expression overlapping with dividing cells (Fig. 4 I,I'). It is important to note the similarities of this growth scenario with that of the male A9 primordium and the A8 migratory cells. The A8 cells can be equated to the null RHG cells and the A9 cells to their twins.

Effect over symmetry

In bilateral structures such as the genital disc, there is normally a fluctuation among left and right growth (Donge, 2006), so the halves are similar but not identical. In the genital disc, we had to include characteristics appropriate for each sex that influence growth /symmetry. For example, we found that in wt discs, females are symmetric in a major proportion compared with males (shown below in Fig. 5 I,J).

We demonstrated in the genital disc death and undead cells secrete proliferative signals such as Wg-Dpp (Figs. 1,2,4). Considering death should vary among each half, the prevention of death that lead to undead cells or genetic conditions that facilitate death with signaling associated should conduct to asymmetrical growths. Examples of asymmetry due to undead cells were derived from the prevention of death in the female A8 primordium (Fig. 5B), in the A10 primordium (Fig. 5D) and in the Dpp domain (Fig. 5F) in both sexes. Examples of asymmetry due to dead and undead cells were produced by the prevention of death in male migratory A8 cells (Fig. 1L) and induction of the RHG null clones (Fig. 5 G-J). Finally asymmetries that corresponded exclusively to difference in the amount of death with signaling associated was that observed in females wt and *pucE69* / + (Fig. 5 I,J; 1D). The asymmetric growth affected the three primordia even though the prevention was executed in one primordium, supporting the long range effects of death signaling and coordinate growth of the three primordia (Milán *et al.*, 1997; Pérez-Garijo and Steller, 2015; Gorfinkield *et al.*, 2003).

