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JNK and JAK/STAT signalling are required for inducing loss of cell fate specification during imaginal wing discs regeneration in *Drosophila melanogaster*

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ABSTRACT

The regenerative process after tissue damage relies on a variety of cellular responses that includes compensatory cell proliferation and cell fate re-specification. The identification of the signalling networks regulating these cellular events is a central question in regenerative biology. Tissue regeneration models in *Drosophila* have shown that two of the signals that play a fundamental role during the early stages of regeneration are the c-Jun N-terminal kinase (JNK) and JAK/STAT signalling pathways. These pathways have been shown to be required for controlling regenerative proliferation, however their contribution to the processes of cellular reprogramming and cell fate re-specification that take place during regeneration are largely unknown. Here, we present evidence for a previously unrecognised function of the cooperative activities of JNK and JAK/STAT signalling pathways in inducing loss of cell fate specification in imaginal discs. We show that co-activation of these signalling pathways induces both the cell fate changes in injured areas, as well as in adjacent cells. We have also found that this function relies on the activity of the Caspase initiator encoded by the gene *dronc*.

1. Introduction

Most animal species have the ability to recover from organ damage by a process known as regeneration (Tanaka and Reddien, 2011; Brockes and Kumar, 2008). Restoration of the damaged organ involves multiple cellular processes, such as regenerative growth, and cellular reprogramming. All of these processes are initiated by the activity of different signalling pathways. There are multiple studies that have analysed the signals that promote regenerative growth from the wound edges (Sun and Irvine, 2014). However, much less is known about the mechanisms that regulate cellular reprogramming and re-patterning during regeneration. Another unresolved question is how the different pathways that operate in regeneration act in coordination to regulate all the events involved in this complex process.

The imaginal discs of *Drosophila melanogaster* are one of the model systems that have been extensively used to study the genetic mechanisms that regulate regeneration (Bergantinos et al., 2010b; Bryant, 1971, 1975; Hadorn and Buck, 1962; Schubiger, 1971; Worley et al., 2012). During larval stages, until the beginning of metamorphosis, the imaginal discs maintain the ability to regenerate (Bergantinos et al., 2010b; Worley et al., 2012; Diaz-Garcia and Baonza, 2013; Smith-Bolton et al., 2009). It is also during larval stages when the characteristic veins and intervein pattern observed in the adult wings are defined. The restricted function of different genes in specific regions of the larval wing disc promotes intervein or vein cell fate (De Celis, 2003). It has been shown that in regenerating discs the vein/intervein pattern defined by the expression of different patterning genes is temporary eliminated in the wound edges as well as in large regions of the discs (Smith-Bolton et al., 2009; Diaz-Garcia and Baonza, 2013; Repiso et al., 2013). Accordingly, it has been proposed that a re-patterning process takes place in the early stages of disc regeneration, which implies the temporary loss of cell fate commitment (Smith-Bolton et al., 2009; Diaz-Garcia and Baonza, 2013; Repiso et al., 2013; Herrera and Morata, 2014; Morata and Herrera, 2016). The signals required to induce cell fate changes are largely unknown.

One of the signals that has been shown to play a fundamental role during regeneration is the c-Jun N-terminal kinase (JNK). This pathway is initially triggered at the wound site in response to cellular stress (Bosch et al., 2005), and plays a fundamental role in regulating many biological processes involved in regeneration (Lee et al., 2005; Bogoyevitch et al., 2010; Bosch et al., 2005, 2008; Bergantinos et al., 2010a; Pastor-Pareja et al., 2008; Chen, 2012). Inhibition of JNK

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function during disc regeneration impairs wound healing and reduces regenerative proliferation (Ramet et al., 2002; Mattila et al., 2005; Bosch et al., 2005, 2008). During disc regeneration, JNK signalling promotes the activation of at least two downstream pathways: JAK/STAT and Wingless (*wg*) (Harris et al., 2016; Katsuyama et al., 2015; Pastor-Pareja et al., 2008; Smith-Bolton et al., 2009). *Drosophila* has three leptin-like (IL-6 family) cytokine ligands known as Unpaireds (Upd, Upd2, Upd3). These proteins bind to the IL-6R type receptor, *Domeless (dome)*, that activates a Janus kinase (JAK). In Drosophila, this kinase is encoded by the gene *hopscotch (hop)*. This phosphorylation cascade promotes the translocation of STAT92E transcription factor to the nucleus (Arbouzova and Zeidler, 2006). During disc regeneration it has been shown that the reduction of JAK/STAT function impairs cell proliferation and prevents normal recovery (Katsuyama et al., 2015; Santabarbara-Ruiz et al., 2015; La Fortezza et al., 2016). Accordingly, it has been proposed that JAK/STAT signalling functions downstream of JNK signalling by mediating compensatory cell proliferation (Katsuyama et al., 2015). However, it has recently been proposed that instead of promoting compensatory cell proliferation, JAK/STAT might be necessary to restrain excessive tissue damage caused by the activation of the JNK pathway, and may facilitate initiation of compensatory responses (La Fortezza et al., 2016). Therefore, the contribution of JAK/STAT signalling to the compensatory cell proliferation induced by JNK signalling is unclear. It is also unknown whether JAK/STAT and JNK signalling are involved in the processes of tissue re-patterning and loss of cell fate commitment associated with regeneration.

To better understand the role of JNK and JAK/STAT signalling in tissue re-patterning and loss of cell fate commitment in response to damage, we have used a combination of genetic techniques and surgical excision. The results presented here identify a previously unrecognised function for cooperative JNK and JAK/STAT signalling in the induction of loss of cell fate specification. Our data indicate that the ectopic activation of JNK signalling is sufficient to induce loss of cell fate commitment markers autonomously, as well as non-autonomously, in surrounding non JNK-expressing cells, independently of the apoptotic function of this pathway. These effects are strongly enhanced when JNK and JAK/STAT are co-activated, suggesting that both pathways synergize in the process of inducing loss of cell fate specification. Interestingly, we have found that the Caspase initiator Dronc is necessary to promote non-autonomous cell fate changes in response to the activation of JNK signalling. All together our data provide important insights into the mechanisms through which the activities of these two signalling pathways can regulate cell fate decisions in response to tissue damage.

2. Materials and methods

2.1. Drosophila stocks and genetics

The following stocks and *Gal4* lines were used:

The *UAS* lines used included: *UAS-GFP* (Bloomington stock center), *UAS-hep^{CA} (II) (III)* (Adachi-Yamada et al., 1999), *UAS-dome^{DN}* (II) (Sotillos et al., 2008), *UAS-hop^{tum-l}* (II) {Harrison et al. (1995) #883}, *UAS-hop$^{RNAi\ (TRIP-v20)}$* (Bloomington Drosophila Stock Center: 32966), *UAS-microRNARHG (II)* (Siegrist et al., 2010) (a gift from C. Estella) and *UAS-reaper* (Bloomington Drosophila Stock Center: 5823). Mutant *dronc^{i29} and dronc^{i24}* (a gift from G. Morata) (Xu et al., 2005).

We used the Gal4 lines: *en-Gal4 UAS-GFP/CyO; Tub-Gal 80 ^{TS}/TM6B, UAS-hep^{CA}UAS-GFP Tub-Gal80^{TS}; dronc^{i29} hh-Gal4/TM6B, and w; Tub-Gal80^{TS} /CyO; hh-Gal4/TM6B*. We obtained these lines from Gines Morata (CBM, Madrid) (Perez-Garijo et al., 2009). All of these stocks have been previously described in FlyBase (http://flybase.bio.indiana.edu/).

The reporter lines: *puc-LacZ* line (Martín-Blanco et al., 1998), *bs^{P1292}* [lac-Z rosy+] insertion *l(2)03267* (Roch et al., 1998), TRE-DsRed (Chatterjee and Bohmann, 2012) and 10X*STAT92E-GFP* (Bach et al., 2007).

Stocks and crosses were maintained on yeast food at 25 °C before and after the transitory inhibition of Gal80^{TS} at 29 °C.

2.2. Genetic analysis

This analysis was performed by crossing *en-Gal4 UAS-GFP /CyO; Tub-Gal80^{TS}/TM6B* to the following stocks:

UAS-dome^{DN}; UAS-hep^{CA}
UAS-dome^{DN}; UAS-GFP/TM6B
UAS-GFP; UAS-hep^{CA}
UAS-hop^{Tum-l}; UAS-hep^{CA}
UAShop^{Tum-l}; UAS-GFP/TM6B
UAS-hop$^{RNAi\ (TRIP\ -v20)}$ (Bloomington Drosophila Stock Center: 32966)
UAS-hep^{CA}; UAS-hop$^{RNAi\ (TRIP-V20)}$
UAS-microRNARHG; UAS-hep^{CA}
UAS-microRNARHG; MKRS/TM6B

en-Gal4 Tub-Gal80^{TS} bs^{P1292}

Analysis of the expression pattern of different markers in control animals were performed by crossing *en-Gal4 UAS-GFP/CyO* flies to *UAS-GFP*.

