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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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Programmed cell death (apoptosis) is a conserved process aimed to eliminate unwanted cells. The 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 proapoptotic genes (*hid, rpr, grim*), which in turn activate caspases. In stress-induced apoptosis, the *dp53* (*Drosophila p53*) gene and the Jun N-terminal kinase (JNK) pathway function upstream in the activation of the proapoptotic genes. Here we demonstrate that *dp53* and JNK also function downstream of proapoptotic genes and the initiator caspase Dronc (*Drosophila* NEDD2-like caspase) 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 proapoptotic proteins Hid and Rpr. Thus, our results indicate that stress-induced apoptosis in *Drosophila* is dependant on an amplification loop mediated by *dp53* and JNK. Furthermore, they also demonstrate a mechanism of mutual activation of proapoptotic genes.
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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 death.[3] 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 proapoptotic 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 caspases.[6] A target of Diap1 is the initiator caspase-9 ortholog Dronc (*Drosophila* NEDD2-like caspase).[7] The only known Dronc substrates to date are effector caspases Dcp-1, Drice (*Drosophila* interleukin converting enzyme) and Diap1 itself.[6]

The *p53* gene is a major proapoptotic factor in both *Drosophila* and vertebrates.[8] In mammalian cells, the P53 protein has other nonapoptotic functions; it is involved in antiproliferative responses, including cell-cycle arrest and senescence.[9,10] In contrast, the *Drosophila* homolog of *p53*, called *dp53*, has mostly a proapoptotic role, although some effect on cell-cycle regulation has been noticed in over-expression experiments.[11] It has been reported that *dp53* activates the transcription of *rpr* in response to radiation-induced DNA damage to initiate apoptosis,[12] and that over-expression of *dp53* in the eye induces Hid-mediated apoptosis.[11] Thus, *dp53* is currently depicted upstream of the apoptotic cascade (Figure 1a). Another major proapoptotic factor in *Drosophila* and vertebrates is the Jun N-terminal kinase (JNK) pathway.[13] In *Drosophila*, this pathway plays a prominent role in inducing apoptosis and is activated in a variety of contexts, including tumor 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 P35.[16] The presence of P35 does not change the apoptotic nature of these cells (called 'undead' cells), but they remain alive, and this is what facilitates the study of their properties.

A significant observation is that after a brief apoptotic stimulus, X-radiation or a 2 to 3 h 37°C heat shock, cells containing P35 remain in a prolonged apoptotic status: 72–96 h after the end of the stimulus, when normally cell death is already undetectable, they still show expression of the proapoptotic gene *hid* and active Drice.[17] Furthermore, they show persistent expression of the signalling genes