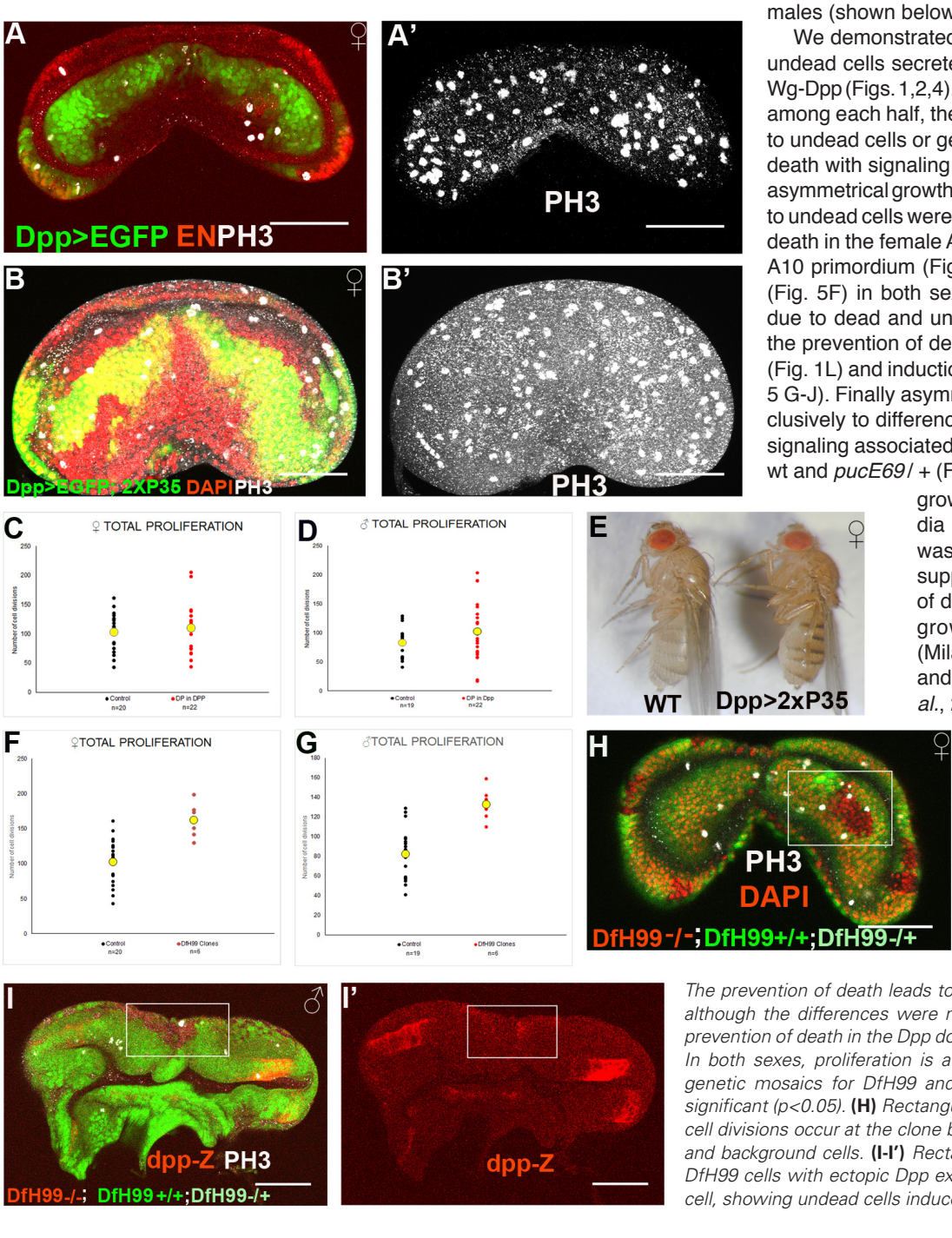

Fig. 4. Competitive death with proliferative signaling. **(A,B)** *The Dpp domain is shown by EGFP.* **(A)** *Control.* **(B)** *Experimental.* **(A'-B')** *Z projections. The cell divisions are augmented due to death prevention in* **(B')**. **(C,D)** *The prevention of death leads to much more variable proliferation although the differences were not significative (p>0.01).* **(E)** *The prevention of death in the Dpp domain reduces the adult size.* **(F,G)** *In both sexes, proliferation is augmented in discs with induced genetic mosaics for DfH99 and the differences are statistically significant (p<0.05).* **(H)** *Rectangular highlighted area showing that cell divisions occur at the clone border and involve the clone, twin and background cells.* **(I-I')** *Rectangular highlighted area showing DfH99 cells with ectopic Dpp expression together with a dividing cell, showing undead cells induced proliferation.*

To corroborate these findings, we proposed to interfere with the signaling and not with death. We based on the following: JNK is necessary for death signaling (Ryoo *et al.*, 2004), and JNK-independent death has been demonstrated (de la Cova *et al.,* 2004; review in Igaki, 2009). In accordance, the induction of Bsk$^-$ clones gave rise to completely symmetric discs in males and a high proportion in females (Fig. 5 I-L), the opposite to the induction of DfH99 clones (Fig. 5 G,H; compare with K,L; see also I,J). The symmetric growth supported a JNK-independent death without signaling. Considering Bsk$^-$ clones grew but to a reduced extant than their twins (analyzed in next section, Fig. 6A), even they were eliminated, competitive death was directed to the clone cells, but this necessary occurred without signaling. In agreement, clone cells died without signaling, cell-free spaces were observed among the clones (Fig. 5 N-N").

JNK's growth function

Recently it has being demonstrated this role of JNK in the wing and the antenna discs (Willsey *et al.*, 2016), so we decided to test this function in the genital disc. We perturbed directly the JNK activity inducing Bsk- clones or indirectly affecting JNK's regulators: RHG, Egr or *puc*.

We observed differences and similarities in the growth of clones *vs* twins in relation to the genotypes, sex, region of growth and time of clone induction. Respect to the sex, the females' genitalia (A8) was the most affected due to less or null JNK activity, in accordance with, the downregulation of *hid* in those cells. The time of clones' induction resulted relevant due to the JNK pre-existing protein, especially because the JNK growth function is exerted exclusively in the cytoplasm (Willsey *et al.,* 2016). In accordance, the late-induced Bsk- clones grew widely (Fig. 5 K,L) while the early ones were not present so died (Fig. 6A, white highlighted area and arrow)

