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A *dp53*-JNK-dependant feedback amplification loop is essential for the apoptotic

response to stress in Drosophila

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Summary

Programmed cell death (apoptosis) is a conserved process aimed to eliminate unwanted cells. Key molecules are a group of proteases called caspases that cleave vital proteins, which leads to the death of cells. In Drosophila the apoptotic pathway is usually represented as a cascade of events in which an initial stimulus activates one or more of the pro-apoptotic genes (hid, rpr, grim), which in turn activate caspases. In stress-induced apoptosis the *dp53* gene and the JNK pathway function upstream in the activation of the pro-apoptotic genes. Here we demonstrate that *dp53* and JNK also function downstream of proapoptotic genes and the initiator caspase Dronc and that they establish a feedback loop that amplifies the initial apoptotic stimulus. This loop plays a critical role in the apoptotic response because in its absence there is a dramatic decrease in the amount of cell death after a pulse of the pro-apoptotic proteins Hid and Rpr. Thus our results indicate that stress-induced apoptosis in *Drosophila* is dependent on an amplification loop mediated by *dp53* and JNK. Furthermore, they also demonstrate a mechanism of mutual activation of proapoptotic genes.

Introduction

Apoptosis is a process of major physiological importance, conserved in the Animal Kingdom, aimed to eliminate cells. It performs developmental roles, reported in insects and vertebrates, and also functions as a safeguard mechanism to remove abnormal cells or cells damaged by stress events^{1,2}. The executive role in apoptosis is played by a group of cysteine proteases, termed caspases, which destroy many vital cell proteins, thus causing cell death³. All animal cells contain them, but their activity is normally prevented by the Inhibitor of Apoptosis Proteins (IAPs)^{4,5}. Thus a key regulatory step in apoptosis is the control of IAP function.

In *Drosophila* the initiation of apoptosis is achieved by the activation of one or more of a group of pro-apoptotic genes *reaper (rpr), head involution defective (hid)*

and *grim* (Figure 1a). Their products bind to *Drosophila* IAP-1 (Diap1) and interfere with its function, releasing the caspases⁶. A target of Diap1 is the initiator caspase-9 ortholog Dronc⁷. The only known Dronc substrates to date are effector caspases Dcp-1, Drice and Diap1 itself⁶.

The *p53* gene is a major pro-apoptotic factor, both in *Drosophila* and in vertebrates⁸. In mammalian cells the P53 protein has other non-apoptotic functions; it is involved in anti-proliferative responses, including cell-cycle arrest and senescence^{9,10}. In contrast, the *Drosophila* homolog of *p53*, called *dp53*, has mostly a pro-apoptotic role, although some effect on cell cycle regulation has been noticed in overexpression experiments¹¹. It has been reported that *dp53* activates the transcription of *rpr* in response to radiation-induced DNA damage to initiate apoptosis¹², and that overexpression of *dp53* in the eye induces Hid-mediated apoptosis¹¹. Thus *dp53* is currently depicted upstream of the apoptotic cascade (Figure 1a). Another major pro-apoptotic factor in *Drosophila* and vertebrates is the Jun N-terminal kinase (JNK) pathway¹³. In *Drosophila* this pathway plays a prominent role inducing apoptosis and is activated in a variety of contexts, including tumour suppression^{14,15}.

The scheme in Figure 1a accounts for most of the features of *Drosophila* apoptosis, but recent findings have suggested the existence of additional complexity. These originate from experiments in which effector caspase activity was blocked by the baculovirus protein P35¹⁶. The presence of P35 does not change the apoptotic nature of these cells (called "undead" cells), but they remain alive, what facilitates the study of their properties.

A significant observation is that after a brief apoptotic stimulus, X-radiation or a 2-3 hours 37^0 C heat shock, cells containing P35 remain in a prolonged apoptotic status: 72-96 hrs after the end of the stimulus, when normally cell death is already undetectable, they still show expression of the pro-apoptotic gene *hid* and active Drice¹⁷. Furthermore, they show persistent expression of the signalling genes *dpp* and *wg*¹⁸, another feature of apoptotic cells.

The persistence of the apoptotic program in undead cells suggests a maintenance mechanism. In this context it is of interest to consider the function of

dp53 and the JNK pathway. *dp53* becomes activated in response to irradiation and in turn directly activates the expression of rpr^{12} . However, there is evidence that in undead cells *dp53* gene is activated by Dronc¹⁹. The function of *dp53* both upstream and downstream Dronc suggested that it is part of a feedback loop aimed to maintain the apoptotic program¹⁹. A similar argument can be made with the JNK pathway: irradiation induces JNK, which in turn induces rpr^{20} , but JNK functions also downstream Dronc to activate *dpp* and *wg* in undead cells^{21,22}. These findings place JNK function genetically both upstream and downstream Dronc.