To study the effect of ectopic expression of the different *UAS* lines used in our analysis *en–Gal4 Tub-Gal80^{TS} UAS- X* larvae (where X indicates the different transgenes used in our assay) were raised at 25 °C until 132± 12 hs AEL, at which point the larvae were shifted to 29 °C for 24 hs and then shifted back to 25 °C. We analysed the effects caused by the overexpression of the different UAS lines immediately after the end of the shift to 29 C° (T0), and 24 hs later (T1) (Fig. S1).

To activate JNK signalling in *dronc* mutants, *UAS-GFP UAS-hep^{CA} Tub-Gal80^{TS}; dronc^{i29} hh-Gal4/TM6B* flies were crossed to *w; If/CyO; dronc^{i24} /TM6B or w; Tub-Gal80^{TS}; hh-Gal4 to UAS-hep^{CA}; UAS-GFP* as a control.

2.3. Immunocytochemistry

Immunostaining of the wing discs was performed according to standard protocols. The following primary antibodies were used: rabbit anti-Phospho-histone 3 1:200 (Cell Signalling Technology), rabbit anti-cleaved Dcp1 1:200 (Cell Signalling Technology), rat anti-Araucan 1:200 (kindly provided by Sonsoles Campuzano), guinea pig anti-Knirps 1:500 and rabbit anti-Spalt major 1:200 (Gift from José Félix de Celis), mouse anti-ß Galactosidase 1:200 (Promega), rat anti-Phospho-Mad 1:100 (from Ginés Morata), and mouse anti-Wg (4D4; 1:100), were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Secondary antibodies (Molecular Probes) were used at dilutions of 1:200.

Imaginal discs were mounted in Vectashield mounting fluorescent medium (Vector Laboratories, Inc.).

2.4. Surgical amputation of Imaginal disc and analysis

en–Gal4 Tub-Gal80^{TS} UAS- X larvae were raised at 25 °C until the third instar larval stage (96± 12 hs after egg lay), at which time the larvae were shifted to 29 °C for 24 hs and then a fragment of the posterior compartment was surgically amputated (108–132 hs AEL) as described in Diaz-Garcia and Baonza (2013).

The larvae used in this assay were:

en-Gal4 UAS-GFP/ UAS-GFP; Tub-Gal80^{TS}/+, *en-Gal4 UAS-GFP UAS-puc Tub-Gal80^{TS}* /+, and *en-Gal4 UAS-GFP/ UAS-dome^{DN}; Tub-Gal80^{TS}*/+.

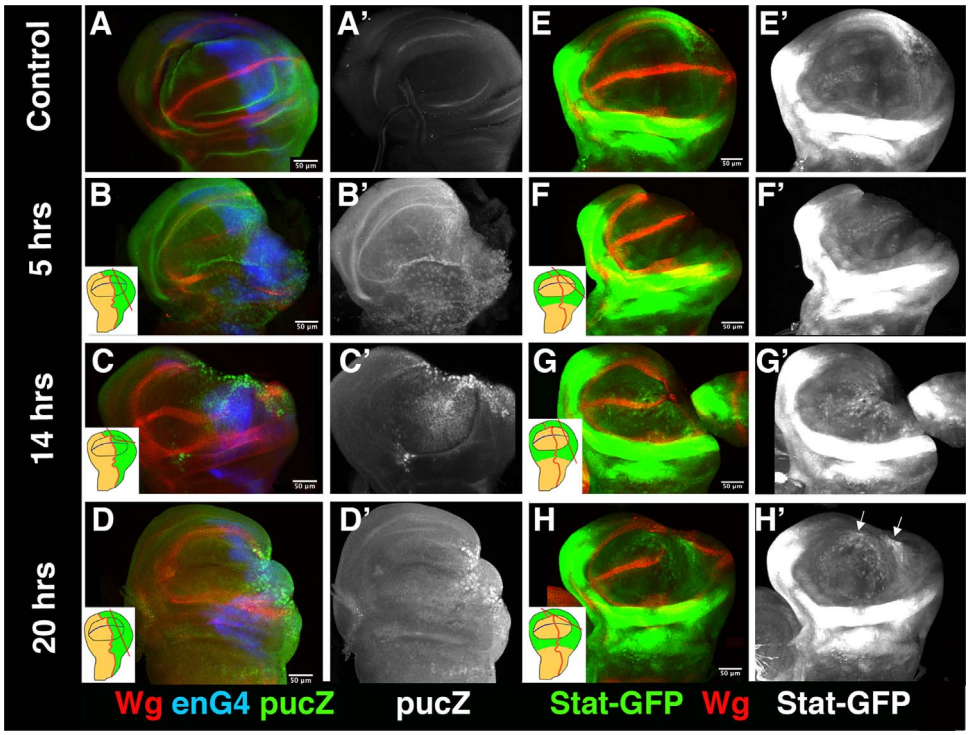

Fig. 1. Pattern of activation of JNK and STAT during wing disc regeneration. (A-D′) Third instar *en-Gal4* UAS-*GFP puc-lacZ*/+ wing imaginal discs stained for anti- ß-Galactosidase to detect the *puc-lacZ* expression pattern (green in A-D, and grey in A′-D′) and anti-Wg (red in A-D). (E-H′) Third instar wing imaginal discs carrying the *10XSTATGFP* reporter, stained for anti-Wg (red E-H). (A-A′) Non-amputated control *en-Gal4* UAS-*GFP puc-lacZ*/+ and (E-E′) 10*XSTATGFP* disc. (B-B′ and F-F′) Regenerating wing disc at 5 h AC. We observed activation of JNK signalling pathway in the cells of the wound edge (B-B′). The expression of the *10XSTATGFP* reporter is similar to the control disc (F-F′). (C-C′ and G-G′) Regenerating wing disc at 14 h AC. At this time the activation of JNK in the disc cells is detected not only near the wound edge but also in regions far away from it (C-C′). Some cells near the wound edge expressed high levels of *10XSTATGFP* (G-G′). Note that the expression of Wg disappears at the dorsal/ventral (D/V) boundary close to the wound edge. (D-D′) Regenerating wing disc at 20 h AC. At this time there is a reduction in the number of cells that activate JNK signalling pathway, but there are still a few ones adjacent to the wound edge. The expression of the *10XSTATGFP* reporter increases in some cells near the wound edges (H-H′, arrows in H′). Schematic illustrations on the left indicate the cutting lines and the region eliminated in each case.

2.5. In vitro culture

Imaginal discs were cultured as described (Aldaz et al., 2010) except that we do not add Ecdysone to the medium. The discs cultivated were *puc-Lac-Z* /10X*STAT92E-GFP*.

2.6. Statistical analysis

Suppression of effects is determined based on a shift in the percentages of the observed phenotypes, from Ara expression altered, either absent or down-regulated (L3 altered) to wild-type (discs without any observable effect or normal), that is significantly different based on a Pearson's chi-squared test. Degrees of freedom= 1, $\chi 2 = 10.83$ at $p = 0.001$, and for Degrees of freedom = 2, $\chi^2 = 13.82$ at $p = 0.001$, expected values for phenotypes derived from replicate controls.

In all the data included in the text the variance indicated refers to Standard deviation (SD).

2.7. Quantitative analysis

Images were processed using the ImageJ software (NUH,Bethesda,USA). Each image analysed 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 posterior compartment and calculated the percentage of the ROI covered by the staining using the Area Fraction option in Set Measurements. The P-values were calculated using two-tailed Student's *t*-test.

2.8. Microscopy

Images were captured using a Confocal LSM510 Vertical Zeiss and processed with ImageJ or Adobe Photoshop CS3.

2.9. qPCR analysis

RNA from wild type, *UAS-GFP Tub-Gal80^{TS} UAS-hep^{CA}; dronc^{i29} hh-Gal4/ dronc^{i24}*, *UAS-GFP Tub-Gal80^{TS} UAS-hep^{CA}; hh-Gal4/UAS-GFP* and *dronc^{I24}/dronc^{I24}* discs was extracted from +/−70 wing imaginal discs using Qiagen RNA later buffer, Zymo Research RNA MicroPrep and RNA Clean and Concentrator kits. RT reactions were performed using the iScript cDNA Synthesis kit (Biorad PN170–8891) following manufacturer´s instructions. qPCR reactions were performed in a CX384 Real Time System C1000 Thermal Cycler (Bio-Rad). The relative quantification data process was carried out with GenEx v. 5.4.4 (MultiD Analyses AB, Gothenburg, Sweden) software. Data were normalized to two housekeeping genes.