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Abbreviations: Diap1, *Drosophila* inhibitor of apoptosis protein 1; *dp53*, *Drosophila p53*; *dp53-i*, *dp53* RNA interference; *dpp*, *decapenthaplegic*; Drice, *Drosophila* interleukin converting enzyme; Dronc, *Drosophila* NEDD2-like caspase; *dronc-i*, *dronc* RNA-interference; *en*, *engrailed*; *gfp*, *green fluorescent protein*; *hep*, *hemipterous*; *hid*, *head involution defective*; *hs-hid*, *heat shock-head involution defective*; *hs-rpr*, *heat shock-reaper*; IAP, inhibitor of apoptosis protein; JNK, Jun N-terminal kinase; *puc*, *puckered*; ROI, region of interest; *rpr*, *reaper*; *sal*, *spalt*; TUNEL, TdT-mediated fluoroscein-dUTP nick end labelling; UAS, upstream activation sequence; *wg*, *wingless*
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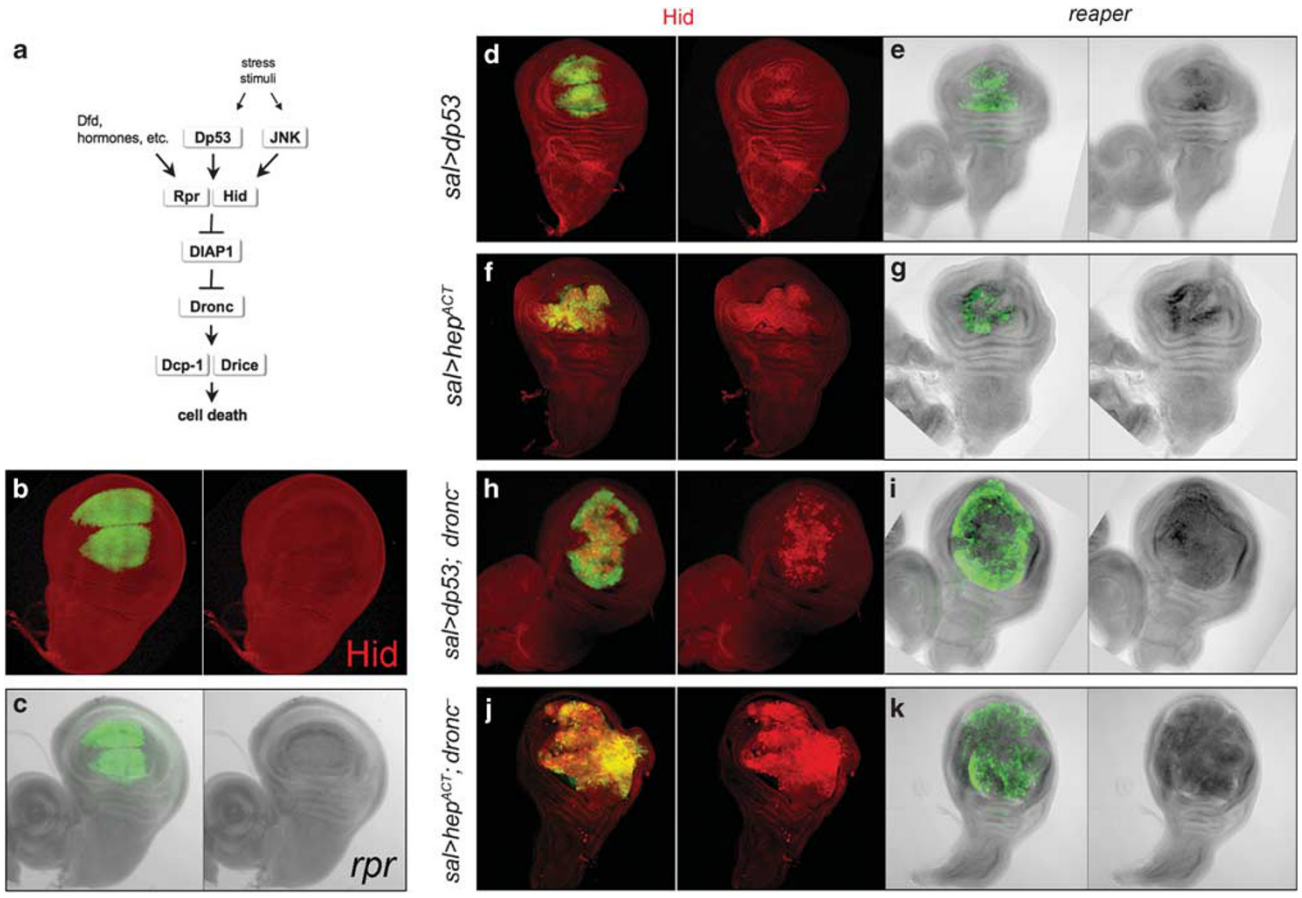

Figure 1 Proapoptotic genes are activated by Dp53 and the JNK pathway. (**a**) Simplified scheme of the apoptotic pathway in *Drosophila*. Upstream activators like Dp53 and the JNK pathway induce one or more of the proapoptotic genes (only *hid* and *rpr* are shown in the scheme but there are other related genes, *grim* and *sickle*). The inactivation of the apoptosis inhibitor Diap1 by the proapoptotic proteins allows the proteolytic activation of the Dronc, Dcp-1 and Drice caspases and subsequent cell death (see Hay and Guo[6] for a more detailed description). (**b** and **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** and **e**) Discs of genotype $spalt^{EPv}$-*Gal4*> *UAS-GFP UAS-dp53* showing Hid protein (**d**) and *rpr* transcription (**e**) in the *sal* domain. (**f** and **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** and **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 because of the ectopic expression of *wg* and *dpp* induced by JNK[22]

decapenthaplegic (*dpp*) and *wingless* (*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 Dronc.[19] The function of *dp53* both upstream and downstream Dronc suggested that it is part of a feedback loop aimed to maintain the apoptotic program.[19] A similar argument can be made with the JNK pathway: irradiation induces JNK, which in turn induces *rpr*,[20] but JNK also functions 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*/JNK-mediated 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 method[23] to drive expression of the different genes involved. As a routine we used the *sal^{EPv}-Gal4* line,[24] 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 behavior.

As *dp53* and the JNK pathway appear to be key factors in apoptosis, we first examined their ability to induce proapoptotic 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 pathway[26]), the two proapoptotic genes become expressed in the *spalt* (*sal*) domain (Figures 1d–g). The induction also occurs in discs of the null allelic combination *dronc^{I24}/dronc^{I29}* (hereafter referred to as *dronc^{-}*), indicating that *hid* and *rpr* induction by *dp53* and JNK is not mediated by a feedback loop (Figures 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 (Figures 2c and d). However, and importantly, the activation of both *dp53* and JNK by Hid does not occur in *dronc^{-}* mutants (Figures 2f and 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 proapoptotic genes. Complementary experiments forcing *rpr* expression yielded similar results, included in Supplementary Figure S1.