Together, the preceding observations indicated the existence in undead cells of a feedback loop that involves dp53 and JNK and that maintains the apoptotic program. However, the biological significance of this loop was unclear because the undead cells are aberrant apoptotic cells generated by artificial interference with effector caspase activity. We have performed experiments to test whether there is a similar feedback loop involving dp53 and the JNK pathway in cells embarked in a normal apoptotic program and if so, which is its biological function. Our results show that a dp53/JNKmediated feedback loop indeed occurs in stress-induced apoptosis. Furthermore, they demonstrate that it is essential for the completion of the cell death program, for interfering with the loop results in a dramatic reduction of cell death after an apoptotic stimulus.

Results

A feedback loop in apoptotic cells

We have investigated the possible existence of a feedback loop by inducing apoptosis in cells in which the apoptotic program is not interfered with P35. We have made use of the Gal4/UAS method²³ to drive expression of the different genes involved. As a routine we used the *sal^{EPv}-Gal4* line²⁴, which directs expression only in part of the wing imaginal disc and acts relatively late in the development. It allows normal larval viability after apoptotic induction. Moreover, apoptotic cells can be visualized in third instar discs, what facilitates the study of their behaviour.

As *dp53* and the JNK pathway appear to be key factors in apoptosis we first examined their ability to induce pro-apoptotic genes. As expected from previous work^{11,12,20,25} both *dp53* and JNK can activate *hid* and *rpr*: in *sal>dp53* and in *sal> hep^{ACT}* discs (where *hep^{ACT}* provides constitutive activity of the JNK pathway²⁶) the two pro-apoptotic genes become expressed in the *sal* domain (Figure 1d-g). The induction also occurs in discs of the null allelic combination *dronc*¹²⁴/*dronc*¹²⁹ (hereto after referred to as *dronc*-), indicating that *hid* and *rpr* induction by *dp53* and JNK is not mediated by a feedback loop (Figure 1h-k).

Having shown that *dp53* and JNK can activate *hid* and *rpr*, we then checked whether the latter might reciprocally induce *dp53* and JNK. The results are illustrated in Figure 2: in *sal>hid* discs there is transcription of *dp53* and JNK activity in the *sal* domain (Figure 2c, d). However, and importantly, the activation of both *dp53* and JNK by Hid does not occur in *dronc*⁻ mutants (Figure 2f, g) indicating that it is downstream of and requires *dronc* function. These results demonstrate that the activation of *dp53* and JNK downstream *dronc* is a component of the apoptotic program.

The results of the preceding experiments indicate that *dp53* and JNK can activate *hid* and *rpr*, and that *hid* can in turn induce *dp53* and JNK through Dronc. It follows that both *hid* and *rpr* should be able to activate each other. This is indeed the case: forcing expression of *hid* in the *sal* domain gives rise to *rpr* activity (Figure 2e), and this activation requires *dronc* function (Figure 2h). This finding suggests that *dp53* and JNK pathway function downstream *dronc* to feedback the apoptotic program by increasing the levels of Hid and Rpr products. In addition, this feedback provides a mechanism of mutual activation among pro-apoptotic genes. Complementary experiments forcing *rpr* expression yielded similar results, included in Figure S1.

We finally tested the existence of interactions between *dp53* and JNK. The experiments consisted of forcing expression of either *dp53* or activating the JNK pathway in the *sal* domain and then checking JNK and *dp53* activity respectively. The experiments were performed in *dronc*⁺ and *dronc*⁻ mutant background to test whether their interactions were mediated by the loop. The unexpected result is illustrated in Figure 3 and shows that these two factors can activate each other even in *dronc*⁻ mutant discs.

The feedback loop is essential for the completion of the apoptotic program

Having demonstrated the existence of a feedback loop involving *dp53* and JNK in cells undergoing the normal apoptotic program the next step was to test whether it has a functional role. The rationale of the experiments was to induce a brief pulse of pro-apoptotic genes such as *hid* or *rpr* and then to study the apoptotic response of the wing disc in the presence and in the absence of *dronc*, *dp53* or JNK.

We have used two gene constructs bearing a minimal heat shock promoter directing expression of either *hid* or *rpr* coding regions. They allow forcing pro-apoptotic activity simply by a temperature shift (pulse of 30 minutes at 37°C) and independently of upstream controls. The apoptotic response was measured by examining Drice protein levels and TUNEL staining 4 hours after the end of the heat pulse, and also the accumulation of the Hid protein.

The experiments were performed in parallel with the two heat shock constructs. They involved the generation of the following genotypes: 1) *en>gfp*; *hs-hid* (control), 2) *en>dronc-i*; *hs-hid*, 3) *en>dp53-i*; *hs-hid*, and 4) *en>puc*; *hs-hid* and also the same series with the *hs-rpr* construct. The usage of the *en-Gal4* driver in these experiments permits to interfere with the loop in the posterior compartments leaving the anterior ones as controls. In genotypes 2 and 3 we interfered with transcription of *dronc* or *dp53* using RNA-i (see Materials and Methods). In the case of *dp53* we observed that it has the same effect as the dominant negative form (Figure S2), thus confirming its specificity. In genotype 4 the overexpression of the negative regulator *puckered (puc)* causes the strong reduction of JNK activity²⁷. As the results obtained in the two series of experiments are very similar we only describe in detail the *hs-hid* series (Figures 4 and 5). The results of the *hs-rpr* experiments are shown in Figure S3.