Primers used:

Housekeeping *RpL32*	Fwr	ATCGATATGCTAAGCTGTCGCAC
	Rev	GGCGACGCACTCTGTTGTC
Housekeeping *RpL13A*	Fwr	ATGACTGGTTTAACGAACAGGACC
	Rev	CACGGAGGCCAGGCG
upd2	Fwr	ACGAGTTATCAAGCGCAAGCA
	Rev	ATATCTTGGTATTCGCTCATCGTG
upd3	Fwr	ACAGATTCCTGCCCCGTCT
	Rev	GGTCGCGATGGGCGT

3. Results

3.1. Pattern of activation of JNK and JAK/STAT signalling during regeneration

In an attempt to better understand the requirement of Jun N-terminal Kinase (JNK) and JAK/STAT signalling pathways during wing disc regeneration, we first established the temporary pattern of activity of these pathways in amputated wing discs. To that end we used a method developed by us that allows the study of disc regeneration under physiological conditions (Diaz-Garcia and Baonza, 2013). To monitor the activity of JNK signalling we used a Lac-Z insertion in the gene *puckered (puc)* (Martin-Blanco et al., 1998). To examine JAK/STAT signalling activity we used the 10xSTAT-GFP transcriptional reporter, which contains multimerized copies of a Stat92E-responsive element (Bach et al., 2007).

We removed a fragment of the posterior compartment of *en-Gal4 UAS-GFP puc-Lac-Z* or *10 × Stat-GFP* third instar larvae wing discs (120–140 hs AEL) (Diaz-Garcia and Baonza, 2013), and analysed the expression of these reporters at different times after amputation (Fig. 1). Consistent with previous reports using other methods (Bosch et al., 2005) (Katsuyama et al., 2015; Pastor-Pareja et al., 2008; La Fortezza et al., 2016), we found that the JNK and JAK/STAT signalling pathways were activated during regeneration. At 5 h after cut (AC) JNK signalling was already active in the cells at the wound edge, (Fig. 1B-B′). As regeneration progressed (14 hs AC) the activity of the pathway strongly increased at the wound edge, and in regions adjacent to it, extending also to the anterior compartment, even though we only eliminated a fragment of the posterior compartment (Fig. 1 C-C′). The activity of the reporter declined in most of the cells of the disc at 20 hs and it was only maintained at high levels in the region adjacent to the wound edge (Fig. 1D-D′). In control contralateral non-amputated discs we never observed expression of this reporter in the wing pouch region (Fig. 1). In the same interval of time, between 5 and 20 h AC, we found that the levels of expression of the 10xSTAT-GFP reporter increased in some cells close to the wound site between 14 and 20 hs AC (Fig. 1). These data indicate that during discs regeneration, JNK and JAK/STAT are activated, although whereas the activity of JNK signalling spreads throughout a large region of the discs, JAK/STAT signalling is restricted to some cells near the wound edge, as assayed by 10xSTAT-GFP reporter. We have confirmed this observation by analysing the expression of *puc-LaZ* and *10xSTAT-GFP* reporters at the same time in amputated discs that have been cultured in vitro (Fig. S2).

3.2. Down-regulation of JAK/STAT and JNK signalling partially prevents the loss of cell fate commitment in regenerating discs

In the third instar wing discs the characteristic vein/intervein pattern of the adult *Drosophila* wing is defined by the expression of several genes, such as Iro-Complex genes, that are specifically expressed in the pro-veins L3 and L5, or knirps (Kni) in the pro-vein L2 (De Celis, 2003) (Fig. 2A). In regenerating wing discs the expression of vein fate commitment markers is temporary lost, not only in the cells at the wound edge but also in adjacent cells (Fig. 2B) (Diaz-Garcia and Baonza, 2013). To examine whether the activation of JNK and/or JAK/STAT signalling is involved in this down-regulation, we have analysed the expression of Araucan (Ara) (a component of the Iro-Complex) and Kni in regenerating discs in which the function of JNK or JAK/STAT signalling pathways was compromised. Using the Gal4/UAS/Gal80^{Ts} system we have blocked the activity of JNK signalling by over-expressing the phosphatase *puckered (puc)*, which controls the activity of JNK signalling by a negative feedback loop. To eliminate the function of the JAK/STAT pathway we have over-expressed a dominant negative form of *Domeless* (*dome*) under the control of *engrailed-Gal4* line to restrict the expression of the transgenes to the cells of the posterior compartment (see M & M). This approach enabled us to define the autonomous effects (posterior compartment), as well as any other possible non-autonomous effects in adjacent cells of the anterior compartment.

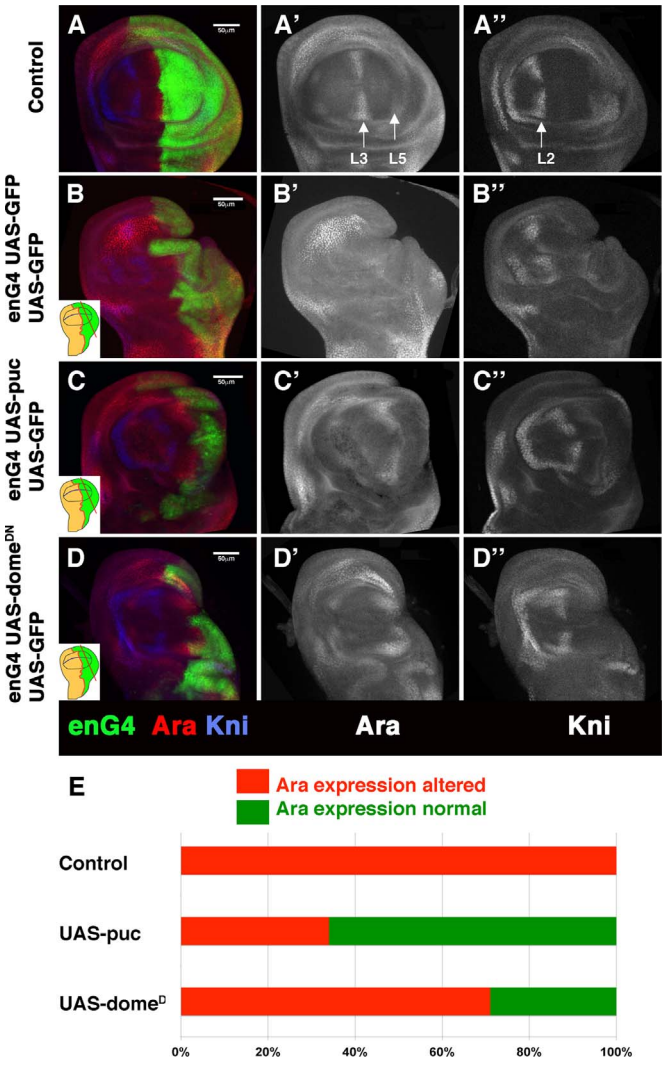

Fig. 2. JNK and JAK/STAT signalling are involved in regulating the expression of markers of cell fate commitment during regeneration. (A-D′′) Third instar wing discs stained for anti-Araucan (Ara) (red in A-D, and grey in A′-D′) and anti-Knirps (Kni) (blue in A-D, and grey in A′′-D′′). (A-A′) *en-Gal4 UAS-GFP/ UAS-GFP; Tub-Gal80^{TS}/+* control non regenerating discs. (B-B") *en-Gal4 UAS-GFP; Tub-Gal80^{TS}* regenerating wing disc at 18 hs after cut (AC). In these discs, although we have only amputated a fragment of the P compartment, the expression of Ara in the anterior compartment (pro-vein L3) is either eliminated or reduced. The expression of Kni is not altered. (C-C′′) Regenerating *en-Gal4 UAS-GFP/+; Tub-Gal80^{TS}/+UAS-puc* wing discs. In most of these discs the expression of Ara is not altered during regeneration (see E). (D-D′′) In more than 30% of the *en-Gal4 UAS-GFP/ UAS-dome^{DN}; Tub-Gal80^{TS}/+* regenerating discs analysed the expression of Ara in the pro-vein L3 is not altered (E). Insets indicate the cutting lines and the region eliminated in each disc. (E) Bar chart shows the percentage of regenerating discs in which the expression of Ara in pro-vein L3 is either eliminated or reduced (Ara expression altered, in red) or non-affected (Ara expression normal, in green) in the different genotypes analysed. *en-Gal4 UAS-GFP/ UAS-GFP; Tub-Gal80^{TS}/+*(Control); *en-Gal4 UAS-GFP/+; Tub-Gal80^{TS}/UAS-puc* (*UAS-puc*); *en-Gal4 UAS-GFP/ UAS-dome^{DN}; Tub-Gal80^{TS}/+* (*UAS-dome^{DN}*). Suppression is determined based on a shift in the percentage to wild-type (Ara expression normal) from Ara expression altered that is significantly different based on a Pearson's chi-square test for degrees of freedom = 1, $\chi^2 = 10.83$ at $p = 0.001$. See also the detailed statistical analysis in Fig. S3).