We finally tested the existence of interactions between *dp53* and JNK. The experiments consisted of forcing

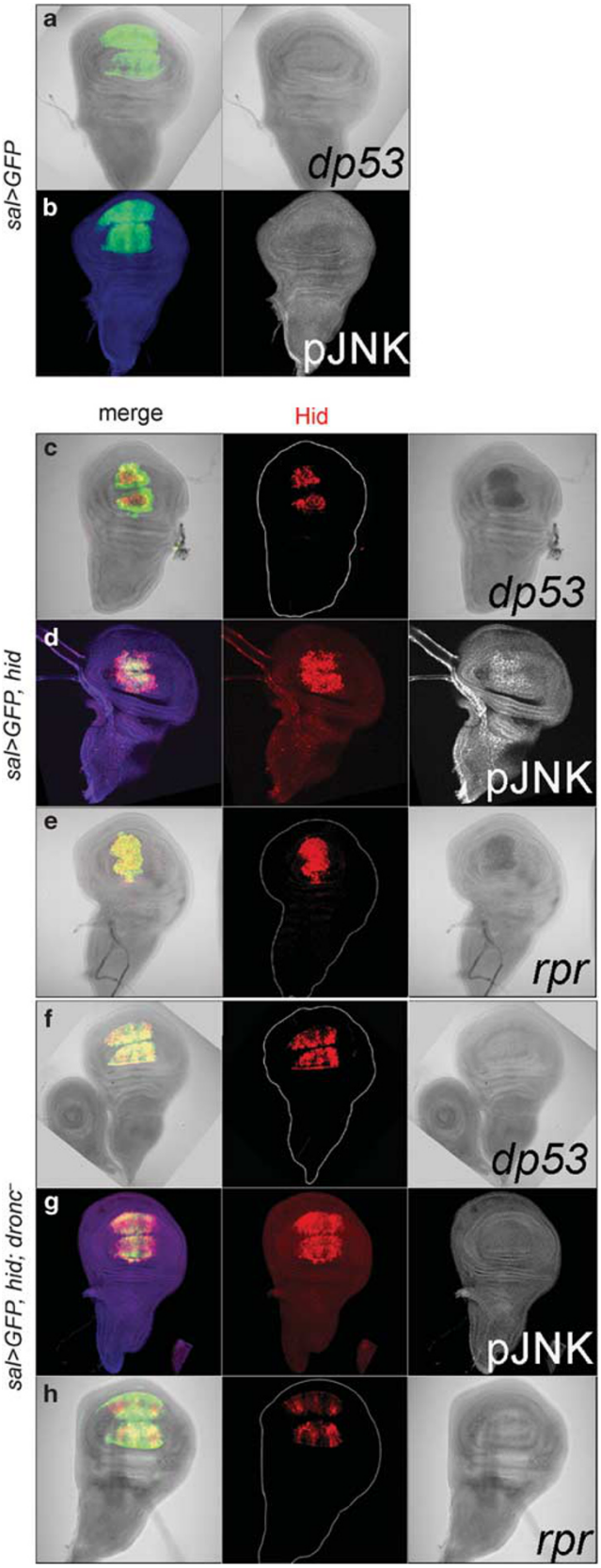

Figure 2 The proapoptotic factor Hid activates *dp53*, the JNK pathway and *rpr* in a *dronc*-dependant manner. (**a** and **b**) Control discs of genotype *spalt^{EPv}-Gal4> UAS-GFP* doubly stained for GFP and *dp53* transcript (**a**) and for GFP and the phosphorylated form of JNK/Basket (**b**). (**c** and **d**) 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 coexpression with Hid, as revealed with the antibody that recognizes the phosphorylated form of JNK/Basket (gray). 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–h**) The result of forcing *hid* in *dronc^{-}* discs is shown. (**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**) that in *dronc* mutant discs *rpr* remains silent

