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The role of Dpp and Wg in compensatory proliferation and in the formation of hyperplastic overgrowths caused by apoptotic cells in the *Drosophila* wing disc

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Non-lethal stress treatments (X-radiation or heat shock) administered to *Drosophila* imaginal discs induce massive apoptosis, which may eliminate more that 50% of the cells. Yet the discs are able to recover to form final structures of normal size and pattern. Thus, the surviving cells have to undergo additional proliferation to compensate for the cell loss. The finding that apoptotic cells ectopically express *dpp* and *wg* suggested that ectopic Dpp/Wg signalling might be responsible for compensatory proliferation. We have tested this hypothesis by analysing the response to irradiation-induced apoptosis of disc compartments that are mutant for *dpp*, for *wg*, or for both. We find that there is compensatory proliferation in these compartments, indicating that the ectopic Dpp/Wg signalling generated by apoptotic cells is not involved. However, we demonstrate that this ectopic Dpp/Wg signalling is responsible for the hyperplastic overgrowths that appear when apoptotic ('undead') cells are kept alive with the caspase inhibitor P35. We also show that the ectopic Dpp/Wg signalling and the overgrowths caused by undead cells are due to a non-apoptotic function of the JNK pathway. We propose that the compensatory growth is simply a homeostatic response of wing compartments, which resume growth after massive cellular loss until they reach the final correct size. The ectopic Dpp/Wg signalling associated with apoptosis is inconsequential in compartments with normal apoptotic cells, which die soon after the stress event. In compartments containing undead cells, the adventitious Dpp/Wg signalling results in hyperplastic overgrowths.

KEY WORDS: Apoptosis, Compensatory proliferation, Hyperplastic overgrowths, JNK, *dpp*, *wg*

INTRODUCTION

Apoptosis, e.g. programmed cell death, is a powerful biological process implicated in various developmental events. In *Drosophila*, as in other organisms, there are two operational aspects of this phenomenon. (1) The apoptosis involved in the elimination of cells that is necessary for normal morphogenesis; for example, sculpting the larval head (Lohmann et al., 2002) or the adult leg articulations (Manjon et al., 2007). This type of apoptosis has to be regulated during development. (2) The apoptosis involved in the elimination of cells that have been damaged or injured during development. This is a stress response function, which is not developmentally regulated in a strict sense, as it does not occur normally unless the organism is subjected to tissue damage or to various forms of physiological stress (Ollmann et al., 2000; Brodsky et al., 2000).

The molecular/genetic mechanism implicated in *Drosophila* apoptosis is well known: after an apoptotic stimulus, one or several of the pro-apoptotic genes *reaper*, *head involution defective* (*hid*; *Wrinkled* – FlyBase), *grim* or *sickle* (*skl*) are activated and, in turn, their products inactivate that of the *diap1* gene (*thread* – FlyBase) (Goyal et al., 2000; Ryoo et al., 2002; Wang et al., 1999; Yoo et al., 2002), whose function is essential for cell viability. The loss of DIAP1 activity allows the catalytic activation of the caspases, which are responsible for dismantling the cell substrates, causing the death of the cells.

The imaginal discs of *Drosophila* provide a convenient system in which to study the properties of apoptotic cells. The wing disc shows very little apoptosis during development (Milan et al., 1997), but responds with elevated apoptotic levels after irradiation or heat-shock treatments (Perez-Garijo et al., 2004). It has been estimated that the proportion of cell death after such treatments is greater than 50% (Haynie and Bryant, 1977; Pérez-Garijo et al., 2004). In spite of this massive cell elimination, the disc recovers and eventually forms adult structures of normal size. The implication is that surviving cells undergo additional proliferation to compensate for the cell loss.

Several reports (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004) described unexpected properties of apoptotic cells that suggested a mechanism for compensatory proliferation. In those experiments apoptosis was induced by various stimuli (forced activation of pro-apoptotic genes or stress treatments such as X-rays or heat shock), but the death of apoptotic cells was prevented by the presence of the baculovirus caspase inhibitor P35 (Hay et al., 1994). Under these conditions, these 'undead' cells remain alive, while retaining all the features of apoptosis (reviewed by Martin et al., 2009).

Those experiments reported two crucial observations. The first was that undead cells appear to stimulate the proliferation of non-apoptotic cells in their vicinity. The second was that undead cells exhibit ectopic expression of the *dpp* and *wg* signalling genes, which are known to act as mitogens in the imaginal discs (Burke and Basler, 1996; Giraldez and Cohen, 2003). Moreover, Ryoo et al. and Pérez-Garijo et al. provided evidence that induction of *dpp*/*wg* expression also occurs in normal (e.g. not containing P35) apoptotic cells (Ryoo et al., 2004; Pérez-Garijo et al., 2004).

These two observations suggested a mechanism for compensatory proliferation: before dying, the apoptotic cells secrete Wg and Dpp, which stimulate the proliferation of non-apoptotic cells located nearby that would restore the normal size of the disc. It is clear from this definition that the additional proliferation needed for size compensation would be caused by the adventitious activation of the Dpp and Wg pathways in the proximity of the apoptotic cells, and

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not by the normal Dpp and Wg activities of the disc. These are of course required for the normal growth of the disc, but would not be involved in compensatory proliferation.

It was also observed that keeping apoptotic cells alive with P35 results in abnormal development of the affected compartments. This was especially clear in discs in which the posterior compartment contained P35 but the anterior one did not; the anterior compartment recovered after massive apoptosis to form a structure of normal size and pattern, but the posterior compartment grew in excess and showed morphological aberrations (Ryoo et al., 2004; Perez-Garijo et al., 2004). Thus, although there is normal compensatory proliferation in the anterior compartment, the presence of undead cells in the posterior compartment gives rise to hyperplastic overgrowths. Measurements of cell division levels (Pérez-Garijo et al., 2004) indicated abnormally high proliferation rates in the posterior compartments, which would account for the excess of growth.

The discovery that apoptotic cells emit Dpp and Wg signals also suggested an explanation for these overgrowths. As undead cells retain *dpp* and *wg* expression indefinitely after the initiation of apoptosis (Perez-Garijo et al., 2004; Martin et al., 2009), it appears likely that the excess of growth is due to the continuous supply of these signals. In support of this, Ryoo et al. showed that the activity of the Wg pathway contributes to the developmental anomalies induced by undead cells (Ryoo et al., 2004).

In the experiments reported here, we aimed to test the role of the Dpp and Wg signals emitted by apoptotic cells in the following two processes: (1) the compensatory proliferation that occurs after massive apoptosis in response to irradiation; and (2) the formation of hyperplastic overgrowths when apoptotic (undead) cells are kept alive with P35.