In the control genotype *en>gfp; hs-hid* the heat shock pulse generates a strong apoptotic response, indicated by the high levels of co-extensive anti-Caspase-3 and TUNEL staining in the entire disc (Figure 4a, a'). Expectedly, in *en>dronc-i; hs-hid* discs Caspase-3 and TUNEL levels in the posterior compartment after heat shock are low (Figure 4b), because apoptosis is blocked by the lack of *dronc* activity.

The significant result is that in *en>dp53-i*; *hs-hid* and *en>puc*; *hs-hid* discs there is a very clear reduction of the amount of cell death in the posterior compartment in comparison with the control anterior compartments (Figure 4c, d). This finding suggested that most of the cell death caused by *hid* is mediated by the amplification loop established by *dp53* and JNK. It also suggested that the loop might feed back to the endogenous hid gene. Therefore, we examined Hid protein levels in the same genotypes of above. These results are shown in Figure 5. Whereas in *en>gfp*; *hs-hid* discs the heat shock generates high levels of Hid protein (Figure 5a, e), in *en>dronc-i*; *hs-hid* Hid is almost undetectable in the posterior compartment (Figure 5b, e). Similarly, there is a clear reduction of Hid protein in the posterior compartments of *en>dp53-i; hs-hid* and *en>puc; hs-hid* discs (Figure 5c-e), suggesting that the accumulation of Hid protein after heat shock is mediated by the *dronc/dp53/*JNK loop. The loop-mediated accumulation in Hid protein can also be detected in the *hs-rpr* experimental series: in *en>dronc-i*; *hs-rpr* and *en>puc*; *hs-rpr* discs there is a drastic reduction of Hid protein in the posterior compartments in comparison with the anterior ones (Figure S3b, c). We also examined the apoptotic response in haltere and leg discs, seeking to confirm the results in the wing disc. As shown in Figure S4, we obtain similar results.

The preceding experiments strongly suggest that the pro-apoptotic proteins are able to induce their own genes through the loop. However, there was the possibility that the low levels of Hid protein in the posterior compartments in which *dronc/dp53/JNK* are inhibited, are due either to increased degradation of the exogenous Hid protein or decreased translation of the exogenous *hid* transcript. We have tested these possibilities by introducing the P-element insert *hid*⁰⁵⁰¹⁴-*lacZ*²⁸ in the *hs*-*hid* experiment and examining the *lacZ* activity in anterior and posterior compartments after heat shock. The higher *lacZ* expression levels in the anterior compartment clearly indicate transcriptional up regulation of the endogenous gene (Figure 6a). Furthermore, we have tested the response to *hs*-*hid* of the *hid*²⁰⁻¹⁰-*lacZ*, a construct that can be activated by Dp53¹¹. As shown in Figure 6c, *lacZ* expression is induced by the heat shock, supporting the idea that Dp53 is involved in the activation of the endogenous *hid* gene after a pulse of *hid*. Moreover, we find that the Hid protein

is present at high levels immediately after the end of the heat shock and that those levels are similar in the anterior and posterior compartments of *en>dronc-i, hs-hid* discs (Figure S5). This rules out the possibility that interference with the loop components affects the synthesis of the exogenous Hid protein. Together, these experiments clearly establish that the *dp53/JNK* feedback loop amplifies the apoptotic program by inducing additional pro-apoptotic genes.

We also analysed the involvement of the feedback loop in the apoptotic response to physiological stress. Previous work²² has shown that irradiation induces high levels of JNK activity in the wing disc, as indicated by the activity of the target gene *puc*. We reasoned that at least part of the JNK activity should be due to its activation downstream *dronc*, and then designed experiments to measure and compare JNK activity levels after irradiation in the presence and in the absence of *dronc* activity. We have tested JNK activity four hours after irradiation in two different genotypes. 1) Directly in *dronc⁻*, *puc*^{E69}-*lacZ/+* mutant discs. This is possible because the null trans heterozygous combination *dronc*¹²⁹/*dronc*¹²⁴ allows normal larval viability up to the pupal stage²⁹, and 2) In discs of genotype *en>diap1*; *puc*^{E69}-*lacZ/+*, in which the overexpression of *diap1* in the posterior compartment results in partial suppression of *dronc* function³⁰. In this experiment the anterior compartments

The results are illustrated in Figure 7. In *dronc*- discs *puc* expression after irradiation (Figure 7b, d) is much lower than in the *dronc*+ controls (Figure 7a). Similarly, in the *en>diap1* discs the expression levels of *puc* in the posterior compartment, where *dronc* activity is compromised, is much lower than in the control anterior compartment (Figure 7c). Taken together these experiments establish that much of JNK function in stress-induced apoptosis derives from its activation downstream *dronc*, thus illustrating the role of the feedback loop in a physiological context. In addition they bear on the mechanism by which the JNK pathway is activated after stress. It has been shown³¹ that the physiological response of JNK to stress depends on dTraf1 function. Our results therefore suggest an interaction between Dronc and dTraf1 in order to activate JNK.