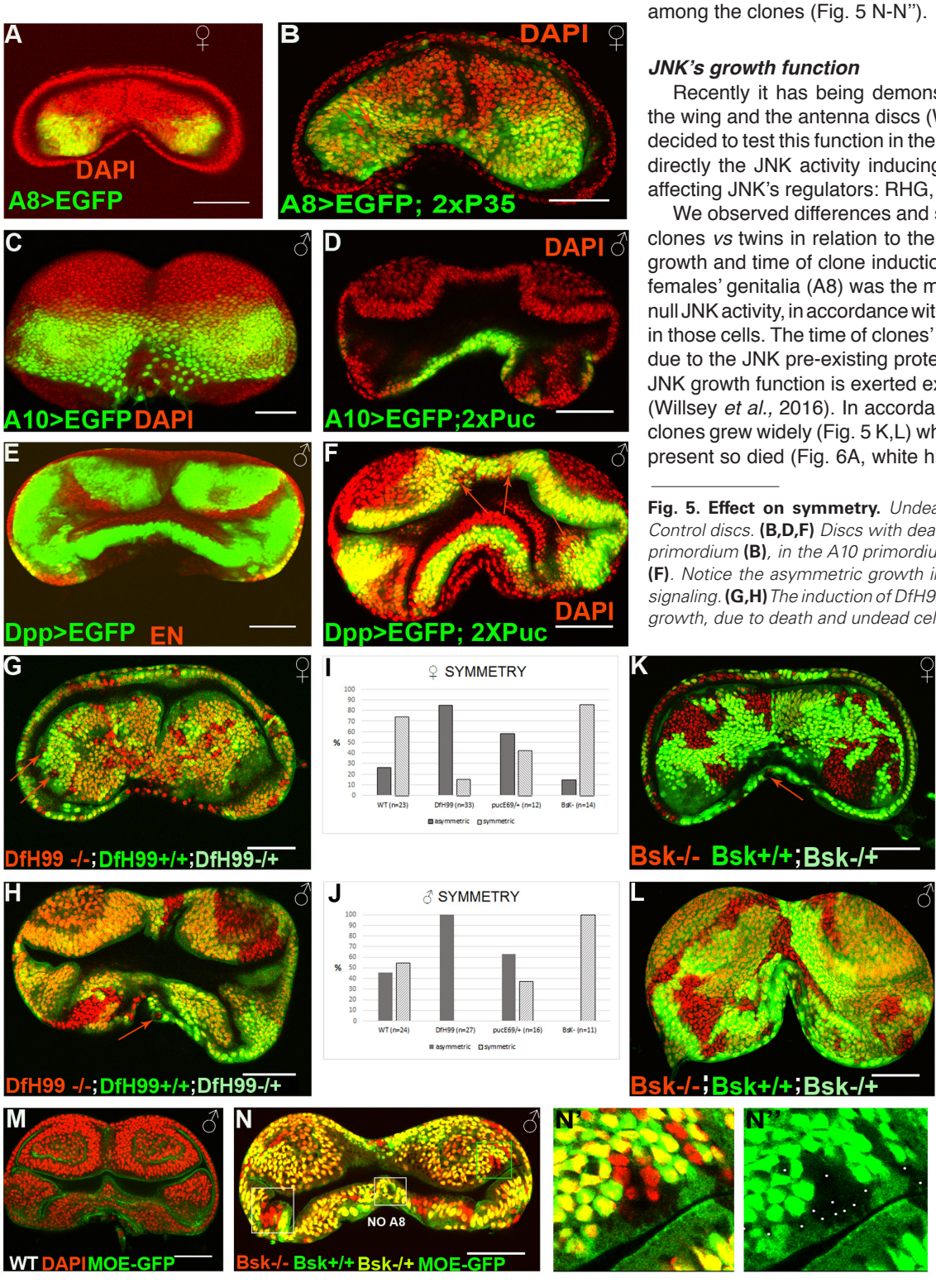

Fig. 5. Effect on symmetry. *Undead cells (red arrows).* **(A,C,E)** *Control discs.* **(B,D,F)** *Discs with death prevented in the female A8 primordium* **(B)**, *in the A10 primordium* **(D)** *and in the Dpp domain* **(F)**. *Notice the asymmetric growth in* **(B,D,F)** *due to undead cells signaling.* **(G,H)** *The induction of DfH99 clones provokes asymmetric growth, due to death and undead cells signaling.* **(I,J)** *Comparative percentage graphics of left-right asymmetry/symmetry. Note the opposite bilateral growth among DfH99 and Bsk$^-$induced clone discs, and that the* puc^{E69}*/+ background increases the asymmetry in both sexes.* **(K,L)** *The induction of Bsk$^-$ clones provokes symmetric growth.* **(M)** *Wt tissue organization principally demonstrating by the nuclei disposition (DAPI) and the plasma membrane (construct Moe-GFP).* **(N)** *Tissue organization in a disc with induced Bsk$^-$ clones, which carries the Moe-GFP construct. Notice the cell-free spaces (rectangular highlighted areas). These spaces correspond to clones areas.* **(N'-N")** *Amplified rectangular green highlighted area in* **(N)**. **(N")** *The white dots indicate cell-free spaces, compare with* **(N')**.