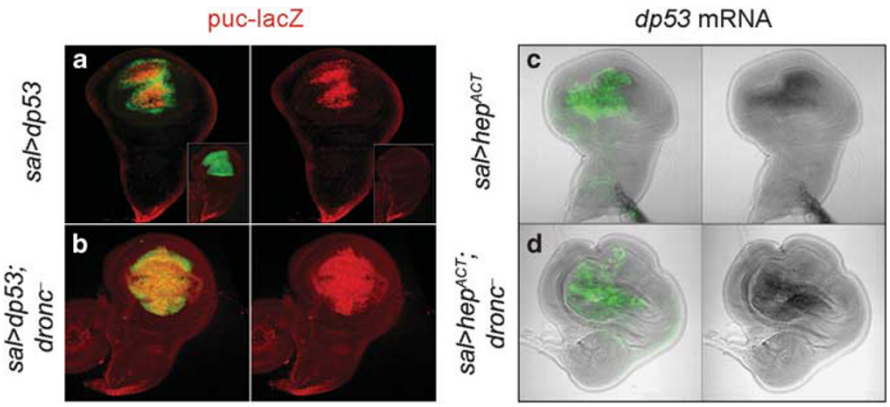

Figure 3 *dp53* and JNK are able to activate each other independently of dronc function. (**a** and **b**) In *sal*> *dp53* (*spalt*^{EPv}-*Gal4*> *UAS-dp53 UAS-GFP; puc-lacZ*/+) and *sal*> *dp53 dronc*$^{-}$ (*spalt*^{EPv}-*Gal4*> *UAS-dp53 UAS-GFP; puc*^{E69}-*lacZ dronc*^{124}/*dronc*^{129}) discs, the JNK pathway becomes activated, as revealed by the puc-LacZ marker. In otherwise wild-type 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** and **d**)

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 proapoptotic genes such as *hid* or *rpr* and then to study the apoptotic response of the wing disc in the presence and 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 proapoptotic activity simply by a temperature shift (pulse of 30 min at 37°C) and independently of upstream controls. The apoptotic response was measured by examining Drice protein levels and TUNEL (TdT-mediated fluoroscein-dUTP nick end labeling) staining 4 h 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 *heat shock-reaper* (*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 (Supplementary Figure S2), thus confirming its specificity. In genotype 4, the overexpression of the negative regulator *puckered (puc)* causes the strong reduction of JNK activity.[27] As the results obtained in the two series of experiments are very similar, we only describe in detail the *heat shock-head involution defective* (*hs-hid*) series (Figures 4 and 5). The results of the *hs-rpr* experiments are shown in Supplementary 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 (Figures 4a and 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 (Figures 4c and 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 (Figures 5a and e), in *en*> *dronc-i*; *hs-hid* Hid is almost undetectable in the posterior compartment (Figure 5b and 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 (Figures 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 (Supplementary Figures S3b and c). We also examined the apoptotic response in haltere and leg discs, seeking to confirm the results in the wing disc. As shown in Supplementary Figure S4, we obtain similar results.

The preceding experiments strongly suggest that the proapoptotic proteins are able to induce their own genes through the loop. However, there was the possibility that the