In contrast to control regenerating discs, in which the expression of Ara in pro-vein L3 was always altered, either eliminated or strongly down-regulated (Fig. 2B, E and Fig. S3), we found a high proportion of regenerating discs expressing *UAS-puc* (66% Fig. S3), in which the

expression of Ara was normal (Fig. 2 C-C", E and Fig. S3). Similarly, down-regulation of JAK/STAT signalling in the posterior compartment by expressing *UAS-dome^{DN}* also partially suppresses the effects in pro-vein L3 observed in control regenerating discs (Fig. 2D-D′, E and Fig. S3). The expression of kni in pro-vein L2 is not affected either in control regenerating discs or in discs expressing the transgenes (Fig. 2). This result indicates that the loss of pro-vein L3 is not due to a systemic response that delays vein specification. The vein pattern is not affected in contralateral non-regenerating discs expressing the transgenes used in our analysis (data not shown).

All together these results suggest that the function of JNK and JAK/STAT signalling are involved in the induction of loss of cell fate commitment observed during regeneration.

3.3. JAK/STAT signalling mediates the non-autonomous loss of markers of cell fate commitment induced by the activation of JNK-signalling

To further analyse the role of JNK signalling in the loss of cell fate specification, and its functional relationship with JAK/STAT signalling during tissue regeneration, we examined the effects caused by the ectopic activation of JNK signalling, while simultaneously reducing or increasing JAK/STAT signalling. To this end we have transiently expressed a constitutively activated form of the JNK-kinase Hemipterous (*hep^{CA}*) using the Gal4/UAS/Gal80^{Ts} system in different mutant conditions. We used the *engrailed-Gal4* line to limit the expression of these transgenes to the cells of the posterior compartment. *en–Gal4 Tub-Gal80^{TS} UAS- X* larvae (where X indicates the different transgenes used in our assay) were raised at 25 °C until 132± 12 hs AEL, at which point the larvae were shifted to 29 °C for 24 hs (T0) and then shifted back to 25 °C. The discs were analysed at different times (see M & M and Fig. S1). At the time when the larvae were transferred to 29° to induce the expression of the different transgenes used in our assay (132±12 hs AEL) the vein pattern was already established (Fig. 3A-A"). We found that in all discs analysed after over-expressing *UAS-hep^{CA}* (n = 31) the expression of Ara in pro-vein L5 (posterior compartment) was eliminated (Fig. 3C-C and Fig. S3), likely this effect is caused because most of the cells in this region are dying (see below). Interestingly, we observed that the expression of Ara in pro-vein L3 in the anterior compartment was also always either eliminated or strongly down-regulated (Fig. 3 and Fig. S3). These effects are non-autonomously induced by the activation of *UAS-hep^{CA}* in the posterior compartment since *en-Gal4* is not active in pro-vein L3. The expression of Kni in pro-vein L2 was not affected in these discs (Fig. 3C-C"). To confirm the loss of vein/intervein commitment after activation of JNK signalling we analysed the expression of the intervein marker *Blistered* (*bs*) (Roch et al., 1998). In discs over-expressing *UAS-hep^{CA}* under the control of *en-Gal4*, the expression of Bs is reduced in both the posterior compartment, as well as in the region adjacent to the anterior/posterior boundary (Fig. S4). All together these results indicate that the ectopic activation of JNK pathway promotes changes in the fate of the cells in adjacent regions of damaged areas.

We next investigated the possible function of JAK/STAT signalling in mediating the loss of cell fate commitment induced by the ectopic activation of JNK signalling. We first analysed the expression of the 10xSTAT-GFP reporter in discs over-expressing *UAS-hep^{CA}* under the control of *en-Gal4* at different times after *UAS-hep^{CA}* induction. After over-expressing *UAS-hep^{CA}* for 16 hs we found that the 10xSTAT-GFP reporter was already active in the cells of the posterior compartment (Fig. S5C-C′). After 24hs of over-expressing *UAS-hep^{CA}* the activity of the reporter strongly increased in the cells of the posterior compartment (Fig. S5D-D'). These results indicate that in our experimental condition JNK signal activates JAK/STAT signalling pathway. Then we examined the effects caused by the over-expression of *UAS-hep^{CA}*

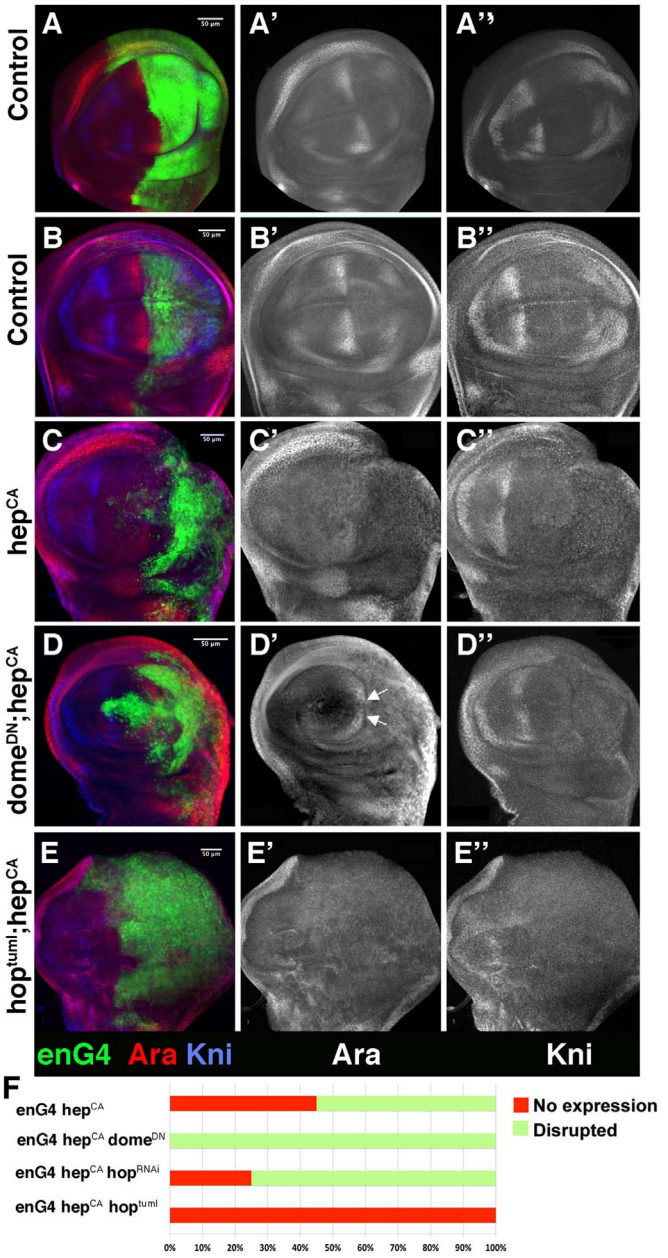

Fig. 3. JNK and JAK/STAT signalling pathways collaborate to induce non-autonomous down-regulation of markers of cell fate commitment. (A-E'') Third instar wing discs stained with anti-Araucan (Ara) (red in A-E, and grey in A′-E′) and anti-Knirps (Kni) (blue in A-E, and grey in A''-E''). (A-A'') Control *en-Gal4 UAS-GFP; Tub-Gal80^{TS}* wing disc stained at 120 hs AEL for Ara and Knirps, showing that the vein patterning defined by the expression of these genes is already specified at this time. (B-B'') Control *en-Gal4 UAS-GFP; Tub-Gal80^{TS}* wing disc. (C-C'') The over-expression of *UAS-hep^{CA}* in the posterior compartment of *en-Gal4 UAS-GFP/ UAS-GFP; UAS-hep^{CA}/Tub-Gal80^{TS}* discs causes the down-regulation of Ara, not only in the cells of the posterior compartment, but also in the cells of the pro-vein L3 in the anterior compartment (C'). knirps expression is not affected (C''). (D-D'') *en-Gal4 UAS-GFP/ UAS-dome^{DN}; Tub-Gal80^{TS}/UAS-hep^{CA}* discs. The ectopic expression of *UAS-dome^{DN}* and *UAS-hep^{CA}* in these discs partially restores the levels of Ara in the cells of the pro-vein L3 (arrows in D'). (E-E") In *en-Gal4 UAS-GFP/UAS-hop^{Tum-l}; UAS-hep^{CA}/Tub-Gal80^{TS}* discs, the co-expression of *UAS-hop^{Tum-l}* and *UAS-hep^{CA}* in the posterior compartment eliminates the expression of Ara in the cells of the pro-vein L3 (E') and strongly down-regulates Kni in the cells of the pro-vein L2 (E''). (F) Bar chart shows the percentage of discs in which the expression of Ara in pro-vein L3 is either eliminated (No expression) or partially eliminated (disrupted) in the different genotypes analysed. The suppression is determined based on a shift in the percentage from No expression to disrupted that is significantly different based on a Pearson's chi-square test for degrees of freedom = 2, χ^2 = 13.82 at p = 0.001. See also the detailed statistical analysis in Fig. S3.