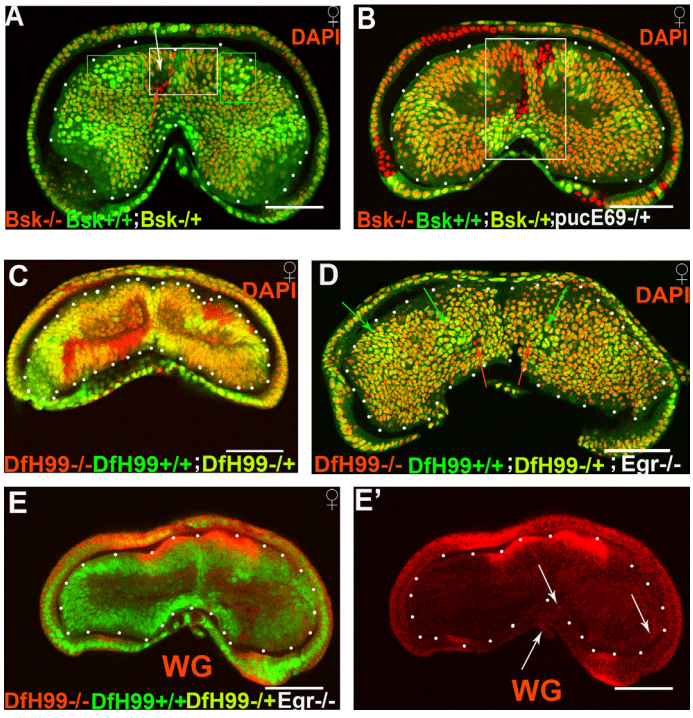

Fig. 6. JNK's growth function. *The A8 primordia highlight with white dots.* **(A)** *The Bsk⁻ clones cells die, leaving cell-free spaces (white rectangular highlighted area and arrow); only few clones cells persist (red arrow), or only twins remain (green rectangular highlighted area).* **(B)** *The* puc^{E69}*-/+ background allows growth of the Bsk⁻ clone cells in a major proportion (white rectangular highlighted area).* **(C)** *Conspicuous growth of DfH99 clones and their respective twins in the female A8 primordium.* **(D)** *The clones DfH99 cannot grow in the female A8 primordium in a null background for Egr. Only few clones cells remain (red arrows), or only the twins are present (green arrows).* **(E-E')** *Undead cells Egr⁻DfH99 expressing Wg (white arrows).*

persisting only the twins (Fig. 6A, green highlighted areas). To confirm these findings, we performed the same experiment in a *pucE69*/+ background. The background was sufficient to allow e.g. in the female A8 primordia more clones cells persisted at the time of analysis (Fig. 6B, highlighted area; compare with A). These results showed the fundamental function of JNK for growth (Willsey *et al.*, 2016) but not for death. Remarkably the Bsk- clones and their respective twins grew in a particular region that involved the bilateral axis (Fig. 6 A,B; see also 5 K,L).

The lack of RHG products as being positive regulators of JNK (review in Igaki, 2009) might impair its activity over growth; however, this did not seem the case as DfH99 clones conspicuously grew (Fig. 6C) (Benitez *et al.*, 2010). However, in a null background for the JNK activator, Egr, the growth of DfH99 clones was severely affected (Fig. 6D, red arrows; compare with C) even were eliminated specially in the female genitalia persisting only the twins (Fig. 6D, green arrows). Although some Egr⁻; DfH99 cells expressed Wg showing they had JNK activity (Fig. 6 E-E', white arrows), the majority of Egr-, DfH99 cells died (Fig. 6D) pointing the lack of Egr and the RHG products are enough in certain contexts to cancel the JNK activity. Interestingly, clones and twins growth of Egr-; DfH99, were circumscribed to the same region as those of Bsk- (Fig. 6 A-D), indicating the JNK's growth function is important at that region but even more outside, where constitutive JNK activity is essential.

Competitive death independent of JNK

Previous data showed that competitive death can occur independently of JNK (de la Cova *et al.*, 2004), so it should occur without signaling (Fig. 5N; 6 A,B). To analyze this, we focus on the male A8 primordium, where death is physiologically prevented at larva (Benitez *et al.*, 2010). Consequently, the physiological growth conditions must necessarily be uniform to prevent cell competition. In fact, the induction of clones that affect growth so break with uniformity, like DfH99 or Bsk⁻, caused the irruption of clones' death but without signaling since it left cell free spaces (Fig. 7 A,B). The first to die were the clones, followed by the rest since the primordium could be eliminated (Fig. 7 C,D). Competitive death without signaling (clones' death) or very scarce signaling (twins and background cells death) which does not induce proliferation could explain the elimination of the primordium. The Bsk⁻ cells die without signaling (Fig. 5 N-N") but not the DfH99 cells (Fig. 4 I,I'). However, in this context the DfH99 cells behaved identical to the Bsk⁻ cells, suggesting in practice they are Bsk⁻. Considering, only the lack of RGH products is enough to cancel the JNK activity, normally in the primordium it must be very low, which explains the scarce growth and eventual scarce signaling associated to death. Indeed in the Egr⁻ null background, the induction of clones DfH99 made the A8 primordium never appeared at L3 discs (data not shown). Since prevention of death impeded cell competition (de la Cova *et al.*, 2004) we induced Bsk⁻ clones only in the A8 primordium with the simultaneous expression of the p35 anti-apoptotic protein. In support, the primordium was preserved, clones, twins and background cells grew together (Fig. 7E), but the clones tended to die (Fig. 7 E',E"). In this case, the clones' death was independent of JNK and caspases (reviewed in Igaki, 2009).