Discussion

In *Drosophila*, like in other organisms, apoptosis may be developmentally regulated, as part of the normal morphogenetic process^{32,33}, or may function as a safeguard mechanism to remove damaged or malignant cells^{12,34}. The latter mechanism is activated after stress events that cause extensive cell damage. Our results bear mainly on stress-induced apoptosis. Currently, the apoptotic program in *Drosophila* is portrayed as a lineal cascade of events, like that in Figure 1a. Apoptotic stimuli are channelled by upstream activators like *dp53* and or JNK, which activate one or more of the pro-apoptotic genes *hid*, *rpr* or *grim*. These in turn initiate the cell death process by suppressing *diap1* and allowing caspase activation.

Our results provide a new picture of the mode of action of *dp53* and JNK in apoptosis; not only they function as upstream activators of the pro-apoptotic genes in response to stress like DNA damage, but they also act downstream *dronc* to amplify the apoptotic program by secondary activation of additional pro-apoptotic genes.

We suggest a model of stress-induced apoptosis (Figure 8) in which most of the cell death is caused by the secondary activation of *dp53* and JNK downstream the apical caspase *dronc*. Because *dp53* and JNK are able to induce *hid* and *rpr*, it results in the establishment of a loop that amplifies the original apoptotic stimulus. The fact that *dp53* and JNK activate each other (Figure 3), also contributes to the loop. Our results also indicate that the loop operates in the physiological response to stress events (Figure 7a-d).

This amplification appears to be an essential and necessary component of the apoptotic program of *Drosophila* because its suppression or reduction causes a dramatic decrease of cell death. This is clearly shown by the heat shock experiments (Figure 4, 5 and S3) in which we force the initiation of the apoptotic program by providing the pro-apoptotic proteins Hid or Rpr: the amount of cell death after the stimulus depends critically on the existence of the amplification loop.

The loop also provides a mechanism of mutual activation of pro-apoptotic genes; as indicated in Figure 2e, h: the activation of *hid* gives rise to *rpr* function, and conversely *rpr* function induces *hid* (Figure S1). This mutual activation may be an

important part of the completion of the program because it has been shown in a recent report³⁰ that the Rpr and Hid proteins interact physically and that their association is critical for their cell killing activity. The loop-mediated mutual activation of *hid* and *rpr* may be instrumental to provide the right amounts of Rpr and Hid proteins needed for the interaction.

We also find that dp53 and JNK are able to activate each other even in discs in which apoptosis is suppressed. The mechanistic aspects of these interactions are not clear; it has been shown that the ligand Eiger is a target of Dp53³⁵, suggesting it may mediate JNK activation by dp53. How dp53 is transcriptionally activated by JNK is not known, although it might contribute to the stabilization of the Dp53 protein (see below).

The amplification loop may be a mechanism to add robustness to the apoptotic program; it is possible to imagine a situation in which a weak apoptotic stimulus may induce the pro-apoptotic genes at low levels. Depending on a threshold of *dronc* function it may result either in the establishment of the amplification loop and the death of the cell, or else the loop is not established and the cell survives.

Although we believe our experiments clearly demonstrate the existence of an amplification loop in stress-induced apoptosis, it is not clear whether it also occurs in developmentally regulated apoptosis. Nevertheless, there are indications that the loop function in some developmental contexts. In the leg disc it has been shown that the correct formation of the leg articulations requires local apoptosis in the intersegmental regions³³, where *rpr* and the JNK pathway are co-expressed. We have observed that this local JNK activity is suppressed in *dronc* mutant discs (Figure S6), indicating it is downstream *dronc*, in good concordance with the loop hypothesis.

There are also some indications of the existence of apoptosis amplification loops in vertebrates. For example, in mammalian cells activated caspases feedback to amplify cytochrome c release and hence augment caspase-9 activity³⁶, a process that could be mediated by $p53^{37}$. It has also been proposed that in mammalian cells phosphorylation of P53 at Ser6 by JNK contributes to its stability and promotes its pro-apoptotic role³⁸. These observations suggest functional interactions between

caspases, *p53* and JNK, similar to those we describe for *Drosophila* that may result in the establishment of an apoptotic loop.

The anti-tumour properties of *p53* and JNK pathway derive in part from their ability to induce apoptosis. Our results indicate that, in *Drosophila* apoptosis, *dp53* and the JNK pathway are activated downstream of caspase-9/Dronc and that this activation is critical for the completion of the process. An intriguing possibility is that in humans much of the pro-apoptotic, and hence tumour-suppressive, function of P53 and JNK is exerted downstream caspase-9. It might have implications in pharmacological aspects of cancer therapies, as altering caspase activity could be a vehicle to modify P53/JNK signalling. In this sense it may be of interest to examine P53/JNK activity levels in human tumours that bear mutations in caspases.