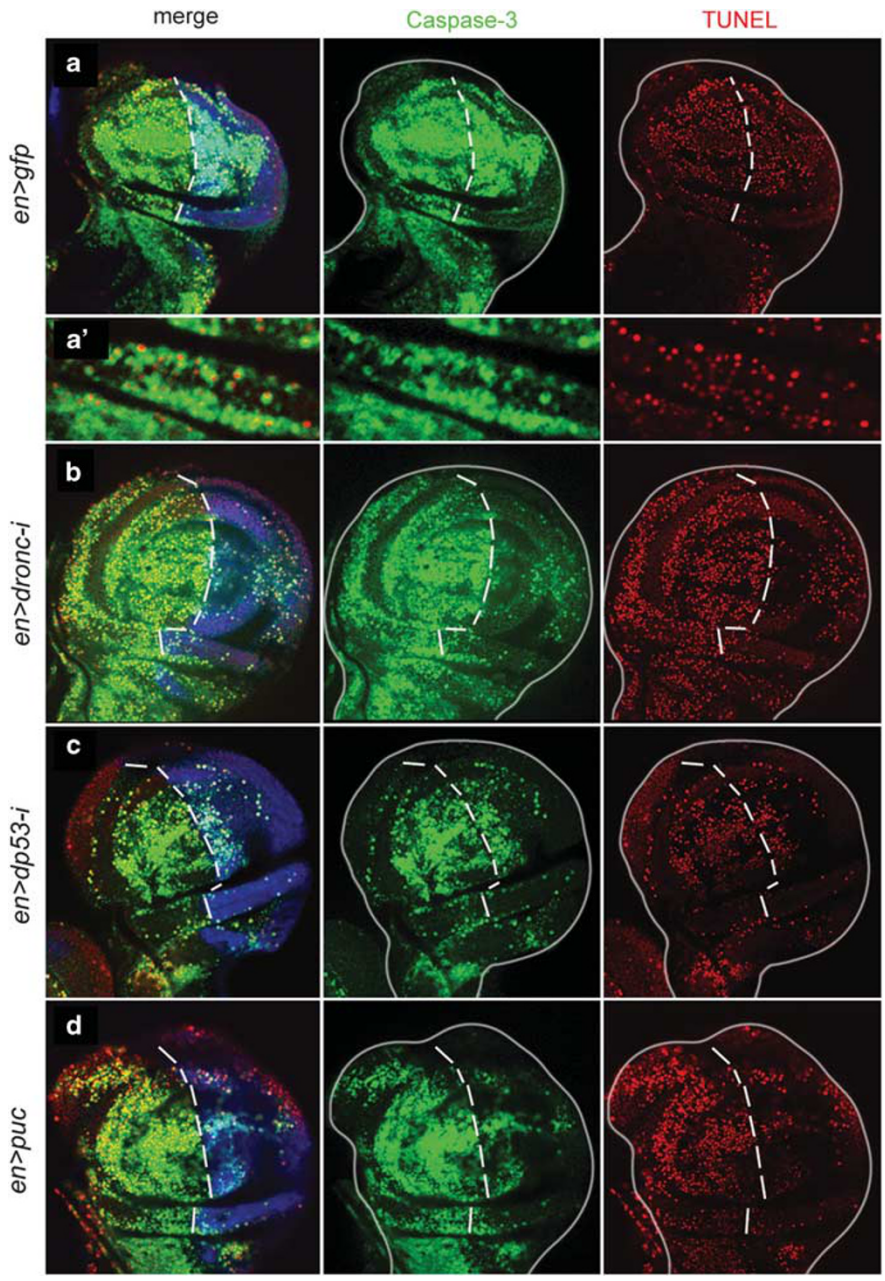

Figure 4 Apoptotic response to a brief pulse of *hid* activity. (**a**) *en-Gal4 > UAS-GFP; hs-hid* wing disc showing high levels of anti-Caspase-3 (green) and TUNEL (red). GFP staining is shown in blue. A magnification is shown in (**a**′) to appreciate the concordance between anti-Caspase-3 and TUNEL staining. (**b**) *en-Gal4 > UAS-GFP UAS-dronc RNA-i; hs-hid* showing a much reduced response in the posterior compartment. In the discs of genotype *en-Gal4 > UAS-GFP UAS-dp53 RNA-i; hs-hid* (**c**) or *en-Gal4 > 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

low levels of Hid protein in the posterior compartments, in which *dronc/dp53*/JNK are inhibited, are because of either 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 upregulation 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 Dp53.[11] 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 (Supplementary Figure S5).