We wish to point out that we addressed the second issue in a previous paper (Pérez-Garijo et al., 2005). We reported that undead cells lacking *wg* but possessing *dpp* activity give rise to neoplastic tumours in the wing disc. Those clones showed higher proliferation rates than the surrounding cells and produced massive overgrowths. We interpreted the overgrowths as being caused by unbalanced signalling from undead cells: the mitogenic influence of Dpp was not counteracted by the growth repressing function of the Wg signal. However, subsequent work showed that the *wg* mutant chromosome used in those experiments inadvertently contained a mutation at the *lethal giant larvae* (*lgl*) gene. *lgl* mutations are known to produce neoplastic tumours (reviewed by Hariharan and Bilder, 2006), suggesting that the *lgl* mutation may be the cause of the appearance of tumours in our experiments. It also indicated that our previous interpretation of the appearance of tumours was incorrect. The role of the *lgl* mutation in the formation of those tumours is presently being studied.

The results that we present in this report indicate that the ectopic Dpp and Wg signals do not mediate compensatory proliferation, because it can occur in compartments in which they cannot be produced. However, ectopic Dpp and Wg are major factors involved in the appearance of the hyperplastic overgrowths caused by keeping apoptotic cells alive. We also present evidence indicating that the activation of *dpp* and *wg* in apoptotic cells, and hence the formation of overgrowths, is caused by a non-apoptotic role of the JNK pathway, which is itself activated by the irradiation.

MATERIALS AND METHODS

Mutations and stocks

Gal4 drivers: *hh-Gal4* and *spalt-Gal4* (a gift of J. F. de Celis, Centro de Biología Molecular Severo Ochoa, Madrid, Spain). UAS stocks: *UAS-p35* (Hay et al., 1994), *UAS-FLP* (Bloomington Stock Center), UAS-GFP, *UAS-hep^{Act}* (Bloomington Stock Center) and *UAS-shmi Dpp2* (Haley at el., 2008). Mutations: the allele *dpp^{d12}* eliminates adult *dpp* disc activity (St. Johnston et al., 1990); the *wg^{RF}* is a null *wg* mutation (gift of Gary Struhl, Columbia University, New York, NY, USA). Mutant *wg^{RF}* embryos exhibit a very strong phenotype identical to that reported for null *wg* alleles. The *dronc^{I29}* mutation effectively inhibits apoptosis (Xu et al., 2005). Other mutations, *dpp-lacZ* (*P{PZ}dpp^{10638}*), *arm-lacZ* (Bloomington Stock Center), *puc-lacZ*, *crinkled* (*ck*), were used as markers. The Minute allele used in the experiments was *M(2L)24F* (FlyBase).

Flies with posterior compartment mutant for *dpp^{d12}* were of the genotype *dpp^{d12} ck FRT40A/M(2L)24F ubi-GFP FRT40A; hh-Gal4/UAS-Flp*. Similarly flies with posterior compartment mutant for *wg* were *wg^{RF} FRT40A/M(2L)24F ubi-GFP FRT40A; hh-Gal4 UAS-Flp*. To generate clones mutant for both *dpp* and *wg* we built a recombinant *dpp^{d12} wg^{RF} FRT40A* chromosome.

Apoptosis induction by irradiation

Larvae arising in the crosses described above were irradiated with 1500 rads at 48-72 hours after egg laying, which corresponds to the second larval period. They were allowed to grow, then were dissected and wing discs extracted when they reached the wandering or prepupal stage, usually 72-96 hours after the irradiation.

Histochemistry

Fixation and immunohistochemistry of imaginal discs were carried out as described previously (Aldaz et al., 2003). The following antibodies were used: anti-casp3 (Cell Signaling), anti-Wg (Hybridoma Bank), anti-Dronc, anti-Hid (gifts of Hermann Steller, Rockefeller University, New York, NY, USA), anti-PH3 (Upstate), rabbit anti-β-Gal (Cappel). Secondary antibodies used were purchased from Jackson ImmunoResearch.

For the double in situ hybridisation/antibody staining, we followed the protocol of Goto and Hayashi (Goto and Hayashi, 1997), with some modifications. After fixation, larvae were washed three times and stained with primary antibody overnight at 4°C in PBTH (DEPC-treated PBS, 0.1% Tween 20, 50 μg/ml heparin, 10 μg/ml salmon sperm) with 0.26 U/ml RNAse inhibitor (Roche). Incubation with secondary antibody was carried in PBTH for 4 hours at 4°C before fixation for 20 minutes in 4% paraformaldehyde. After fixation, larvae were washed for 15 minutes with PBT (1×PBS, 0.1% Tween) + HSS (0.02 M Tris HCl pH 8.2, 0.25 mM EDTA, 0.3 M NaCl, 1×Denhardts, 50% formamide) 1:1, prehybridized for 60 minutes in HSS at 55°C and incubated with the probe overnight at 55°C. After incubation the discs were washed three times with HSS at 55°C and three times with PBT at room temperature before incubation with anti-DIG (Roche Diagnostics; diluted 1:2000) overnight at 4°C. Staining was done with FastRed (Boehringer Mannheim).

Measurement of the P:A size ratio

To measure the size of anterior and posterior compartments, we used the WCIF ImageJ Software. As the posterior compartments were labelled with GFP, we measured the size of the P compartment (in pixels) and also that of the entire disc. The P:A ratio was calculated with Microsoft Excel. To measure the relative size of the *spalt* domain in the experiments involving JNK activation, we used the same method, measuring the size of the *spalt* domain in comparison with that of the entire disc.

RESULTS

Experimental design

We have used the wing disc to investigate the role of the Dpp and Wg signals in two processes: (1) compensatory proliferation, which restores normal size and shape after massive cell death; and (2) the hyperplastic overgrowth produced in compartments in which the death of apoptotic cells is prevented by blocking caspase activity.