Prevention of death and its effect on disc size

First, it is important when considering the disc size to note that males are usually larger than females (Fig. 7 F,G). As undead cells could produce a drastic reduction of normal growth (Fig. 2 K-K", M; 3 C-F; 4E), the death prevented discs were on average, smaller than in wt (Fig. 7 F,G). In accordance, the prevention of death in the A10 primordium in a *pucE69*/+ background had the greatest effect on the reduction of size (Fig. 7 F,G) and proliferation (Fig. 2M). In more ample regions such as prevention in the Dpp domain, it reduced the size of the adults (Fig. 4E). Curiously, the death of Bsk⁻ clones did not affect the size (Fig. 7 F,G); indicating that such losses were compensated in some way.

Discussion

In this work, we showed developmental apoptosis in the genital disc, which included death by cell competition was linked to proliferative signaling and both processes were dependent on JNK. In consonance, the proliferative signaling became more evident when we prevented death and simultaneously des-repressed JNK activity, allowing seeing the ectopic expression of Wg or Dpp overlapping dividing cells or positive point for PH3. Under the latter genetic conditions, the *per se* wt variable proliferation was more variable and growth was markedly asymmetrical. These phenomena were particularity evident in tissue with active growth, such as the genitalia. In contrast, suppression of JNK activity, i.e., elimination of signaling but not death, resulted in symmetrical growth. In our experiments, the variation in proliferation and asymmetry was not

always derived from undead cell signaling, but it also occurred through death signaling. Such came for A9 cells death due to the interaction with A8 migratory cells; twins' death in interaction with DfH99 cells. In the absence of JNK, we detected independent death pathways (review in Igaki, 2009) that proceeded without signaling so they never give rises to undead cells. We corroborated the involvement of JNK in the promotion of growth (Willsey *et al.*, 2016), but we demonstrated that it is fundamental because the cells die in its absence, especially outside of a particular region where constitutive activity is essential.

Competitive and non competitive death

We have detected three genetic growth contexts during development, in which death was used or not and occurred under different circumstances. The first context occurred in the genitalia primordia (female A8 and male A9), the second occurred in the A8 male primordium and the third occurred in the analia primordium (A10).

What are the differences among these events of death? To answer this question, we are going to link the death in the genitalia primordia with its lack in the male A8 primordium compared with those in the analias.

The death in the genitalia primordia occurs at random by cell competition and its signaling may favor growth. The proliferative signaling due to JNK dependent death causes the loss to be replaced by more than one cell, but more importantly, it simultaneously may contribute via extra growth impulses to the established growth discontinuities, leading to more death and more growth.

The cause of the competition was shown, especially in males in which A8 migratory cells surely establish competition with their A9 neighbors due to their prevention of death, which is a growth advantage. This phenomenon was better visualized with the growth behavior of other death prevented cells, the nulls for the RHG. As RHG null clones outgrew their twins, a similar scenario was proposed for the interaction among A8 and A9 cells. The contribution to growth of competitive death was shown by the fact A8 migratory cells proliferation augmented in direct relation to the stringency of their death prevention and cell division were detected in them or their vicinity. Similarly, the clones DfH99 had a huge growth, the proliferation surrounded their borders and the discs showed a significant increase in the proliferation. We show apoptosis induces proliferation since we detected overlapping expressions of ectopic Wg with PH3 in females, wt and *pucE69* / +, which in this sex could only come from death signaling events.

In genital discs, the discontinuity of Dpp activity (Arias *et al.*, 2015) is the site where competitive death signaling may make its growth effects. Additional discontinuities are the A8 migratory cells in the male genitalia (A9). In this sense, the female genitalia are comparatively less discontinuous than male genitalia. In accordance, females are symmetric in a major proportion compared with males, but males are larger than females.