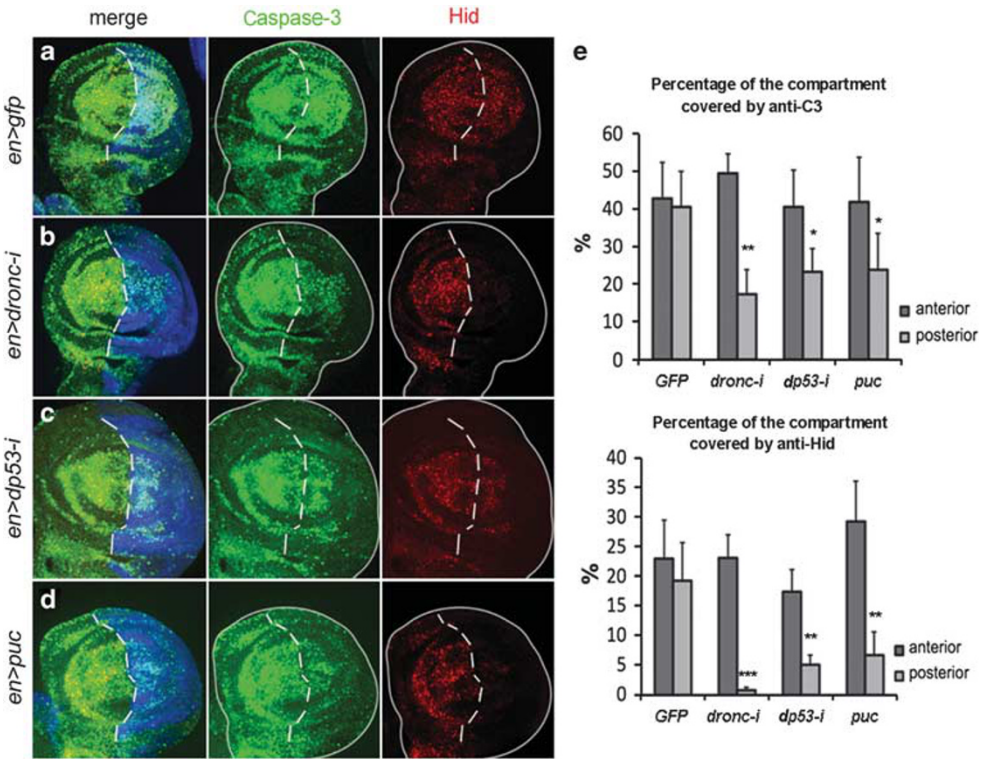

Figure 5 Loop-mediated amplification of Hid levels after a pulse of *hid*. (**a**) Control *en-Gal4> UAS-GFP; hs-hid* flies. The expression of GFP is shown in blue. The 30 min heat shock induces high levels of Caspase-3 (green) and Hid (red), both in the anterior and posterior compartments. (**b**) *en-Gal4> UAS-GFP UAS-dronc RNA-i; hs-hid* disc showing a large reduction of Caspase-3 and Hid activity in the posterior compartment because of 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 feedback loop. (**c**) A *en-Gal4> UAS-GFP UAS-dp53 RNA-i; 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 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-RNA-i* ($n=19$, $P<0.0001$ for both markers), *UAS-dp53-RNA-i* ($n=20$, $P<0.01$ 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. *$P<0.01$, **$P<0.001$, ***$P<0.0001$

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 proapoptotic genes.

We also analyzed the involvement of the feedback loop in the apoptotic response to physiological stress. Previous work[22] 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 because of its activation downstream *dronc*, and then we designed experiments to measure and compare JNK activity levels after irradiation in the presence and absence of *dronc* activity. We have tested JNK activity 4 h 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*^{I29}/*dronc*^{I24} allows normal larval viability up to the pupal stage.[29] (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* function.[30] In this experiment, the anterior compartments serve as controls because *dronc* function is unaffected.

The results are illustrated in Figure 7. In *dronc*$^{-}$ discs, *puc* expression after irradiation (Figure 7b and 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 shown[31] 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

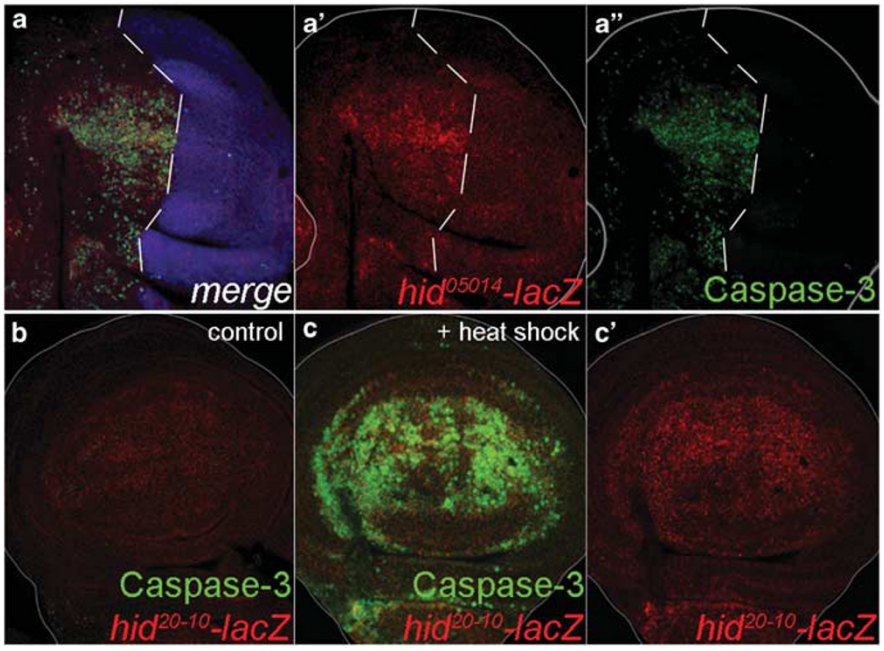

Figure 6 Activation of the endogenous *hid* gene after a pulse of Hid protein. (**a**–**a**″) Disc of the genotype *en-Gal4 > UAS-GFP, UAS-dronc RNA-i, hid*^{05014}*-lacZ; hs-hid* fixed 4 h after the end of the heat shock, labelled for β-gal (red) and caspase (green). The posterior compartment is labelled in blue. The panel (**a'**) shows *lacZ* activity in the anterior compartment, which is largely coextensive 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** and **c**′) Disc of the same genotype fixed 4 h after heat shock. *lacZ* expression is induced and is coextensive with high levels of caspase activity