In the compensatory proliferation experiments the rationale was to induce apoptosis by X-rays in cells that were defective in either *dpp* and/or *wg* function, so that they could not bring about ectopic Dpp/Wg signalling. The region covered by the *dpp* or *wg* defective

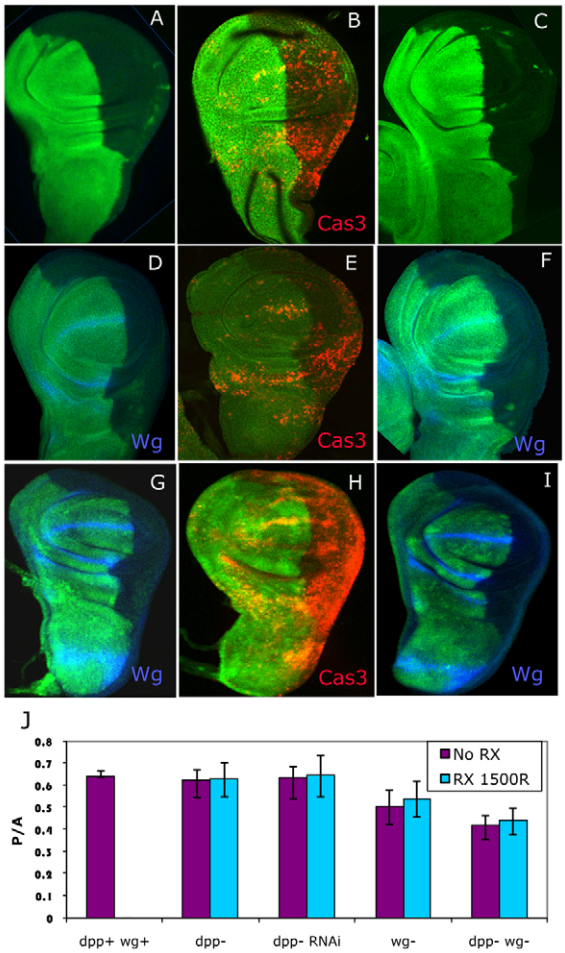

Fig. 1. Effect of radiation-induced apoptosis on the size of mutant posterior wing compartments. Posterior (P) wing compartments were mutant for *dpp* (A-C), *wg* (D-F) or for *dpp* and *wg* (G-I). In all experiments, P compartments are labelled by loss of the GFP marker (green; see Materials and methods). The discs in C, F and I were irradiated in the second instar and fixed when the larvae reached the wandering or prepupal stage. (**A**) Non-irradiated wing disc in which the P compartment is homozygous for dpp^{d12}. The small GFP patches represent the few remaining *M*/+ cells. (**B**) Disc of the same genotype as in A, fixed and stained for caspase activity (red) 3 hours after irradiation. (**C**) Irradiated disc of the same genotype as in A, fixed at the prepupal stage. The size of the P compartment is not significantly altered. (**D**) Non-irradiated wing disc in which the P compartment is mutant for wg^{RF}, double stained for GFP and Wg (blue). Note the small size of the P compartment and that it lacks Wg protein. (**E**) Disc of the same genotype as in D, showing caspase activity (red) 4 hours after irradiation. (**F**) Prepupal disc of the same genotype as in D, fixed and doubly stained for GFP and Wg. The disc was irradiated in the second instar and fixed at the prepupal stage. The irradiation does not affect the size of the P compartment. (**G**) Non-irradiated disc in which the P compartment is doubly mutant for dpp^{d12} and wg^{RF}, double stained for GFP and Wg. (**H**) Disc of the same genotype as in G, fixed and stained for caspase activity (red) 4 hours after irradiation. (**I**) Prepupal disc of the same genotype as in G, fixed and stained for GFP and Wg to show the lack of effect of irradiation on the size of the P compartment. The disc was irradiated in the second instar and fixed at the prepupal stage. (**J**) Posterior:anterior (P:A) size ratio in the various phenotypes. Non-irradiated discs; mauve; irradiated discs, blue. The left bar shows the P:A ratio of a wild-type disc and, from left to right, the various combinations of discs in which the P compartment is mutant for dpp^{d12}, contains the *UAS-shmi Dpp2* construct, is mutant for wg^{RF}, or is mutant for dpp^{d12} wg^{RF}. Note that there is no significant difference in irradiated versus non-irradiated discs in any of these combinations.

cells needed to be sufficiently large, so that the possible effect on size could easily be noticed; we therefore performed our experiments in the posterior (P) compartment.

Because of the high cell death caused by a dose of 1500R (higher than 50%), if the Dpp and/or the Wg signal were required for compensatory proliferation it would be expected that after irradiation the affected compartment would be reduced to at least 50% of its normal size. As the growth of the wing disc requires normal activity of *dpp* and *wg* (Burke and Basler, 1996; Giraldez and Cohen, 2003), it was important to ensure that the normal *dpp* and *wg* expression domains were not altered, or that the alterations introduced were compatible with the growth of the disc.

The elimination of *dpp* activity in the P compartment was not expected to have a developmental effect, as the Dpp signal is synthesised in the A compartment, from where it diffuses to A and P compartment cells. As illustrated in Fig. 1J, this is the case: the P compartment:A compartment size ratio is not affected by the lack of *dpp*. However, we observed that the lack of *wg* causes a diminution of the P compartment size (Fig. 1D-J).

Using mitotic recombination methods, we have generated wing discs in which the P compartment is entirely mutant for dpp^{d12}, which eliminates adult *dpp* function (St Johnston et al., 1990). To abolish *dpp* activity in the P compartment, we have also used a transgenic strain carrying the UAS-shmiR-*dpp*2 construct, which has been shown to degrade the mRNA of *dpp* (Haley et al., 2008).

To remove *wg* function in the P compartment by the same system, we used the wg^{RF} mutation, which behaves as a null allele: wg^{RF} cells show no trace of anti-Wg staining (see Fig. 1F,I) and wg^{RF} embryos display a strong *wg* phenotype (not shown). We also tested the possibility of redundant roles of *dpp* and *wg* by generating discs in which the P compartment was deficient for both gene activities.

The experiments to study the role of *dpp* and *wg* in the hyperplastic overgrowths were very similar to those above, but adding the UAS-p35 transgene to the P compartment cells. The elimination of *dpp* or *wg* activity in the undead cells allowed the contribution of either signal to the overgrowths to be tested.

The wing disc exhibits compensatory proliferation in the absence of Dpp and Wg signalling

To test the compensatory response in dpp^{-} compartments, we used a combination of the FRT/FLP and Minute methods (Martin and Morata, 2006; Foronda et al., 2008) to generate discs in which virtually all of the cells in the P compartment were homozygous for dpp^{d12}. In discs of genotype dpp^{d12} *ck FRT40A/M(2)24F ubi-GFP FRT40A; hh-Gal4 UAS-Flp*, the high levels of Flipase generated by the *hh*-Gal4 driver would induce FRT-mediated mitotic recombination in many cells in the posterior compartment. The dpp^{-} M^{+} clones will have a proliferation advantage (Morata and Ripoll, 1975) and will eventually fill the posterior compartment (see Fig. S1 in the supplementary material). The conversion of the affected