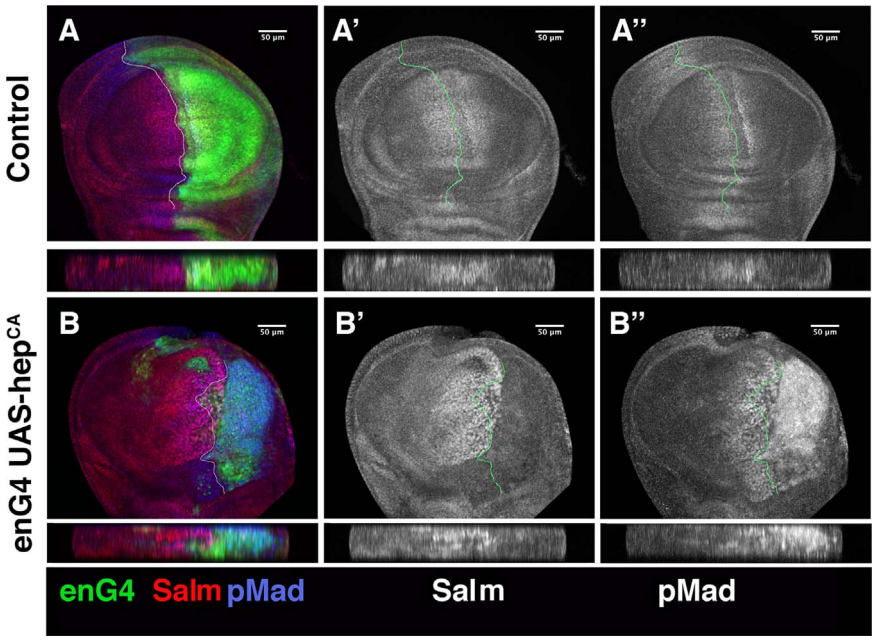

Fig. 4. The ectopic activation of JNK signalling in the posterior compartment does not affect the activity of *dpp* signalling in the a/p boundary. (A-B′′) Third instar wing discs stained with anti-Salm (Salm) (red in A-B, and grey in A′-B′) and anti-pMad (blue in A-B, and grey in A′′-B′′). (A-A") Control *en-Gal4 UAS-GFP; Tub-Gal80^{TS}* wing disc. The white line indicates the a/p boundary. (B-B′′) The over-expression of *UAS-hep^{CA}* in the posterior compartment of *en-Gal4 UAS-GFP/ UAS-GFP; UAS-hep^{CA}/Tub-Gal80^{TS}* discs causes the down-regulation of Salm and the up-regulation of pMad in the posterior compartment, but in the anterior compartment the expression of Salm and pMad are similar to control discs. Note that the range of expression of both proteins is similar in control and discs expressing *UAS-hep^{CA}* (see text). Transversal sections are shown below the figures.

under the control of *en-Gal4* at the same time that the activity of JAK/STAT signalling was blocked by overexpressing either: *UAS-dome^{DN}* or *UAS-hop^{RNAi}* (see M & M). In contrast to discs over-expressing only *UAS-hep^{CA}*, we found that in discs co-overexpressing *UAS-hep^{CA}* and *UAS-dome^{DN}*, the expression of Ara was never completely eliminated in pro-vein L3 (Fig. 3D-D′′, F and Fig. S3). In discs co-expressing *UAS-hep^{CA} and UAS-hop^{RNAi}* the percentage of discs with this phenotype was strongly reduced compared with discs expressing only *UAS-hep^{CA}* (45% *UAS-hep^{CA} vs* 25% *UAS-hep^{CA}/ UAS-hop^{RNAi}*, Fig. 3F and Fig. S3). In concordance with these results, we found that in the anterior compartment of *en-Gal4 UAS-GFP bs-lacZ UAS-dome^{DN}/ UAS-hep^{CA} Tub-Gal 80^{TS}* discs the vein/intervein pattern defined by Bs was only partially altered (Fig. S4D-D′). The temporary ectopic expression of *UAS-dome^{DN}* or *UAS-hop^{RNAi}* alone does not perturb the vein/intervein pattern (Fig. S4C-C′ and E-E'). These results suggest that the autonomous activation of JAK/STAT in *hep^{CA}*-expressing cells is required for inducing the loss of cell fate commitment observed in pro-vein L3.

Next we explored whether the co-activation of JNK and JAK/STAT signalling pathways could enhance these effects. To that end, we temporary co-overexpressed an activated form of *hop (UAS-hop^{Tum-l})* and *UAS-hep^{CA}* for 24 hs under the control of *en-Gal4*. We found that in all the discs examined (n = 26) the co-activation of both pathways in the posterior compartment totally eliminated the expression of Ara in the cells of pro-veins L5 and L3 (Fig. 3E-E′′ and F). Strikingly, we observed that in these discs the expression of Kni in the cells of pro-vein L2 was also reduced (Fig. 3E-E′′). Accordingly, we found that in these discs the expression of Bs was strongly reduced in the intervein cells of the posterior compartment and in a large region of the anterior compartment (Fig. S4F-F′). In discs over-expressing *UAS-hop^{Tum-l}* alone the expression of Ara and Bs was identical to that found in control wild type discs (Fig. S4).

The *decapentaplegic (dpp)* signalling pathway plays a fundamental role in patterning the wing discs along the anterior posterior axis (De Celis, 2003). JNK regulates the activity of this pathway (Perez-Garijo et al., 2009), therefore, it is possible that the effects on vein patterning observed upon JNK activation might be due to the alteration of *dpp* activity. However, different observations and our results, suggest that the fate changes caused by JNK signalling are not due to the alteration on the activity of *dpp* signalling. Firstly, it has been shown that in late third instar (older than 105 after egg lay), vein/intervein pattern could be maintained independently of *dpp* signalling (Repiso et al., 2013). Our experiment were always preformed at 132± 12 hs AEL, therefore when the function of this signal was not required for vein patterning. Moreover, we have analysed using our experimental condition the activity of *dpp* signalling pathways in discs over-expressing *UAS-hep^{CA}*. We examined the expression of pMad, and Spalt major (Salm) as a readout to assess the activity of Dpp signalling activity. We found that the range of expression of these factors and their levels of expression in the anterior compartment are similar in discs expressing *UAS-hep^{CA}* and control discs. The average range of cells expressing Salm and pMad at the a/p boundary in the anterior compartment is 11,8± 1,1 and 7,8 ± 1,03 rows of cells, respectively in control discs (n = 10) vs 10,9 ± 0,9 rows of cells for Salm and 8 ± 0,81 for pMad in discs expressing *UAS-hep^{CA}* (n = 10) (the variance represents the Standard deviation) (Fig. 4). In *en-Gal4 UAS-hep^{CA}* discs the expression of pMad in the posterior compartment is not restricted only to the central region of the wing blade, as in controls, and expand to most of the cells of this compartment (Fig. 4). This is likely as consequences of the ectopic expression of *dpp* that is induced in JNK-activating cells (Perez-Garijo et al., 2009). Finally, we have over-expressed *UAS-hep^{CA}* using *apterous-Gal4*. This Gal4 line drives the expression of Gal4 in the cells of the dorsal compartment. As previously described for *en-Gal4,* temporary activation of JNK under the control of *ap-Gal4* down-regulates the expression of Ara in pro-vein L3 and L5, in the dorsal compartment and also in the ventral compartment. This effect is partially suppressed in *ap–Gal4 UAS-GFP UAS-dome^{DN}; Tub-Gal80^{TS} UAS-hep^{CA}* discs and it is enhanced when JAK/STAT signalling is ectopically activated in *UAS-hop^{Tum-l} UAS-hep^{CA}* (Fig. 5 and Fig. S3).

Collectively our data suggest that the cooperative function of JAK/STAT and JNK signalling is necessary to promote the loss of fate commitment non-autonomously.