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 channeled by upstream activators like *dp53* and/or JNK, which activate one or more of the proapoptotic 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 proapoptotic genes in response to stress like DNA damage, but they also act downstream *dronc* to amplify the apoptotic program by secondary activation of additional proapoptotic 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 (Figures 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 (Figures 4 and 5 and Supplementary Figure S3) in which we force the initiation of the apoptotic program by providing the proapoptotic 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 proapoptotic genes; as indicated in Figures 2e and h, the activation of *hid* gives rise to *rpr* function, and conversely *rpr* function induces *hid* (Supplementary Figure S1). This mutual activation may be an important part of the completion of the program because it has been shown in a recent report[30] 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 Dp53,[35] 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 proapoptotic genes at low levels. Depending on a threshold of *dronc* function, it may result 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 functions in some developmental contexts. In the leg disc, it has been shown that the correct formation of

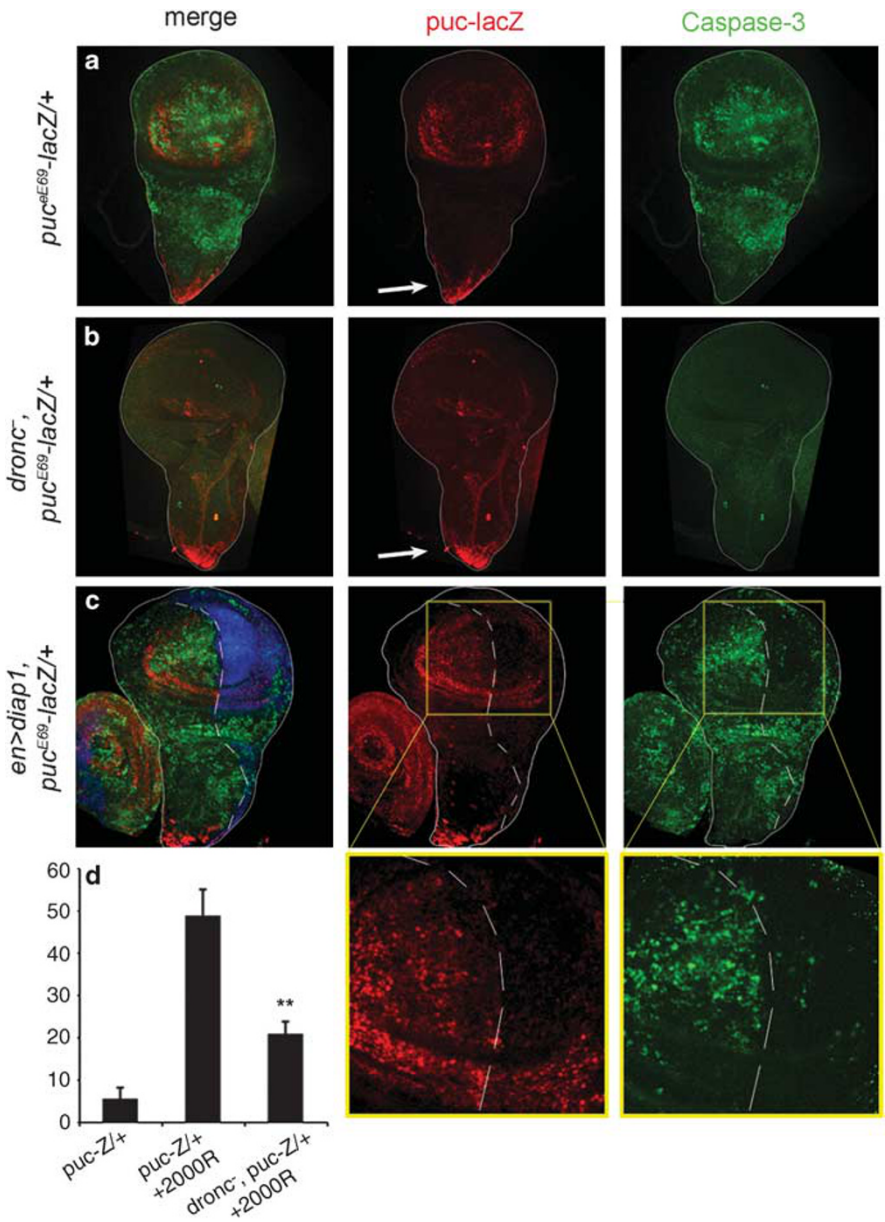

Figure 7 Activation of the JNK pathway after physiological stress. (**a**) Control puc^{E69}-*lacZ*/+ disc showing high levels of *puc* expression and caspase activity in the wing pouch 4 h after an irradiation of 2000R. The expression of *puc* in the proximal region (arrow) is normally present and corresponds 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 nonirradiated puc^{E69}-*lacZ*/+ disc, in an irradiated disc of the same phenotype and in an irradiated $dronc^{I24}$, puc^{E69}-*lacZ*/$dronc^{I29}$ disc. The middle and right panels show amplification of the inset in the corresponding photographs in (**c**). $^{**}P<0.001$

the leg articulations requires local apoptosis in the intersegmental regions,[33] where *rpr* and the JNK pathway are coexpressed. We have observed that this local JNK activity is suppressed in *dronc* mutant discs (Supplementary 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 activity,[36] 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 proapoptotic role.[38] 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 antitumor 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