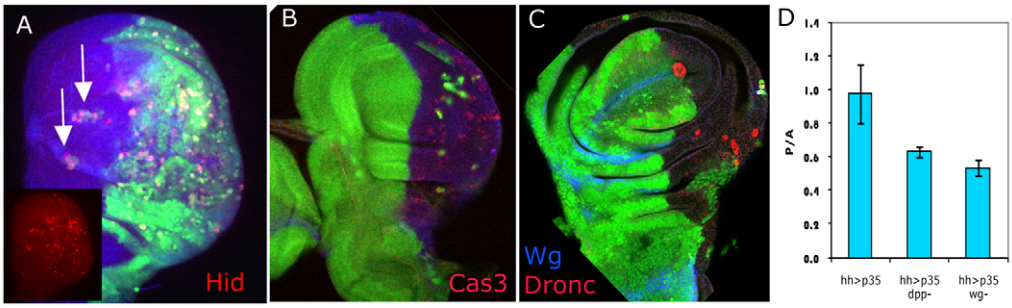

Fig. 2. Role of *wg* and *dpp* in the hyperplastic overgrowths caused by undead apoptotic cells. (**A**) Irradiated disc of genotype *hh-Gal4>UAS-p35 UAS-GFP* fixed and stained for GFP and Hid (red in inset). In this, as in the following cases, the disc was irradiated in the second larval period and then fixed at the prepupal stage. Note the two groups of undead cells of posterior origin (arrows) that have penetrated into the anterior compartment. (**B**) Irradiated prepupal *hh-Gal4>UAS-p35* disc in which the posterior compartment is entirely mutant for dpp^{d12} (see Materials and methods). There are numerous undead cells because they contain the activated form of caspase (although functionally blocked by P35), shown in red. However, the posterior compartment is not enlarged in size. The few green spots are remaining *M/+* cells. (**C**) Prepupal irradiated *hh-Gal4>UAS-p35* disc in which the posterior compartment is mutant for wg^{RF}. The disc was stained with anti-Wg to show there is no *wg* activity in the posterior compartment. It is also stained with anti-Dronc to reveal the presence of undead cells. As in the disc in B, the posterior compartment is not enlarged. (**D**) P:A size ratio in discs of genotypes as in A, B and C. The P:A ratio in *hh>p35* is significantly higher than in *hh>p35* dpp^{d12} and *hh>p35* wg^{RF} discs. The P:A ratio of the latter is similar to that of normal discs (compare with Fig. 1J).

compartment from *M/+* to M^+ occurs early in the development of the disc (Martín and Morata, 2006). The $dpp^- M^+$ cells are identified in the disc by loss of the *ubi-GFP* transgene, and in the adult wing by homozygosity for the cuticular marker *crinkled* (*ck*). One advantage of using the *hh-Gal4* line is that *dpp* is eliminated only in the P compartment, thus the A compartment serves as a control. We also used this line in the experiments to suppress *dpp* activity by RNA interference with the *UAS-shmiR-dpp2* construct (Haley et al., 2008).

An X-ray dose of 1500 rads induced high apoptotic levels in the imaginal discs that were already detectable after 3 hours (Fig. 1B) and remained detectable until 48 hours after irradiation (Pérez-Garijo et al., 2004). Irradiated second instar larvae were allowed to develop until the wandering/prepupal stage before extracting imaginal discs for fixing and staining. Then, the size ratio of P and A compartments was measured and compared with that of non-irradiated controls.

If compensatory proliferation after apoptosis is inhibited in the dpp^- P compartments, the P:A ratio should decrease significantly. Inspection by compound microscopy of discs and adult wings from irradiated and from non-irradiated larvae did not indicate any significant difference in the P:A size ratio of dpp^+ and dpp^- compartments (Fig. 1A,C). Nevertheless, we carried out a careful measurement of the sizes of irradiated compartments and compared the P:A ratio with that of non-irradiated controls. The results are summarised in Fig. 1J and Table S1 in the supplementary material, and show that the P:A ratio is similar in control and irradiated discs. These results clearly indicate that the P compartment is able to compensate for growth in the absence of *dpp* function. This is also supported by the results obtained with discs of the genotype *hh-Gal4>UAS-shmiR-dpp2* in which *dpp* transcripts are degraded, resulting in at least a 75% reduction of *dpp* activity (Haley et al., 2008). In these discs, the P:A ratio is also unaffected by the irradiation (Fig. 1J; see also Table S1 in the supplementary material).

Next, we studied the compensatory response to X-rays of compartments in which *wg* activity was eliminated. The protocol was the same as that used in the dpp^{d12} experiment but in this case the M^+ clones were homozygous for the wg^{RF} mutation. These posterior compartments show no sign of *wg* activity (Fig. 1D,F) and are also smaller than normal P compartments (Fig. 1J). The results are illustrated in Fig. 1D,F,J and in Table S1 in the supplementary material. Although the size of P compartments that are entirely wg^{RF} is smaller than that of normal ones, the irradiation does not affect the P:A ratio, indicating that there is compensatory proliferation.

As there was the possibility of redundant functions of *dpp* and *wg*, we also studied the apoptotic response of P compartments defective in both *dpp* and *wg*, by generating P compartments doubly mutant for dpp^{d12} and wg^{RF}. As illustrated in Fig. 1G,I,J and in Table S1 in the supplementary material, the P:A ratio remains unaltered after irradiation, indicating that there is size compensation.

The overall conclusion from all of the experiments described above is that the elimination of *dpp* and/or *wg* activity in the P compartment does not prevent compensatory proliferation after radiation-induced apoptosis. The implication of this result is that the ectopic activation of *wg* and *dpp* observed in apoptotic cells does not play a significant role in the compensatory proliferation process.

The Dpp and Wg signals are necessary for the hyperplastic overgrowths caused by undead cells

It is known (Perez-Garijo et al., 2004; Kondo et al., 2006; Wells et al., 2006) that preventing the death of apoptotic cells by inhibiting caspase function causes overgrowths and pattern abnormalities. After stress-induced apoptosis (Perez-Garijo et al., 2004) in *hh>p35* discs, the A compartment exhibits high apoptotic levels for about 24 hours, but eventually recovers and forms structures of normal size and pattern. By contrast, the P compartment (Fig. 2A, see Table S2 in the supplementary material) becomes larger than normal and shows aberrant morphology.

The principal difference between the A and the P compartments in these experiments is that apoptotic cells are kept alive in the P compartment. These cells can be identified because they express the pro-apoptotic gene *hid*, as well as other apoptotic markers, such as Dronc and Drice (Martin et al., 2009). In addition, they frequently show ectopic *dpp* and *wg* expression, which persist during the rest of the development. Dpp and Wg function are pattern organizers as well as mitogenic signals in the wing disc (reviewed by Lawrence and Struhl, 1996), suggesting that their inappropriate activities might be responsible for the hyperplastic overgrowths.