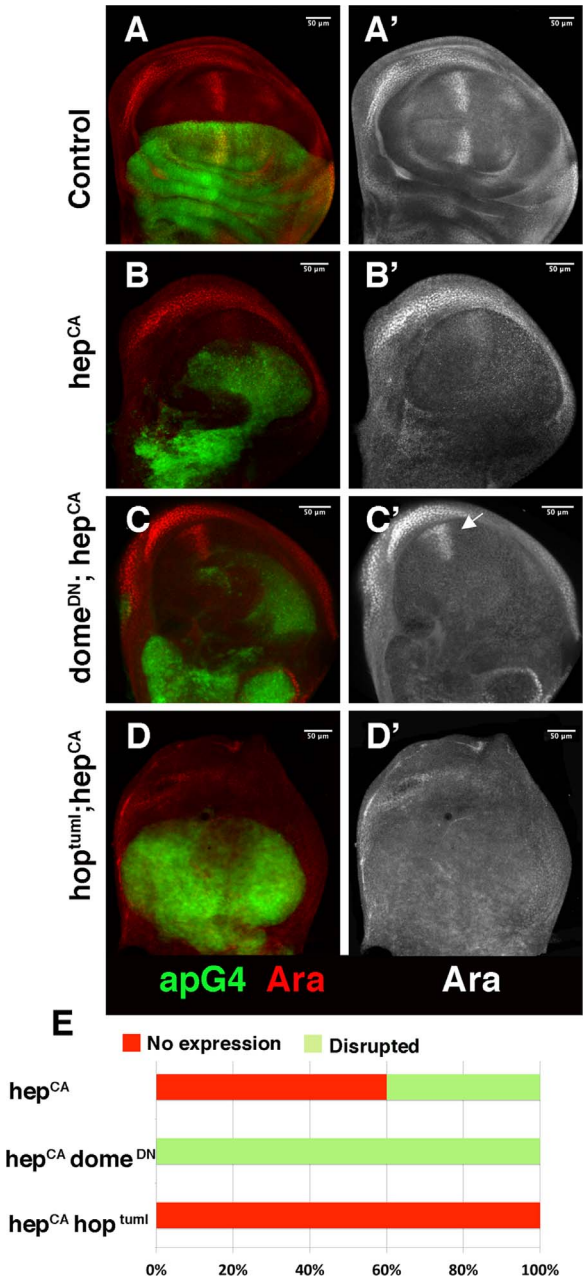

Fig. 5. The ectopic activation of JNK signalling under the control of *apterous*-Gal4 induces both autonomous and non-autonomous changes in the expression of markers of cell fate commitment. (A-D′) Third instar wing discs stained with anti-Araucan (Ara) (red in A-D, and grey in A′-D′). (A-A′) Control *ap-Gal4 UAS-GFP/Cyo; Tub-Gal80^{TS} /TM6* wing discs. (B-B′) *ap-Gal4 UAS-GFP/+; Tub-Gal80^{TS} /UAS-hep^{CA}* discs. The expression of Ara in the cells of the pro-veins L3 and L5 is down-regulated in the dorsal compartment as well as in ventral compartment. (C-C′). The ectopic expression of *UAS-dome^{DN}* and *UAS-hep^{CA}* in the dorsal compartment in *ap-Gal4 UAS-GFP/ UAS-dome^{DN}; Tub-Gal80^{TS}/UAS-hep^{CA}* discs partially restores the levels of Ara in the cells of ventral compartment (see arrow in C′). (D-D′) In *ap-Gal4 UAS-GFP/ UAS-hop^{Tum-l}; UAS-hep^{CA} /Tub-Gal80^{TS}* discs, the expression of Ara in the pro-vein cells is eliminated in both the dorsal and ventral compartments. (E) Bar chart shows the percentage of discs in which the expression of Ara in pro-vein L3 is affected. See the detailed statistical analysis in Fig. S3.

3.4. JNK signalling regulates cell fate specification through a non-apoptotic function of the pathway

The activation of JNK signalling promotes several cellular processes, one of which is to trigger apoptosis. It has been shown that apoptotic cells can induce different signals that affect several cellular processes in surrounding tissues (Perez-Garijo and Steller, 2015). Therefore, it is possible that the changes in cell fate commitment caused by the activation of JNK might be due to the induction of cell death. Recently, it has been shown that down-regulation of JAK/STAT signalling affects the viability of JNK-expressing cells (La Fortezza et al., 2016). We have confirmed these results, as we find that in our experimental conditions Caspase activation increases when the function of JAK/STAT is impaired in JNK-expressing cells and is dramatically reduced when *hop^{Tum-l}* is combined with *UAS-hep^{CA}*, as assayed with anti-Dcp1 (Figs. S6 and S7). We previously described that in this latter mutant condition there is a strong induction of cell fate re-specification throughout the disc (Figs. 3, 5 and Fig. S4). This result indicates that JNK activity is able to induce cell fates changes, autonomously as well as non-autonomously, even in condition in which cell death is reduced. Therefore, this suggests that JNK signalling might be at least partially regulating cell fate specification through a non-apoptotic function of the pathway.

To further examine this idea, we examined the effects of co-expressing *UAS-hep^{CA}* and *UAS-RHG microRNA* (miRNA). This transgene generates miRNAs that simultaneously inhibit the function of the pro-apoptotic genes *reaper*, *hid*, and *grim* (Siegrist et al., 2010). Cell death is totally suppressed in discs co-expressing *UAS-hep^{CA}* and *UAS-RHG* for 24 hs under the control of *en-Gal4* (compare Fig. 6A-A′′ with Fig. S6C-C′′). However, we still find that in these discs the expression of Ara in pro-veins L5 and L3 was altered, although the effects on the anterior compartment were slightly weaker than those observed is discs over-expressing only *UAS-hep^{CA}* (Fig. 6D-D′′ and G, and Fig. S3). These results suggest that JNK signalling induces loss of cell fate specification by a mechanism that is partially apoptosis-independent. Supporting this hypothesis we found that the transient over-expression of *UAS-rpr* under the control of *en-Gal4* during 24 hs, although was sufficient to induce massive cell death (Fig. 6E-E′′), did not affect the vein/intervein pattern defined by the expression of Ara and kni in the anterior compartment. We only observed that the expression of Ara in the pro-vein L5 in the posterior compartment disappears (Fig. 6F-F′′). This result seems contradictory with the observation that the induction of cell death promotes the activation of JNK (Shlevkov and Morata, 2012). We have analysed whether the ectopic expression of *UAS-rpr* under the control of *en–Gal4* for 24hs was sufficient to induce the activity of JNK signalling. To this end we examined the expression of *puc-Lac-Z* reporter in *en-Gal4 Tub-Gal80^{TS} UAS-rpr puc-Lac-Z discs*. We find that in these discs *puc-LacZ* is ectopically activated in some cells of the posterior compartment (Fig. S8). However, compared to discs expressing *UAS-hep^{CA}*, in which *puc-LacZ* is expressed in most of the cells of the posterior compartment (Figs. S8 and S9), in *rpr*-expressing discs the number of cells expressing the reporter is much lower (Fig. S8) Thus, the ectopic expression of *UAS-rpr* in our experimental condition (24 hs of cell death induction) might not be sufficient for generating enough number of JNK signalling cells for inducing the loss of cell fate commitment, although other explanations are possible (see discussion).

Altogether our results support a model in which JNK signalling exerts at least part of its function inducing cell fate changes by a mechanism that is apoptosis-independent.

It has been shown that in some experimental conditions the activation of JNK can drive its own activation non-autonomously in surrounding cells (Perez-Garijo and Steller, 2015). Therefore, a possible mechanism to explain the non-autonomous effects that we have observed upon ectopic expression of *UAS-hep^{CA}* in the posterior/dorsal compartments might be that JNK signalling was ectopically activated in some cells of the anterior/ventral compartments by the adjacent *hep^{CA}*-expressing cells. In this context JNK signalling would be required to promote cell fate changes in both compartments. To study this possibility we have examined the activity of *puc-Lac-Z*, after the ectopic over-expression of *UAS-hep^{CA}* for 24 hs under the control of en-Gal4. We found that in all discs analysed (n = 10) the expression of the