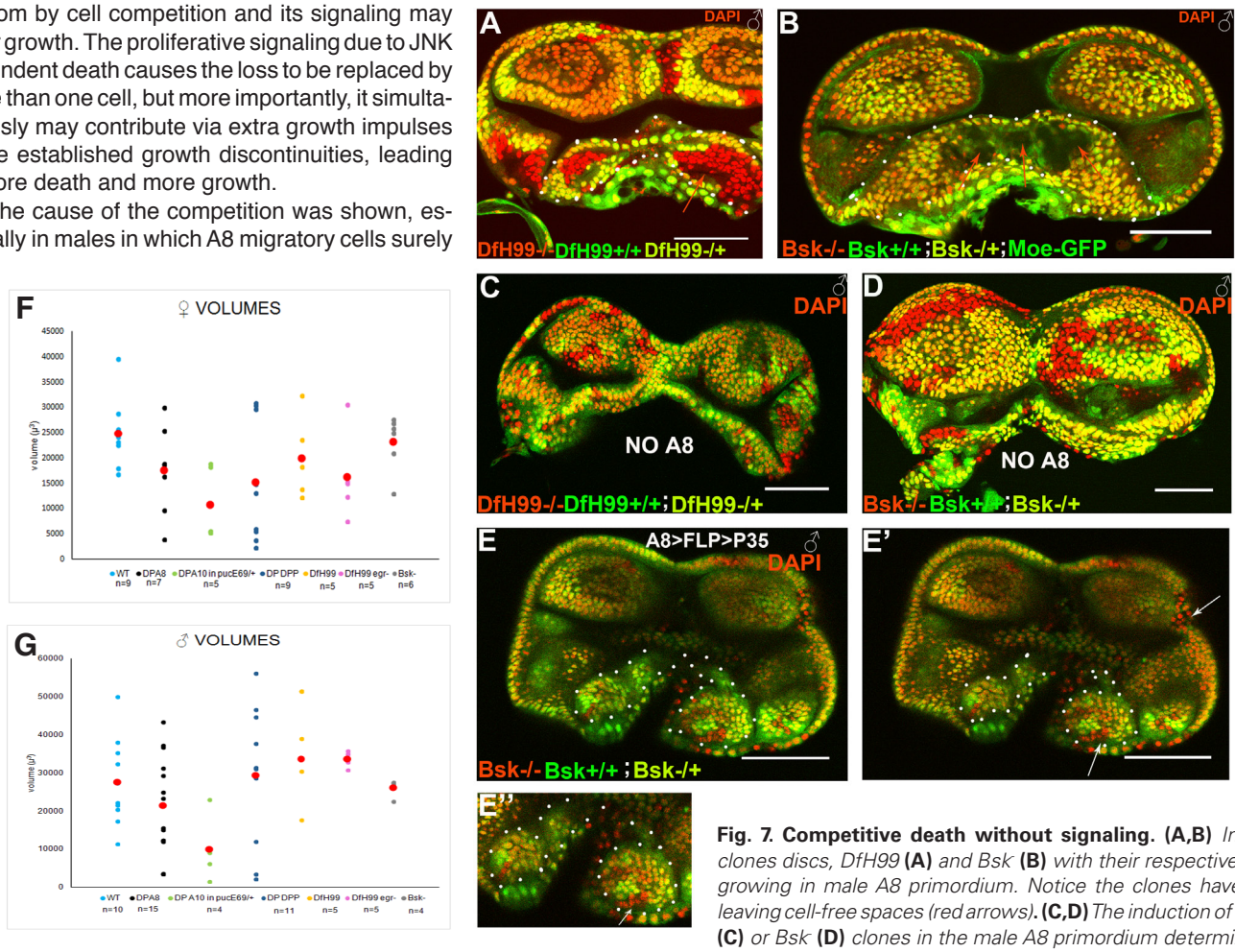

Fig. 7. Competitive death without signaling. (A,B) *Induced clones discs, DfH99* **(A)** *and Bsk*$^{-}$ **(B)** *with their respective twins growing in male A8 primordium. Notice the clones have died, leaving cell-free spaces (red arrows).* **(C,D)** *The induction of DfH99* **(C)** *or Bsk*$^{-}$ **(D)** *clones in the male A8 primordium determines its elimination.* **(E-E")** *The prevention of death by p35 in the A8 male primordium avoided cell competition among the clones Bsk*$^{-}$ *with their respective twins and cells of the background.* **(E')** *Another z section of the same disc showing that the clone cells died, leaving cell-free spaces in the A8 and also in the A9 (white arrows) probably due to Bsk*$^{-}$ *A8 migrating cells death.* **(E")** *Amplification of* **(E')**. **(F,G)** *The prevention of death provoked a reduction in disc size which it was significative only when preventing death in the A10 primordium in a* puc^{E69}*/+ background (p<0.05) and did not change in the Bsk- discs.*

In opposition and to better illustrate the importance of the proliferative signaling by competitive death, it is the male A8 primordium. There the induction of clones DfH99 or Bsk$^-$ provoked cell competition but competitive death occurred without signaling which in turn, ended with growth and eliminated the primordium, highlighting the importance of JNK mediated death in cell competition.