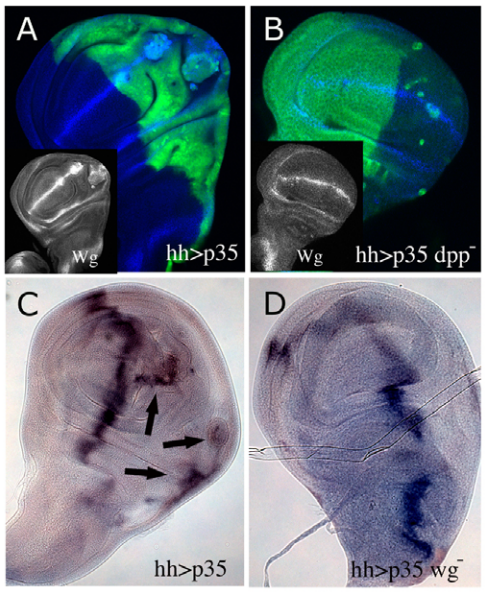

Fig. 3. Expression of *wg* and *dpp* in undead cells lacking *dpp* and *wg*, respectively. (**A**) Control *hh>p35 UAS-GFP* irradiated wing disc doubly marked for GFP (green) and *wg* (blue; white in the inset) showing ectopic *wg* expression in the posterior compartment. (**B**) *hh>p35* disc in which the posterior compartment is mutant for dpp^{d12} (see Materials and methods for details). *wg* expression is labelled blue (white in the inset); the lack of *dpp* is marked by loss of GFP label. Note that there is no ectopic *wg* expression. The small green patches in the posterior compartment correspond to the remaining dpp^{+} cells in the compartment. (**C**) Control *hh>p35* irradiated disc showing ectopic *dpp* expression in the posterior compartment (arrows) after in situ hybridization with a general dpp probe. (**D**) In situ hybridization with the same probe in an *hh>p35* disc in which the posterior compartment is wg^{RF}. Note that there is no ectopic *dpp* expression.

To test the role of *dpp*, we made P compartments containing P35 that were also homozygous for the dpp^{d12} mutation. The actual genotype of the discs was *dpp^{d12} ck FRT40A/M(2)24F ubi-GFP FRT40A; hh-Gal4 UAS-Flp/UAS-p35*.

The effects of the irradiation on the size of *hh>p35* and *hh>p35* dpp^{d12} discs are presented in Fig. 2 and Table S2 in the supplementary material. While in *hh>p35* discs the P compartments showed increased size and pattern defects (Fig. 2A) (Pérez-Garijo et al., 2004), in *hh>p35* dpp^{d12} discs these alterations were much less abundant, and the size and pattern of the P compartments were normalized (Fig. 2B). The P:A size ratio (Fig. 2D; Table S2 in the supplementary material) was similar to that of normal discs. This shows that the Dpp signal is a major factor in the production of the hyperplastic overgrowths in *hh>p35* discs. It is worth noting that we use the dpp^{d12} allele, and that it results in the abolishment of the overgrowths caused by apoptotic dpp^{+} undead cells, indicating that the dpp^{d12} allele eliminates the growth-inducing capacity of apoptotic cells.

The requirement for *wg* was tested by irradiating discs of genotype *wg^{RF}FRT40A/M(2)24F ubi-GFP FRT40A; hh-Gal4 UAS FLP/UAS-p35*, in which the P compartment was defective in *wg* activity. The result was that these discs are of normal aspect; the P compartments showed very few morphological alterations and the P:A ratio was similar to that of discs in which the P compartment was wg^{RF} (compare Fig. 1J with Fig. 2D, see also Table S2 in the supplementary material). This result indicates that *wg* is also required for the appearance of the overgrowths.

The finding that the elimination of either *dpp* or *wg* results in almost complete abolishment of the hyperplastic overgrowths was an unanticipated result. It suggested that both genes are necessary and that there is a mutual requirement. Therefore, we examined *wg* expression in compartments containing undead cells that are mutant for *dpp*, and conversely *dpp* expression in compartments with undead cells mutant for *wg*. The results are shown in Fig. 3 and indicate clearly that the ectopic expression patterns of *wg* and *dpp* are mutually dependent: the function of either gene is required for the activation of the other. These results explain why the elimination of either gene prevents hyperplastic overgrowths, but at the same time it poses the problem of their mutual requirement at the transcriptional level.

Role of the JNK pathway in the activation of *dpp* and *wg*

The activation of *dpp* and *wg* is one of the features of apoptotic cells but the mechanism of activation is not known. A crucial factor in the establishment of apoptosis in *Drosophila* is the JNK pathway. Its activity leads to apoptosis, whereas in absence of JNK function apoptosis is much reduced (Adachi-Yamada et al., 1999; McEwen and Peifer, 2005). In addition, there is no ectopic *dpp* or *wg* activation in undead cells generated by *rpr* induction (Ryoo et al., 2004).

The JNK pathway also has other roles in development that are not connected with apoptosis, such as conferring epithelial cells with the ability to migrate during dorsal closure and disc fusion (Glise et al., 1995; Martin-Blanco et al., 2000), or its involvement with *dpp* activation in the border cells during embryonic dorsal closure (Glise and Noselli, 1997; Hou et al., 1997; Riesgo-Escovar and Hafen, 1997).

We have studied some aspects of JNK activity after X-ray-induced apoptosis, in particular its possible role in the activation of *dpp* and *wg*. First, we checked whether the normal dose of X-rays used in our experiments induced JNK activity. Using the *puc-lacZ* insert to monitor JNK function (Martin-Blanco et al., 1998), we examined *puc* levels 8 hours after irradiation. The results are illustrated in Fig. 4A-C. In non-irradiated discs there was no expression of *puc*, except in a band of cells in the proximal-thoracic region (Fig. 4A), as was previously known (Martin-Blanco et al., 2000). In irradiated discs there was an overall increase of JNK activity in the rest of the disc (Fig. 4B), which was especially clear in the wing pouch and was associated with high apoptotic levels (Fig. 4C). This activation was not unexpected as JNK mediates most or all stress-induced apoptosis in *Drosophila* (McEwen and Peifer, 2005; Luo et al., 2007). We also observed that undead cells in irradiated *hh>UAS-p35* discs co-express JNK and *wg* even 72 hours after irradiation (Fig. 4D-F).