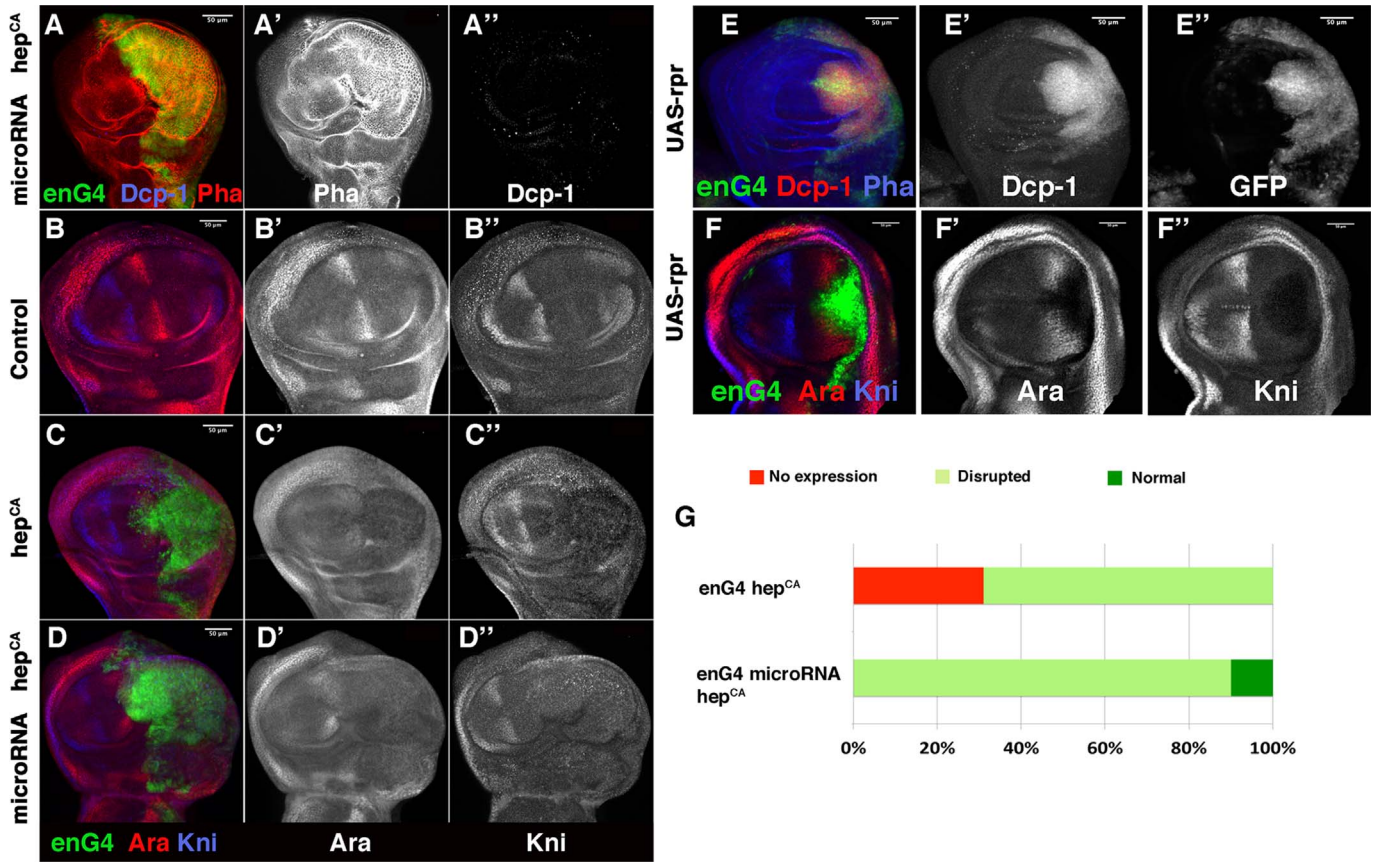

Fig. 6. -The ectopic activation of JNK promotes the loss of markers of cell fate commitment partially independent of its apoptotic function. (A-A'') Third instar *en-Gal4 UAS-GFP/UAS-RHG microRNA; UAS-hep^{CA} /Tub-Gal80^{TS}* wing discs stained for anti-cleaved Dcp1 (blue in A, and grey in A") and Phalloidin (red in A, and grey in A'). The expression of *UAS-RHG microRNA* in *UAS-hep^{CA}*-expressing cells prevents the induction of apoptosis caused by the activation of JNK signalling (compared with Figs. S6 and S7). (B-D and F) Third instar imaginal wing discs stained with anti-Araucan (red in B-D and F, and grey in B'–D' and F') and anti-Knirps (blue in B-D and F, and grey in B''-D'', and F''). (B-B") Control wing discs. (C-C'') In *en-Gal4 UAS-GFP/ UAS-GFP; UAS-hep^{CA} /Tub-Gal80^{TS}* discs the expression of Ara in the cells of the pro-vein L3 is strongly down-regulated. (D-D") In *en-Gal4 UAS-GFP/ UAS-RHG microRNA;UAS-hep^{CA}/Tub-Gal80^{TS}* discs, even though cell death is blocked, we still observed changes in the expression of Ara in pro-vein L3. (E-E'') The over-expression of *UAS-rpr* during 24 hs under the control of *en-Gal4* causes massive cell death in the posterior compartment. The disc is stained for anti-cleaved Dcp1 (red in E and grey in E'), and with Phaloidin (blue). (F-F'') After 24hs of over-expression of *UAS-rpr* in *UAS-rpr*; *en-Gal4 UAS-GFP; Tub-Gal80^{TS}* discs, the expression of Ara in the cells of the pro-vein L3 in the anterior compartment is not affected (F"). (G) Bar chart shows the percentage of discs in which the expression of Ara in pro-vein L3 is affected. See the detailed statistical analysis in Fig. S3.

reporter is autonomously restricted to the *hep^{CA}*-expressing cells in the posterior compartment (Fig. S9). We have obtained similar results analysing the activity of TRE-DsRed. This reporter faithfully reproduces all known patterns of JNK activity (Chatterjee and Bohmann, 2012). In discs over-expressing *UAS-hep^{CA}* the activity of *TRE-DsRed* is mostly restricted to the cells of the posterior compartment, and we only observe few isolated cells expressing *TRE-DsRed* in the anterior compartment (Fig. S10).

These results suggest that the non-autonomous loss of markers of cell fate commitment is not caused by the activation of JNK signalling in the cells of the anterior compartment.

3.5. The non-autonomous loss of markers of cell fate commitment caused by the over-expression of JNK signalling depends on dronc function

The initiator caspase Dronc mediates the apoptotic function of JNK signalling (Igaki, 2009; Ryoo et al., 2004; Shlevkov and Morata, 2012). In addition to trigger apoptosis this factor participates in several non-apoptotic processes (Huh et al., 2004; Ouyang et al., 2011). Interestingly, it has been proposed that Dronc stimulates compensatory proliferation non-autonomously, therefore it is possible that this factor may function to regulate other processes non-autonomously (Huh et al., 2004). To investigate whether *dronc* contributes to the cell fate changes induced by JNK, we expressed *UAS-hep^{CA}* in animals mutant for *dronc*. To this end, we have over-expressed *UAS-hep^{CA}* for 24 hs under the control of *hh-GAL4* in mutant flies for the heteroallelic combination *dron^{i29}/ dronc^{i24}*. We confirmed that in this mutant combination the JNK-induced apoptosis is reduced, although we still found multiple dying cells, as assayed with anti-Dcp1 (Fig. S11). We found that in none of the *UAS-hep^{CA} UAS-GFP Tub-Gal80^{TS}; hh-GAL4 dronc ^{i29}/ dronc^{i24}*discs analysed (n = 20) the expression of Ara in the cells of the pro-vein L3 was altered (Fig. 7B-B''). In the posterior compartment the expression of Ara in pro-vein L5 was absent. These data imply that the non-autonomous effects of JNK signalling in inducing loss of markers of cell fate commitment depend on Dronc.

The activation of JNK signalling promotes the expression of Upd cytokines (Pastor-Pareja et al., 2008; Santabarbara-Ruiz et al., 2015; La Fortezza et al., 2016). A possible mechanism to explain the suppression of the effects caused by the over-expression of *UAS-hep^{CA}* in the *dronc* mutant condition is that Dronc might be necessary to promote the expression of *upd* upon JNK activation. To test this possibility, we have examined the levels of *upd* ligands in *dronc* mutant discs upon the ectopic activation of JNK signalling using a qPCR assay. We found that in *UAS-GFP UAS-hep^{CA} Tub-Gal80^{TS}/+; hh-Gal4 dronc^{i29}/dronc^{i24}*discs the levels of *upd* ligands are even higher than those observed in discs only over-expressing *UAS-hep^{CA}* (Fig. 7C). Therefore, the suppression of the effects caused by the ectopic expression of *UAS-hep^{CA}* in *dronc* mutant background are not due to the inability of JNK signalling to activate JAK/STAT in *dronc* mutant condition.