Materials and Methods

Drosophila stocks

hh-Gal4 and *en-Gal4* (T. Tabata, IMBC, Tokyo, Japan) direct expression to the posterior compartment, and the *sal*^{*EPv*}-*Gal4* in part of the wing pouch (gift of J.F. de Celis, CBMSO, Madrid, Spain). *UAS-dronc-RNA-i* (#23035) and *UAS-dp53-RNA-i* (#10692) were obtained from Viena *Drosophila* RNA-i Center. *UAS-puc*^{2A}, *UAS-p35*, *UAS-dp53.EX 2*, *UAS-dp53*^{*DN*}, *UAS-hep*^{*ACT*}, *UAS-hid* and *UAS-rpr* are described in Flybase.

The puc^{E69} -lacZ line ²⁷ was used as a reporter of JNK pathway activity. We obtained from T. Adachi-Yamada (Kobe University, Kobe, Japan) the hid^{05014} -lacZ, a P-element insertion in *hid* coding region²⁸, because it has been used previously as a reporter of *hid* activity³⁹. As this line has a non-apoptotic expression in some anterior cells close to the dorsal-ventral boundary in late third instar larval stage, we used the hid^{20-10} -lacZ¹¹, that lacks non-specific expression (see Fig. 6b), to confirm our results. We obtained this line from H. D. Ryoo (NYU, New York, NY). *dronc*¹²⁴ and *dronc*¹²⁹ are considered null alleles²⁹, and were contributed by A. Bergmann (MD Anderson Center, Houston, TX). We recombined puc^{E69} with *dronc*¹²⁴. *hs-hid*³ and *hs-rpr*^{37B} are gifts of H. Steller (HHMI, New York, NY) and have been previously described^{28,40}.

Quantitative analysis

In each analysis all the discs were treated in the same conditions (see Inmunostaining section) and images were processed using the ImageJ software as follows. Each image analyzed was a representative section of the wing disc. A high intensity threshold that corresponded to the labeling with the antibody was adjusted for each image. Then we generated a region of interest (ROI) that encompassed the compartment under study and calculated the percentage of the ROI covered by the staining (pixels that are above of the threshold level) using the Area Fraction option in Set Measurements. The number of discs analyzed in each experiment is given in the figure legends. The *p*-values were calculated using two-tailed t-Student test in Microsoft Excel.

Inmunostaining and in situ hybridization

Inmunostaining was performed largely as described previously²², with slight modifications. Wing imaginal discs from wandering third instar larvae were dissected in ice-cold PBS and fixed in 4% paraformaldehyde, 0.1% Triton, 0.1% DOC for 30 minutes at room temperature. Discs were subsequently blocked in blocking buffer (PBS, 0.3% Triton, 1% BSA) and incubated overnight at 4°C with the primary antibody diluted in PBT (PBS, 0.3% Triton). Washes were performed in PBT and incubation with the secondary antibody was carried for two hours at room temperature. Following further washing discs were mounted in Vectashield (Vector Laboratories Inc.). All images were acquired with a Leica DB5500 B confocal microscope except double antibody staining and *in-situ* hybridization images, which were acquired with a LSM510 Meta (Zeiss) confocal microscope.

Primary antibodies were used at the following dilutions: mouse anti-b-gal (Promega) 1:1000; guinea pig anti-Hid (a gift from Hyung Don Ryoo, NYMC, NY), 1:200; rabbit anticleaved Caspase-3 (Cell Signaling Technology), 1:50; mouse anti-pJNK (Cell Signaling), 1:100; rabbit anti-GFP (Molecular Probes, Inc.), 1:300 and anti-Digoxigenin-AP (Roche), 1:4000. Molecular Probes Alexa secondary antibodies were used at 1:200.

For double antibody staining and *in situ* hybridization, the *in situ* hybridization was performed as described elsewhere with the following modification: *in situ* hybridization

was followed by incubation with the primary antibody (for example, anti-Caspase-3 in Fig. 6b), anti-GFP and anti-DIG-AP overnight at 4°C. After washing the discs where incubated with secondary antibodies, always including in the mix one that recognized the species of the anti-GFP, and also anti-DIG (a second round of incubation with the primary antibody to amplify the signal), for two hours at room temperature. The hybridization pattern was revealed using Roche Alkaline Phosphatase reagents NBT (#11 383 213 001) and BCIP (#11 383 221 001) in TrisHCl 1M pH9.5.

RNA probe for the *dp53* transcript was obtained using the BDGP EST GH11591 clone as a template. *rpr* probe is a gift of I. Lohmann (MPI for Developmental Biology, Tübingen, Germany).