In the analia, in contrast to the genitalia, the death detected with its proliferative signals occurred at a specific site, far from the A10 Dpp discontinuity, and was not due to cell competition. In accordance, death prevention in A10 did not change the proliferation in the primordium or in the disc. However, when JNK was de-repressed, the undead cells signaling greatly impair normal growth in their benefit. They could do this, by contrary to the genitalia where they provoked overgrowth (Benitez *et al.*, 2010 and present work); because they are concentrated in a site and the secretions of Dpp- Wg disrupt the endogenous growth gradient due to the same molecules (Adachi-Yamada and O'Connor, 2002).

Pleiotropic activities of JNK

We analyzed JNK functions: growth, death and death signaling. All these functions imply different regulatory pathways. There are three proposals concerning how a molecular factor such as JNK displays different activities (review in Stronach, 2005). One comprises intermediate factors, another subcellular location and the last activity levels. Our results pointed to levels, to which it is necessary to add the subcellular location previously demonstrated (Willsey *et al.*, 2016). With respect to levels, Egr is a JNK activator, the loss of which does not affect the phenotype (Igaki *et al.*, 2002). However, in an Egr$^-$ background, RHG null cells could not grow and died. Since Reaper is also a JNK activator, we searched for the intermediate factors for Rpr and Egr, and they are different (review in Igaki, 2009). Consequently, because the lack of both affected growth, the activation levels seemed to be important. In agreement with the level mechanism, the female genitalia in which the levels of JNK were lower than in males due to the downregulation of *hid*, showed major effects of a downregulation or derepression of JNK.

From our data, it follows that the highest to the lowest JNK levels required for the different activities are death, death signaling and growth. We based on these findings: repression of JNK by directing permanent expression of the phosphatase Puc even in two doses, death was the only function that was impeded e.g., the direction of 2x Puc in A10 in both sexes. To abrogate death signaling and growth functions, it was necessary to eliminate JNK. When JNK was absent, the cells could die but without signaling. In summary, since cells can die independently of JNK, the fundamental roles of JNK are as follows: growth and death signaling. However, the signaling resulted from JNK activation and is not necessary associated to death (Morata *et al.*, 2011). In this sense, stalk cells which eventually could die have high expression of JNK and express Wg. However, we showed JNK mediated death is very common among developmental death in the genital disc and the signaling resulted essential under cell competition.

Finally and to highlight the importance of JNK as a factor for growth and death we want to stand out the opposite growth behavior of Egr$^-$ RHG cells with RHG alone. It is probable that when the majority of the pro-apoptotic products are absent (highly modulate at wt conditions) (review in Ayra and White, 2015) like in the RHG$^-$ cells, the action of Egr- JNK is aimed at activating Hippo's growth mechanism. In this sense the RHG$^-$ cells may owe their competitiveness to a route that favors growth and protects them from dying, respect to their neighbors RHG+/+ or RHG-/+ less protected and more prone to die. Consequently, the regulation of JNK and the modulation of the RHG genes and products appeared fundamental to grow or die; to generate or not cell competition and in the control of size.

Materials and Methods

Genetic strains

The directed gene expression experiments were performed using the Gal4/UAS method (Brand and Perrimon, 1993). The Gal4 insertions used were *Abd-B*^{LDN} (de Navas *et al.*, 2006) referred to as A8 and used to prevent death in A8 cells, *caudal* (Calleja *et al.*, 1996) referred to as A10 and used to prevent death in A10 cells, and Decapentaplegic (Bloomington Center). The UAS transgenes were UAS-EGFP (Bloomington Center); UAS-Puc (Martín-Blanco *et al.*, 1998); UAS P35 (gift of G. Morata) and UAS-RNAi lines against Dronc, Drice and Hid (Vienna *Drosophila* RNAi Center). The mutant alleles were the *pucE69* allele (Martín-Blanco *et al.*, 1998), *egr1,* and *egr3* (Igaki *et al.*, 2002). There were no phenotypic differences among the *egr* alleles. The allele *pucE69* is a βgal reporter gene for *puc* and indirectly for JNK (Martín-Blanco *et al.*, 1998), called *puc-Z*. The reporter of Dpp is a construct: the βgal gene with the Dpp promoter (Bloomington Center).