Because JNK becomes active during apoptosis but also has other non-apoptotic functions, there was the possibility that the activation of *dpp* and *wg* in apoptotic cells might be independent of apoptosis. To test this possibility, we made use of the *UAS-hep^{act}* construct (Adachi-Yamada et al., 1999) to force JNK activity in *dronc* mutant discs in which apoptosis is greatly reduced (Daish et al., 2004; Chew et al., 2004; Xu et al., 2005). We have confirmed that $dronc^{I29}$ mutant discs show a very low apoptotic response to X-rays. The line *spalt*-Gal4 drives expression in the wing pouch (Fig. 5A) and, when directing hep^{act} in $dronc^{+}$ discs, induces high levels of caspase and *wg* activity in the *spalt* domain. Moreover, in *spalt>hep^{act} $dronc^{I29}$* discs the amount of apoptosis is much reduced (see Fig. S2 in the supplementary material).

The significant result is shown in Fig. 5C-E: wing discs of genotype *spalt>hep^{act}; $dronc^{I29}$* exhibit ectopic activation of both *wg* and *dpp* in the region of the wing pouch corresponding to the *spalt* domain.

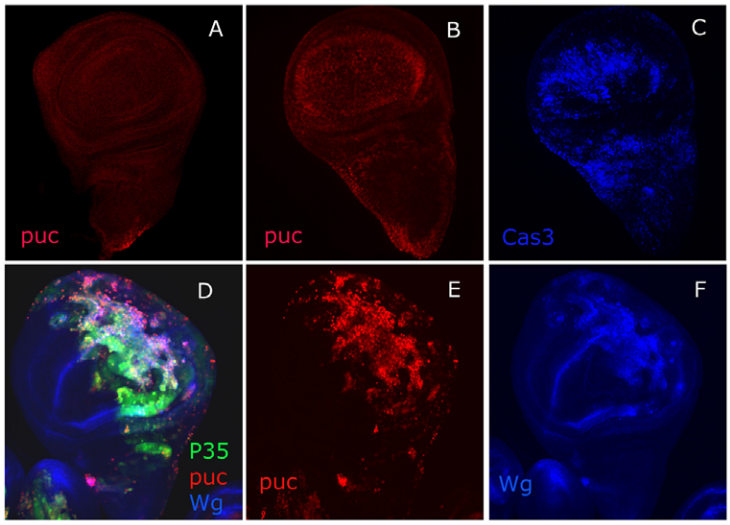

Fig. 4. Response of the JNK pathway to irradiation. (**A**) Non-irradiated wing stained to reveal JNK activity (red, monitored by *puc-LacZ*). JNK expression is restricted to a few cells in the proximal region of the disc. (**B**) Irradiated wing disc stained for *puc-LacZ* 8 hours after irradiation, showing overall activation of the JNK pathway. (**C**) The same disc as is portrayed in B displaying high levels of caspase activity (blue). (**D-F**) Irradiated disc of genotype *hh-Gal4>UAS-p35 UAS-GFP; puc-LacZ,* fixed 96 hours after irradiation and stained for *lacZ* (red) and *wg* (blue). Note the high levels of JNK activity in the posterior compartment (E) associated with ectopic *wg* expression (F).

The expression domains of *wg* and *dpp* appear to be co-extensive in most cells of the *spalt* domain. This experiment strongly suggests that the expression of *dpp* and *wg* in apoptotic cells is not a consequence of apoptosis, but of the activation of JNK. The mechanism by which the JNK pathway induces *wg* and *dpp* is not known.

The hyperplastic overgrowths are due to persistent JNK activity in undead cells

There are two significant observations described in the preceding sections. One is that the induction of hyperplastic overgrowths by undead cells is dependent on the activation of *wg* and *dpp*. The second is that the activation of *wg* and *dpp* is a consequence of JNK function and is independent of apoptosis.

It follows from these two observations that ectopic JNK activity should be able to cause excess growth in the absence of apoptosis. We tested this by examining the size and morphology of discs of genotype *spalt>hep^{act} GFP dronc^{-}*. The results are illustrated in Fig. 6 and see Table S3 in the supplementary material. In these discs there is a clear overgrowth in the *spalt* domain in comparison with the control *spalt>GFP dronc^{I29}* discs. These overgrowths are associated with folding and pattern abnormalities.

DISCUSSION

The involvement of Dpp/Wg signalling in compensatory proliferation was suggested by the finding that *dpp* and *wg* are expressed in apoptotic cells. This, together with the observation of increased proliferation in the vicinity of the apoptotic cells (Ryoo et al., 2004; Perez-Garijo et al., 2004; Huh et al., 2004), led to the model that compensatory proliferation is caused by the mitogenic activity of the ectopic Dpp and Wg signals emitted by apoptotic cells.

In irradiated discs, the ectopic Dpp/Wg signalling generated by the apoptotic cells is superimposed on the normal Dpp/Wg signalling. The latter is essential for the normal growth of the wing compartments; in *dpp^{d12}* homozygous discs the wings are reduced to a rudiment (St Johnston et al., 1990), and as we show here (Fig. 1F,I) the lack of *wg* activity results in smaller compartments. Our experiments have tested the role of the ectopic Dpp/Wg signalling in size restoration of irradiated discs, that is, the contribution of the apoptotic cells to the process.

We have examined the ability of P compartments to compensate growth in conditions in which apoptotic cells can produce neither the Dpp nor the Wg signal, or are defective in both signals. The results indicate that the model of compensatory proliferation mentioned above is incorrect. The elimination of ectopic *dpp* and *wg* functions in wing discs subjected to massive apoptosis does not impede the restoration of normal size and pattern; in other words, there is compensatory growth without contribution of the Dpp and Wg signals emitted by the apoptotic cells.

Having studied compensatory growth only in P compartments, it is just conceivable that Dpp and Wg originated by apoptotic cells in the A compartment might diffuse to the P compartment

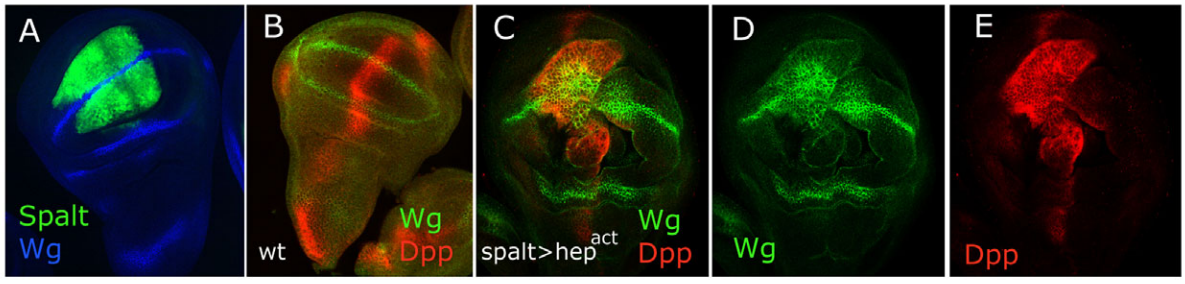

Fig. 5. Induction of *wg* and *dpp* by JNK activity in absence of apoptosis. (**A**) Wing disc of genotype *spalt-Gal4>UAS-GFP* showing the *spalt* domain (green). The disc is also stained for *wg* activity (blue). (**B**) Wild-type disc with double in situ/antibody fluorescent staining for *wg* (green) and *dpp* (red). (**C-E**) Similar staining of a disc of genotype *spalt-Gal4>UAS-hep^{Act}; dronc^{-}*. There is ectopic *wg* (D) and *dpp* (E) activity in the region corresponding to the *spalt* domain.