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Competing financial interests

The authors declare no competing financial interests.

Figure legends

Figure 1

Pro-apoptotic genes are activated by Dp53 and the JNK pathway

a) Simplified scheme of the apoptotic pathway in *Drosophila*. Upstream activators like Dp53 and the JNK pathway induce one or more of the pro-apoptotic genes (only *hid* and *rpr* are shown in the scheme but there are other related genes, *grim* and *sickle*). The inactivation of the apoptosis inhibitor Diap1 by the pro-apoptotic proteins allows the proteolytic activation of the Dronc, Dcp-1 and Drice caspases and subsequent cell death (see⁶ for a more detailed description).

b, **c**) Control discs of genotype $spalt^{EPv}$ -Gal4>UAS-GFP doubly stained for GFP and Hid and for GFP and *rpr* transcripts. There is neither *hid* nor *rpr* activity in the *sal* domain, labelled green.

d-g) Activation of *hid* and *rpr* by Dp53 and JNK pathway. **d**, **e**) discs of genotype *spalt*^{*EPv*}-*Gal4*>*UAS-GFP UAS-dp53* showing Hid protein (**d**) and *rpr* transcription (**e**) in the *sal* domain. **f**, **g**) disc of genotype *spalt*^{*EPv*}-*Gal4*>*UAS-GFP UAS-hep*^{*ACT*} showing accumulation of the Hid protein (**f**) and *rpr* activity (**g**) in the *sal* domain.

h-k) The activation of *hid* and *rpr* by Dp53 and JNK pathway in *dronc* mutant discs. **h**) disc of genotype *spalt*^{*EPv*}-*Gal4*>*UAS-GFP UAS-dp53 dronc*⁻ demonstrating the presence of the Hid protein in the expanded *sal* domain. **i**) Disc of the same genotype as in **h** also showing *rpr* transcription in the expanded *sal* domain. **j**, **k**) *spalt*^{*EPv*}-*Gal4*>*UAS-GFP UAS-hep*^{*ACT} dronc*⁻ discs also showing gain of Hid (**j**) and *rpr* (**k**) in the *sal* domain. The *sal* domain in the *dronc* mutant discs becomes enlarged due to the ectopic expression of *wg* and *dpp* induced by JNK²².</sup>

Figure 2

The pro-apoptotic factor Hid activates *dp53*, the JNK pathway and *rpr* in a *dronc* dependant manner

a, **b**) Control discs of genotype *spalt*^{EPv}-*Gal4*>*UAS*-*GFP* doubly stained for GFP and *dp53* transcript (**a**) and for GFP and the phosphorylated form of JNK/Basket (**b**).

c, **d**) illustrates the effect of Hid on *dp53* transcription and JNK pathway activity. **c**) $spalt^{EPv}$ -Gal4>UAS-GFP UAS-hid disc showing *dp53* expression, revealed by *in situ* hybridization, in cells expressing *hid*, labelled by anti-Hid in red. **d**) In the *spalt*^{EPv}-Gal4>UAS-GFP UAS-hid disc there is also JNK activity in the *sal* domain in co-expression with Hid, as revealed with the antibody that recognizes the phosphorylated form of JNK/Basket (grey).

The **e**) row illustrates the activation of *rpr* by Hid. Following the same scheme as in the first two rows, forcing expression of *hid* in the *sal* domain results in *rpr* activation, revealed by *in situ* hybridization.

f, **g**, **h**) shows the result of forcing *hid* in *dronc*⁻ discs. (**f**) Disc of genotype *spalt*^{*EPv*}-*Gal4*>*UAS-GFP UAS-hid dronc*⁻ showing Hid expression in the *sal* domain but no *dp53* transcription. The (**g**) row demonstrates that JNK activity is absent in the *spalt*^{*EPv*}-*Gal4*>*UAS-GFP UAS-hid dronc*⁻ disc, and (**h**) shows that in *dronc* mutant discs *rpr* remains silent.

Figure 3

dp53 and JNK are able to activate each other independently of dronc function

a, b) In *sal>dp53* (*spalt^{EPv}-Gal4>UAS-dp53* UAS-GFP; *puc-lacZ/+*) and *sal>dp53* dronc⁻ (*spalt^{EPv}-Gal4>UAS-dp53* UAS-GFP; *puc^{E69}-lacZ* dronc¹²⁴/dronc¹²⁹) discs the JNK pathway becomes activated, as revealed by the *puc-LacZ* marker. In otherwise wildtype discs *puc-lacZ* expression is restricted to the most proximal cells (inset). Conversely, driving JNK expression in the *sal* domain activates *dp53* transcription in both *dronc*⁺ and *dronc*⁻ discs (**c, d**).