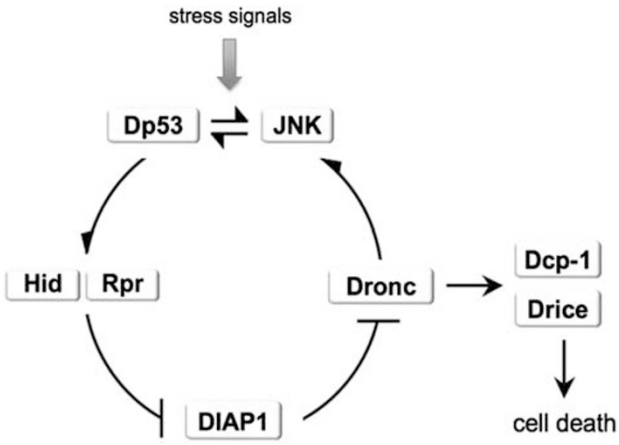

Figure 8 Amplification loop model of stress-induced apoptosis. We envisage stress-induced apoptosis as follows: an original stress event (irradiation, heat shock) induces *dp53* function, which activates *hid* and *rpr* transcription and also the JNK pathway. The JNK pathway is also able to amplify *dp53* transcription (and probably to induce *rpr* and *hid* as well). The activation of *dronc* because of Hid/Rpr function gives rise, on 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

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 proapoptotic and hence tumor-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 tumors 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 JF de Celis, CBMSO, Madrid, Spain). *UAS-dronc-RNA-i* (no. 23035) and *UAS-dp53-RNA-i* (no. 10692) were obtained from Vienna *Drosophila* RNA-i Center (Vienna, Austria). *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 region,[28] because it has been used previously as a reporter of *hid* activity.[39] As this line has a nonapoptotic expression in some anterior cells close to the dorsal–ventral boundary in late third-instar larval stage, we used the *hid^{l20-10}-lacZ*,[11] which lacks nonspecific expression (see Figure 6b), to confirm our results. We obtained this line from HD Ryoo (NYU, New York, NY, USA). *dronc^{I24}* and *dronc^{I29}* are considered null alleles,[29] and were contributed by A Bergmann (MD Anderson Center, Houston, TX, USA). We recombined *puc^{E69}* with *dronc^{I24}*, and *hs-hid^{3}* and *hs-rpr^{37B}* are gifts of H Steller (HHMI, New York, NY, USA) and have been previously described.[28,40]

Quantitative analysis. In each analysis, all the discs were treated in the same condition (see Inmunostaining section) and images were processed using the ImageJ software (NIH, Bethesda, MD, USA) as follows. Each image analyzed was a representative section of the wing disc. A high-intensity threshold that corresponded to the labelling 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 Student's *t*-test in Microsoft Excel.

Inmunostaining and *in situ* hybridization. Inmunostaining was performed largely as described previously,[22] 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 and 0.1% DOC for 30 min at room temperature. Discs were subsequently blocked in blocking buffer (PBS, 0.3% Triton and 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 2 h at room temperature. Following further washing, discs were mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). All images were acquired with a Leica (Solms, Germany) DB5500 B confocal microscope except double antibody staining and *in situ* hybridization images, which were acquired with a LSM510 Meta (Zeiss, Thornwood, NY, USA) confocal microscope.

Primary antibodies were used in the following dilutions: mouse anti-β-gal (Promega, Madison, WI, USA) 1 : 1000; guinea pig anti-Hid (a gift from Hyung Don Ryoo, NYMC, New York, NY, USA), 1 : 200; rabbit anti-cleaved Caspase-3 (Cell Signaling Technology, Danvers, MA, USA), 1 : 50; mouse anti-pJNK (Cell Signaling), 1 : 100; rabbit anti-GFP (Molecular Probes, Inc., Eugene, OR, USA), 1 : 300; and anti-Digoxigenin-AP (Roche, Basel, Switzerland), 1 : 4000. Molecular Probes Alexa secondary antibodies were used in a 1 : 200 dilution.

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 (e.g., anti-Caspase-3 in Figure 6b), anti-GFP and anti-DIG-AP overnight at 4°C After washing the discs were 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 2 h at room temperature. The hybridization pattern was revealed using Roche Alkaline Phosphatase reagents NBT (no. 11 383 213 001) and BCIP (no. 11 383 221 001) in 1 M Tris-HCl, pH 9.5.

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).

Conflict of Interest

The authors declare no conflict of interest.

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