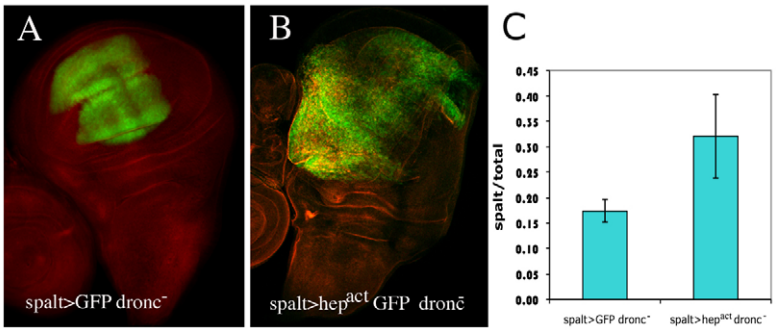

Fig. 6. Induction of hyperplastic overgrowths by JNK activity. (**A**) A *spalt>GFP dronc*^{I19} mutant disc showing the normal *spalt* domain (green) in the wing pouch. (**B**) Forced activity of JNK, using the activated form of *hemipterous* (*spalt>hep*^{Act}*GFP dronc*^{I19}), produces hyperplastic overgrowth in the spalt-expressing region. (**C**) Ratio of the normal *spalt* domain relative to the total size of the disc in *dronc* mutant discs in the absence (left bar) or presence (right bar) of JNK activity. The size of the *spalt* domain in the B is enlarged and also shows abnormal morphology, when compared with that of the disc in A.

where they could induce the additional growth necessary to compensate size. In our view this is very unlikely for two reasons. (1) The undead apoptotic cells induce additional proliferation only in their own vicinity (see Ryoo et al., 2004; Perez-Garijo et al., 2004). Thus, it is hard to imagine that Dpp/Wg of anterior origin could have an affect on proliferation extending to the entire posterior compartment. Moreover, in our experiments the cells are not protected by P35; they are not undead cells but regular apoptotic cells that die soon after initiating apoptosis. Therefore the proliferation stimulus they provide would be very short lived. (2) If the Dpp and Wg of apoptotic origin were able to travel a long way across compartment borders, it would be expected that the overgrowths produced by undead cells were not restricted to compartments. For example, in irradiated *hh>p35* discs or in *en>hid + p35* (and other similar genotypes), in which undead cells belong to the P compartment, the A compartment should also overgrow, stimulated by the Dpp and Wg of posterior origin. In all cases reported (Ryoo et al., 2004; Perez-Garijo et al., 2004; Kondo et al., 2006) (Fig. 2A; Fig. 3A), the effect is essentially restricted to the posterior compartment.

Thus, although the *dpp* and *wg* genes are activated in apoptotic cells, their function appears to be inconsequential. So what is the mechanism responsible for the compensatory growth? One possibility is the existence of some other hitherto undetected signal with mitogenic properties. Although this possibility cannot be ruled out, it appears unlikely because Dpp and Wg are the major growth signals identified in the wing disc after many years of studies. The Dpp pathway has been shown to play a major role in inducing growth in the wing disc; in absence of Dpp activity wing growth is much reduced (St Johnston et al., 1990; Burke and Basler, 1996) and an excess of Dpp activity causes additional growth (Martín-Castellanos and Edgar, 2002; Martín et al., 2004). Moreover, in the experiments in which apoptotic cells are protected with P35, we find that the absence of Dpp and Wg prevents the appearance of overgrowths, strongly suggesting that these signals are responsible for the additional growth associated with apoptotic cells.

We outline our ideas for compensatory growth in Fig. 7A. We believe that it does not require any special mechanism involving the participation of apoptotic cells. It is the normal process that regulates compartment size that is responsible for restoring normal size after massive apoptosis. It has been shown recently (Martín and Morata, 2006) that A and P compartments are autonomous units of size control in the wing disc, i.e. A and P compartments grow autonomously until they reach the final correct size. It has also been shown that the size control mechanism is highly homeostatic. It can adjust to changes in cell size and number (Neufeld et al., 1998; Johnston et al., 1999), and to differential cell division rates (de la Cova et al., 2004; Moreno and Basler, 2004) – alterations in any of these parameters do not produce changes in the final compartment size. As stated above, only the overproduction of Dpp results in breakdown of the size control mechanism.

In our view, the compensatory growth after the loss of cells because of irradiation (or any other stress event) is another example of the versatility of the size control mechanism. As illustrated in Fig. 7A, we propose that the massive cell death caused by the irradiation would be equivalent to making the compartment smaller. The irradiated compartment would then restore the correct size simply by performing some additional division. It would be, in effect, an overall regeneration process of the entire blastema, which would be achieved by lengthening the proliferation period, an idea that is supported by observations such that damage to growing discs results in a prolonged growth period (Wells et al., 2006). Even a loss of 50% of the cells can be restored if all of the surviving cells divided once. In the wing disc, the length of the division period is about 8-12 hours (Garcia-Bellido and Merriam, 1971; Johnston and Sanders, 2003; Neufeld et al., 1998) and therefore only a short delay may be sufficient to allow time for recovery. Thus, irradiated discs would, after some delay caused by the stress, resume growth and the normal control mechanism would stop growth once compartments have reached the final size (Martin and Morata, 2006).

Although the ectopic Dpp and Wg signals do not have a role in compensatory proliferation, they are required for the appearance of overgrowths caused by undead cells (Fig. 2). A key difference between undead cells and normal apoptotic cells is that the former persistently express Dpp and Wg (probably as a result of JNK activity, see below). In irradiated posterior compartments that comprise undead and non-apoptotic cells, such as, for example, in irradiated *hh>p35* discs, the undead cells keep producing the Dpp and Wg signals from shortly after the irradiation and until the end of the proliferation period of the disc (illustrated in Fig. 7B). Thus, the non-apoptotic cells receive a continuous supply of the Dpp and Wg mitogens from the undead ones. The result is an overgrowth, which is also associated with abnormal cell differentiation. Both additional growth and abnormal differentiation would be expected in these circumstances, as Dpp and Wg are growth inducers as well as morphogens determining cell pattern and differentiation.