Figure 4

Apoptotic response to a brief pulse of *hid* activity

a) *en-Gal4* >*UAS-GFP; hs-hid* wing disc showing high levels of anti-Caspase-3 (green) and TUNEL (red). GFP staining is shown in blue. A magnification is shown in **a**' to appreciate the concordance between anti-Caspase-3 and TUNEL staining. **b**) *en-Gal4*>*UAS-GFP UAS-dronc RNAi; hs-hid*, showing a much reduced response in the posterior compartment. In the discs of genotype *en-Gal4*>*UAS-GFP UAS-dp53 RNAi; hs-hid* (**c**) or *en-Gal4*>*UAS-GFP UAS-GFP UAS-puc; hs-hid* (**d**) the apoptotic response in the posterior compartments is also much reduced. Note that TUNEL and anti-Caspase-3 staining are highly concordant in all cases.

Figure 5

Loop-mediated amplification of Hid levels after a pulse of hid

a) Control *en-Gal4>UAS-GFP; hs-hid* flies. The expression of GFP is shown in blue. The 30 min heat shock induces high levels of Caspase-3 (green) and Hid (red) both in the anterior and posterior compartments.
b) *en-Gal4>UAS-GFP UAS-dronc RNAi; hs-hid* disc showing a large reduction of Caspase-3 and Hid activity in the posterior compartment, due to the suppression of *dronc* function. The third panel clearly indicates that most of the Hid

protein visible after the heat shock is generated by the feed back loop. **c)** A *en-Gal4*> UAS-*GFP UAS-dp53 RNAi; hs-hid* disc showing reduction of Caspase-3 and Hid in the posterior compartment, where *dp53* function is diminished. **d)** *en-Gal4*> UAS-GFP UAS-puc; hs-hid disc with reduced levels of Caspase-3 and Hid in the posterior compartment. **e)** The panel shows the results of quantitative measurements of Caspase-3 and Hid activities in the genotypes studied. The percentage of the area of each compartment covered by the staining with anti-Caspase-3 or anti-Hid was calculated as indicated in the Materials and Methods section. There is no statistically significant difference between the values in UAS-GFP expressing discs (n = 23, p > 0.05 both for anti-Caspase-3 and anti-Hid). A statistically significant reduction in the values of the posterior compartment is observed when UAS*dronc-RNAi* (n = 19, p < 0.0001 for both markers), UAS-dp53-RNAi (n = 20, p < 0.01 for anti-Caspase-3 and p<0.001 for anti-Hid) or UAS-puc (n = 27, p < 0.01 for anti-Caspase-3 and p < 0,001 for anti-Hid) are crossed to *en-Gal4 UAS-GFP* flies.

Figure 6

Activation of the endogenous *hid* gene after a pulse of Hid protein

a- a'') Disc of the genotype *en-Gal4*>*UAS-GFP*, *UAS-dronc RNAi*, *hid*⁰⁵⁰¹⁴-*lacZ*; *hs-hid* fixed four hours after the end of the heat shock, labelled for β -gal (red) and caspase (green). The posterior compartment is labelled in blue. The panel **a'** shows *lacZ* activity in the anterior compartment, which is largely co-extensive with caspase activity, shown in **a''**. b) Non-heat shocked disc of genotype *hs-hid/hid*²⁰⁻¹⁰-*lacZ* showing background levels of *lacZ* expression. **c-c'**) Disc of the same genotype fixed four hours after heat shock. *lacZ* expression is induced and is co-extensive with high levels of caspase activity.

Figure 7

Activation of the JNK pathway after physiological stress

a) Control puc^{E69} -lacZ/+ disc showing high levels of *puc* expression and caspase activity in the wing pouch four hours after an irradiation of 2000R. The expression of *puc* in the proximal region (arrow) is normally present and corresponds to the midline cells. **b**) Disc of genotype $dronc^{I24}$, puc^{E69} -lacZ/dronc^{I29} showing very low expression of *puc* and caspase activity after the same dose of irradiation as in a). Note (arrow) the normal *puc* expression

in the midline cells. c) Disc of genotype en>diap1; $puc^{E69}-lacZ/+$. The low *dronc* activity in the posterior compartment results in low level of *puc* expression and caspase activity after a 2000R radiation. d) The left panel shows a quantification of *puc* levels (see Materials and Methods) in a normal non-irradiated $puc^{E69}-lacZ/+$ disc, in an irradiated disc of the same phenotype, and in an irradiated $dronc^{124}$, $puc^{E69}-lacZ/dronc^{129}$ disc. The middle and right panels show amplification of the inset in the corresponding photographs in c).

Figure 8

Amplification loop model of stress induced apoptosis.

We envisage stress-induced apoptosis as follows: an original stress event (irradiation, heat shock) induces dp53 function, which activates *hid* and *rpr* transcription and also the JNK pathway. The JNK pathway is also able to amplify dp53 transcription (and probably to induce *rpr* and *hid* as well). The activation of *dronc* due to Hid/Rpr function gives rise on the one hand to the activation of effector caspases, and on the other to an increase of both Dp53 and JNK levels, thus completing the amplification loop. This feedback is necessary for the completion of the apoptotic program, because in its absence there is a dramatic reduction in the amount of cell death. The model implies that the cell killing function of Dronc requires amplification of its own levels. This is achieved by its ability to induce dp53/JNK function.