Death prevention conditions tested by the Gal4/UAS method

The directed death prevention conditions were ordered in relation to their effects, from the highest to the lowest, as determined by the discs and adult phenotypes. The conditions highlighted in bold were also tested in a *pucE69*/+ background.

A8>2xPuc; A8>Puc; **A8>RNAi hid***; A8 > P35; A8>2x P35; A8>RNAis Dronc and Drice; **A8>RNAi Dronc; A8> RNAi Drice**. (*) Used only in males.

A10>RNAi hid; A10> 2xPuc; A10>Puc; A10>P35; A10>2xP35; A10>RNAis Dronc and Drice; A10>RNAi Dronc; A10>RNAi Drice.

Dpp>2xPuc; Dpp>Puc; Dpp>2P35; Dpp>Puc and RNAi Dronc; Dpp>RNAi Dronc.

Overexpression and clonal analysis

The late L3 (crawling larvae) discs were analyzed by immunohistochemistry. The UAS constructs were induced from the larval stage at 31°C.

Mutant clones for *Df(3L)H99* (Grether *et al.*, 1995) and Bsk^1 (Riesgo-Escovar *et al.*, 1996) were induced with FRT-FLP and FLP-FRT-Gal4/UAS combined methods (review in Blair, 2003). L3 discs of the following genotypes were analyzed: 1) *y, w, hs-flp/+*; *Df(3L)H99* FRT2A/*Ubi*–GFP FRT2A; 2) *y, w, hs-flp/+*; *egr-*; *Df(3L)H99* FRT2A/*Ubi*–GFP FRT2A; 3) *y,w, hs flp*/+; *bsk1* FRT40/*Ubi*-GFPFRT40; 4) *y,w, hs flp*/+; *bsk1* FRT40/*Ubi*-GFPFRT40; *pucE69*/+ 5) A8Gal4>UASFLP>UASp35; *bsk1* FRT40/*Ubi*-GFPFRT40. The *bsk1*$^-$ FRT40 recombinant chromosome was corroborated by the embryonic Bsk$^-$ phenotype. The crosses were maintained at RT, and the clones were induced at L2 by heat shock at 37°C for 15'. For the combined Gal4/FLP methods, the larvae were grown at 30°C.

Antibodies and immunohistochemistry

The following primary antibodies were used: rabbit anti-PH3 (Cell Signaling Technology), monoclonal anti-Wingless (Developmental Studies Hybridoma Bank); monoclonal anti-βgal (Sigma) or rabbit anti-βgal (Promega). We used fluorescent secondary antibodies from Invitrogen. Nuclei were detected by DAPI and the mounting medium was provided by Vectashield-Vector Labs. The TUNEL reaction was performed using an *in situ* apoptotic detection kit from Roche.

Image collection and processing

Fluorescent images were obtained using an Olympus FV1000; FV1200 confocal microscope during multitrack sessions and processed using the

FV10-ASW4.0 Viewer; Mac Bio photonics collection of the ImageJ plugin; Imagen J. Jar and Adobe Photoshop CS4 software programs.

Estimation of volume and statistical analysis

The volume was estimated by multiplying the average area of five cutting planes (x and y-axes), which were equally spaced, by the thickness of the structure (axe z), with subsequent transformation to microns. The area of each plane was measured with the function available in ImageJ, manually trimming the outline of the structure on images obtained with the Olympus FV1000- FV1200 confocal microscope.

Cell divisions, PH3 positive spots, were counted using ImageJ tool Multipoint selection, plane by plane along the z-axes. The planes were separated by 4 μm. The number of cell divisions was compared using the Mann-Whitney test for independent samples implemented in INFOSTAT (Di Rienzo *et al*., 2018).

Acknowledgments

We wish to thank Ginés Morata, Ernesto Sánchez-Herrero, Angélica Cantarero, and the Bloomington and Vienna Stock Centers for providing us with flies. We thank the ServicioNacional de Microsopía *for the use of their confocal facilities and Cecilia Sampedro, Pilar Crespo and Carlos Mas for their technical assistance with microscopy. Finally, we want to thank the anonymous referees for their constructive comments. This work was supported in part by grants from SECYT-UNC (R. 162/12; 124/13; 1565/14) and CONICET (R. 4541/12; 3646/14) to Ana Macías. Ana Macías is supported by CONICET-UNC, Carolina Arias, Adriana Zapata and Francisco Ludueña-Almeida by UNC and Marcelo Zacharonok by Sunrise Scholl.*

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