The JNK pathway is responsible for the ectopic Dpp/Wg signalling and the hyperplastic overgrowths caused by undead cells

The overall conclusion from the above is that the ectopic Dpp and Wg signals generated by apoptotic cells are irrelevant for compensatory proliferation, but are prime factors in the generation of hyperplastic overgrowths caused by undead cells. The question then is why are *dpp* and *wg* activated in normal

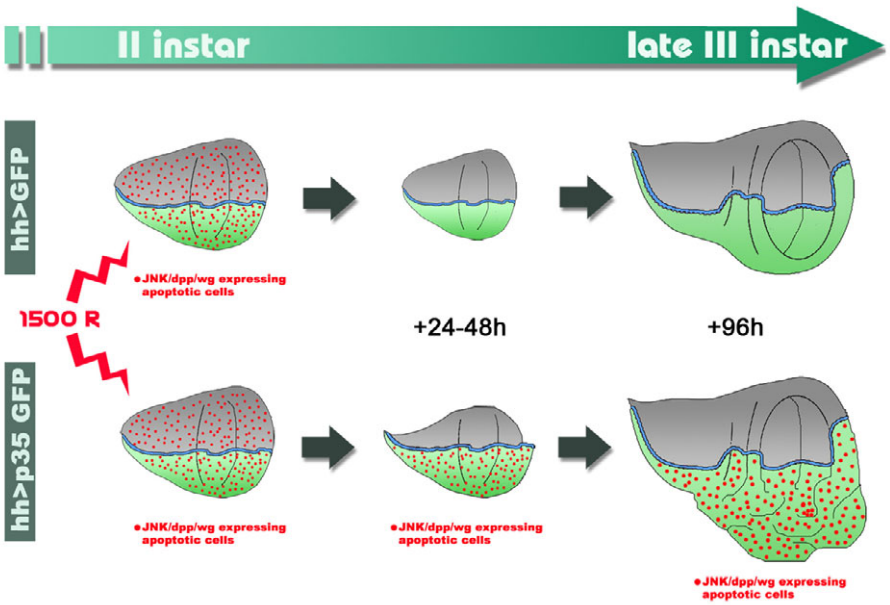

Fig. 7. Compensatory growth versus hyperplastic overgrowths after massive apoptosis in the wing disc. Our view of the events that occur after X-ray-induced massive apoptosis in an early disc. (Top) In an *hh-Gal4>UAS-GFP* wing disc, cells entering apoptosis are not protected by P35; (bottom) cells entering apoptosis in a *hh>UAS-p35 UAS-GFP* wing disc contain P35. In the disc in which cells are not protected by P35, many cells acquire JNK pathway activity, which triggers apoptosis and other JNK-related functions, such as Dpp/Wg signalling and the ability to migrate. However, the latter functions are normally inconsequential because apoptotic cells die very quickly. After 24-48 hours all apoptotic cells have disappeared. This probably causes a transient diminution of size, but eventually the surviving cells proliferate to achieve the stereotyped size of each compartment. In the *hh-Gal4>UAS-p35 UAS-GFP* disc, the irradiation causes JNK-mediated cell death in the anterior and posterior compartment. The anterior compartment behaves as in the disc above, but in the posterior one the cells acquiring JNK/Dpp/Wg signalling cannot be eliminated because of the presence of P35. They remain in the compartment, persistently emitting the Dpp and Wg mitogens during the rest of the growth phase of the disc, in effect causing a hyperplastic overgrowth. Some of the undead cells in the posterior compartment can penetrate into the anterior one, a property that possibly derives from their JNK activity.

apoptotic cells. In our view, their activity is a collateral effect of the activation of the JNK pathway after an apoptotic stimulus: γ-irradiation induces JNK activity in the wing disc and radiation-induced apoptosis depends on JNK activity (McEwen and Peifer, 2005). As expected, in our experiments X-irradiation also induced JNK activity (Fig. 4B).

The function of the JNK pathway appears to be required for the ectopic expression of *wg* and *dpp* in apoptotic cells (Ryoo et al., 2004; McEwen and Peifer, 2005). In experiments in which cell death is blocked with P35 after apoptosis induction, the JNK pathway becomes continuously activated in undead cells and appears to be associated with ectopic *wg* expression (Fig. 4E) (Ryoo et al., 2004; McEwen and Peifer, 2005). It is therefore possible that the ectopic activation of *dpp* and *wg* in the apoptotic cells could be a consequence of JNK function, rather than a consequence of the apoptotic program. Our results strongly support this view: direct activation of JNK via the *UAS-hep^{act}* construct in *dronc* mutant discs, in which apoptosis is much reduced, induces *wg* and *dpp* expression (Fig. 5D-F). Furthermore, these mutant discs show hyperplastic overgrowths in the *spalt* domain, where JNK is active (Fig. 6B,C).

It has been shown that JNK activity induces several cellular functions: the initiation of the apoptotic program, and also other non-apoptotic functions, such as the capacity for cell migration (Glise et al., 1995; Martín-Blanco et al., 2000) and the ability to induce *dpp* (Glise and Noselli, 1997; Hou et al., 1997). It is probable that normal apoptotic cells acquire these other JNK-dependent properties, but that they die very quickly and so these other functions have minimal effects. This is different in undead cells because the JNK activity becomes persistent (Fig. 4E) and, therefore, they can manifest some or all of the JNK non-apoptotic functions: these cells can move and invade neighbouring compartments (Pérez-Garijo et al., 2004) (Fig. 2A), and express *dpp* and *wg* continuously (Fig. 3C, Fig. 4C). In our opinion, it is the persistent manifestation of these two non-apoptotic JNK-mediated properties, *dpp*/*wg* activation and the induction of cell migration that causes the hyperplastic overgrowth (Fig. 7B).

The implication of the Dpp and Wg signals in hyperplastic overgrowths in *Drosophila* might have some general significance as their vertebrate homologues, BMP/TGFβ and Wnt, are known to be involved in the generation of tumours in mammals (Katoh, 2007; Polakis, 2007). Moreover, inappropriate function of the JNK pathway is also connected with tumour formation in vertebrates (Heasley and Han, 2006; Kennedy and Davis, 2003). We speculate that situations similar to those described here might also occur in mammalian cells in which caspase activity is blocked, by virus infections or other causes. This could result in continuous activation of the JNK pathway and, subsequently, of BMP/TGFβ and Wnt, and could eventually produce a tumour.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/7/1169/DC1

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