The model incorporates the functional interactions among the various factors involved, but it does not contemplate their interactions at the molecular level. Some of the molecular interactions between Hid, Rpr, Diap1, Dronc and Drice are well known^{6,7}, but the mechanisms of activation of dp53 and JNK by Dronc and the specific molecular interactions between dp53 and JNK have not yet been described.

Supporting figures

Figure legends

Figure S1

The activation of dp53, the JNK pathway and hid by Rpr requires dronc function.

The genotype of the left panels is sal^{EPv} -Gal4>UAS-GFP UAS-rpr and that of the discs in the right panels is sal^{EPv} -Gal4>UAS-GFP UAS-rpr; dronc⁻. The activity of rpr in the sal domain induces dp53 transcription (**a**), JNK activity, monitored by puc-lacZ (**c**) and hid expression, indicated by high levels of Hid protein (**e**). However, in the absence of dronc function Rpr is unable to induce those genes, as illustrated in the **b**, **d**, and **f** panels.

Figure S2

Suppression of *hs-hid* induced apoptosis by a dominant negative form of Dp53

a) Disc of the genotype *en-Gal4*>*UAS-GFP*, *UAS-p53*^{DN}; *hs-hid* stained for TUNEL (red) and Caspase-3 (green) fixed four hours after the heat shock. The posterior compartment is labeled in blue. There is virtually no apoptotic response in the posterior compartment. **b**) Disc of the same genotype as above showing anti-Hid (red) and anti-Caspase-3 (green) staining. **c**) Result of quantitative measurements of Caspase-3 and Hid in the anterior (dark grey) and posterior (light grey) compartments. The data were obtained as indicated in Material and Methods section. More than 20 discs were analyzed; the *p*-values are below 0.0001.

Figure S3

Accumulation of Hid protein after a pulse of Rpr protein

a) Control *en-Gal4>UAS-GFP; hs-rpr* disc showing accumulation of Drice and Hid proteins in the entire disc.
b) *en>Gal4>UAS-GFP UAS-dronc RNAi; hs-rpr* disc in which there is very little Drice and Hid proteins in the posterior compartment (blue).
c) In the *en>Gal4>UAS-GFP UAS-puc; hs-rpr* disc there is also a strong reduction of Drice and Hid in the posterior compartments.

High levels of Hid after a pulse of hid in leg and haltere discs

a) Leg imaginal disc of the genotype *en-Gal4*>*UAS-GFP*, *UAS-dronc RNAi; hs-hid* showing anti-Hid (red) and anti-Caspase-3 (green) labeling four hours after the heat shock treatment. The posterior compartment is in blue. **b**) A haltere disc of the same genotype stained with the same antibodies as in a). In both cases the number of Hid positive cells is less in the posterior compartment when compared to the anterior.

Figure S5

Accumulation of Hid protein after heat shock

a - **a**^{''}) In a non heat shocked disc of the genotype *en-Gal4*>*UAS-GFP*, *UAS-dronc RNAi*; *hs-hid* Hid (red) and caspase (green) protein levels are barely detectable. Posterior compartments are labeled blue. **b** - **b**^{''}) disc of the same genotype fixed the end of a 30 min heat shock and stained for Hid and caspase antibodies. The amounts of Hid protein appear to be similar in the anterior and posterior compartments, but there is little caspase activity, restricted to some few cells in the anterior compartment. **c** - **c**^{''}) Disc of the same genotype fixed 30 minutes after the end of the heat shock. There already is a difference in Hid proteins levels in the two compartments, being higher in the anterior one. Caspase activity is becoming more extensive in the anterior compartment (compare b'' with c'').

Figure S6

JNK activity in the leg imaginal discs in absence of dronc function

a, **a**') Control puc^{E69} -lacZ/+ late third instar leg imaginal disc showing rings of puc activity (green) in the distal region (arrow). The arrowhead indicates the non-apoptotic expression of *puc* in the stalk of the disc. **b**, **b**') panels show the expression of *puc* in *dronc*¹²⁴, *puc*^{E69}- lacZ/dronc¹²⁹ (dronc⁻) leg discs. The distal rings are no longer seen (arrow), although the expression in the stalk remains. **c**, **c'**) Control everted leg disc from an early pupa showing

clear bands of *puc* activity in the presumptive distal joints (arrows). This pattern disappears in *dronc*⁻ mutants (\mathbf{d}, \mathbf{d}').



Figure 1 Shlevkov and Morata



Figure 2 Shlevkov and Morata

rpr

puc-lacZ

dp53 mRNA



Figure 3 Shlevkov and Morata



Figure 4 Shlevkov and Morata



Figure 5 Shlevkov and Morata



Figure 6 Shlevkov and Morata





Figure 7 Shlevkov and Morata

Caspase-3



Figure 8 Shlevkov and Morata