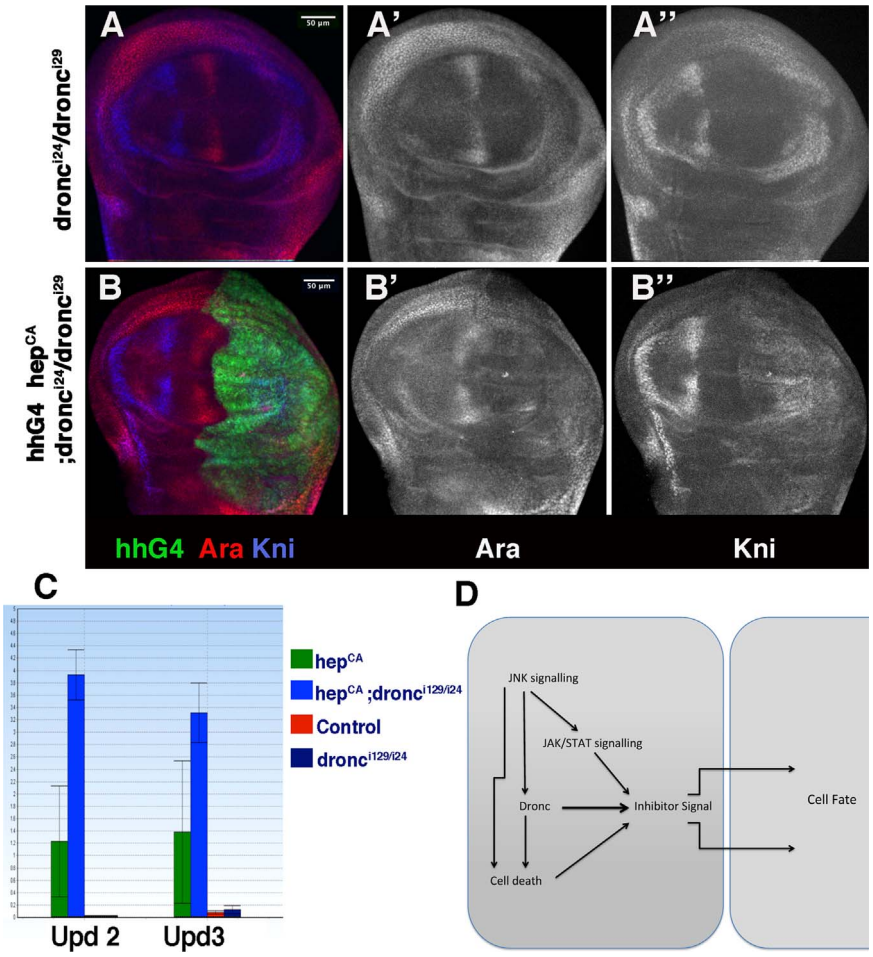

Fig. 7. The non-autonomous down-regulation of vein markers induced by the ectopic activation of JNK depends on Dronc. (A-B'') Third instar imaginal wing discs stained with anti-Araucan (red in A-B, and grey in A′-B′) and anti-Knirps (blue in A-B, and grey in A'' B''). (A-A'') In Control *dronc^{i29}/dronc^{i24}* discs the expression of vein markers is not affected. (B-B'') We observed that in *UAS-GFP Tub-Gal80^{TS} UAS-hep^{CA} /Cyo; hh-Gal4 dronc^{i29}/ dronc^{i24}* discs the expression of Araucan in the cells of the pro-vein L5 in the posterior compartment is eliminated, but in the cells of the pro-vein L3 in the anterior compartment is comparable to control discs. Compared with *Tub-Gal80^{TS} UAS-hep^{CA}; UAS-GFP / hh-Gal4* discs (see also Fig. 3B′). Knirps expression is as in controls (A''). (C) qRT-PCR analysis of upd2 and upd3 transcripts after the ectopic expression of *UAS-hep^{CA}* during 24 hs in *Tub-Gal80^{TS} UAS-hep^{CA}; hh-Gal4/ UAS-GFP* discs (green) and *UAS-GFP UAS-hep^{CA} Tub-Gal80^{TS}/+; hh-Gal4 dronc^{i29}/dronc^{i24}* discs (blue). The levels of expression of these ligands are much higher in these conditions than in control disc (red), and *dronc^{i24}/dronc^{i24}* discs (dark blue). Fold induction relative to control discs (red). Graphs display mean ± s.e.m. for n = 3 biological replicates. (D) Model proposed of synergistic function between JNK, Dronc and JAK/STAT signalling to induce cell fate changes.

4. Discussion

4.1. JAK/STAT is locally activated during Wing disc regeneration

The ectopic activation of JNK signalling is sufficient to promote JAK/STAT function in the wing discs (La Fortezza et al., 2016; Pastor-Pareja et al., 2008). However, here we have shown that in regenerating wing discs JAK/STAT signalling is present only in certain cells in the proximal region of the discs, even though JNK signalling is active in most of the cells of the wing pouch. This could be caused either by the existence of inhibitory signals that during regeneration restrict the domain where JAK/STAT signalling can be activated, or because the transient activation of JNK signalling in regenerating discs is not strong enough to induce JAK/STAT. It is also possible that both mechanisms co-exist. During normal development of the wing disc, multiple signals restrict JAK/STAT signalling activity through the repression of its activating ligand (Upd) and its activated signal transducer (Ayala-Camargo et al., 2013). This process is necessary for normal wing development, as ectopic activation of JAK/STAT signalling in the pouch is deleterious (Ayala-Camargo et al., 2013). Therefore, strict control of JAK/STAT signalling might be essential not only during normal development, but also for a correct regeneration process. In other developmental contexts, such as during leg discs regeneration, where the inhibitory signals that operate in the proximal region of the wing disc do not exist, the Upd ligands are up-regulated only in the early phase of regeneration and fade rapidly after wounding (Katsuyama et al., 2015). Therefore, considering our data and other works previously reported, we propose that during regeneration JAK/STAT signalling is under tight spatial and temporal regulation and is only activated in some of the JNK-expressing cells.

4.2. JNK and JAK/STAT signalling cooperate to non-autonomously repress the expression of cell fate commitment markers

Our work identifies a previously unrecognised function for cooperative JNK and JAK/STAT signalling in regulating cell fate commitment. We have found that ectopic activation of JNK signalling is sufficient to induce the loss of cell fate commitment markers, autonomously, as well as non-autonomously in surrounding non JNK-expressing cells. This non-autonomous effect suggests that the ectopic activation of JNK signalling generates an inhibitory signal/s that can induce cell fate commitment changes in adjacent cells. Our data indicate that the production of this signal/s depends on the function of JAK/STAT signalling.

It has been shown that the JAK/STAT pathway functions downstream of JNK signalling (La Fortezza et al., 2016; Katsuyama et al.,

2015), nevertheless our data indicate, that in contrast to JNK signalling, the transient ectopic activation of JAK/STAT signalling is not sufficient to induce any changes in the expression of patterning genes. These data imply that the JAK/STAT pathway is not the only mechanism through which JNK signalling alters cell fate specification. Therefore, in parallel to JAK/STAT pathway, JNK signalling must induce other signal/s that in combination with JAK/STAT would induce the fate changes caused by the activation of JNK pathway. Interestingly, the intensity of the effects produced by JNK-activated cells increase when JAK/STAT is also activated. This synergistic interaction supports the idea that autonomous induction of the signal/s that regulate cell fate commitment requires cooperative parallel inputs for both signalling pathways (Fig. 7D). Another interesting aspect of the inhibitory signal/s produced by the activation of JNK signalling is that its range of action expands. Thus, the effects caused by the ectopic activation of JNK signalling alone are restricted to the cells immediately adjacent to the JNK-activating cells, even though in these cells the endogenous JAK/STAT signalling is also activated. However, when both pathways are ectopically co-activated at high levels, the cell fate commitment of most of the cells of the anterior compartment are distorted. These observations suggest that the inhibitory signals generated by the co-activation of JAK/STAT and JNK signalling function in a graded manner.

As previously mentioned our results suggest that JNK signalling in parallel to JAK/STAT activates other signal/s that are necessary for inducing the non-autonomously loss of markers of cell fate commitment. Our data suggest that this signal is at least partially independent of the apoptotic function of JNK signalling. We have found that the elimination of Dronc suppress the non-autonomous effects caused by the ectopic activation of JNK signalling. The function of Dronc is activated by JNK signalling (Shlevkov and Morata, 2012). These results are consistent with a function of Dronc mediating the induction of the non-autonomous signal generated by the ectopic activation of JNK signalling. Interestingly, it has been shown that Dronc provides a compensatory proliferation signal to neighbours in different contexts (Huh et al., 2004). Since we have shown that the suppression of apoptosis after the ectopic activation of JNK was not sufficient to completely block the loss of cell fate markers, we proposed that in this context *dronc* provides a signal involved in inducing cell fate re-specification through a non-apoptotic mechanism (Fig. 7D).

It has been previously reported that the induction of apoptosis through the activation of *rpr* promotes the function of JNK signalling (Shlevkov and Morata, 2012). We have found similar results using our experimental condition (Fig. S8). However, we have shown that to difference to the ectopic activation of *UAS-hep^{CA}*, the over-expression of *UAS-rpr* does not alter the expression of Ara in the anterior compartment. We have suggested that these contradictory results might be explained by our observation that the number of JNK signalling cells induced by the ectopic expression of *UAS-rpr* for 24 hs, is smaller than the number JNK signalling cells generated when *UAS-hep^{CA}* is overexpressed. Alternatively, it is also possible that the activation of JNK signalling through the expression of *UAS-hep^{CA}* might directly act over the target genes involved in generating the signals required in changing cell fate, whereas the induction of cell death through *UAS-rpr* would activate JNK signalling, that in turn would induce cell fate changes. In this scenario, the time for promoting cell fate changes would be longer when *UAS-rpr* is over-expressed than when JNK is activated. Supporting this hypothesis, we found that after 48 hs of over-expressing *UAS-rpr*, the expression of Ara is always eliminated (n = 10) in the anterior compartment of the discs analysed (Fig. S12). These data indicate that the time required for promoting cell fate changes after the induction of *UAS-rpr* is longer than the time necessary when *UAS-hep^{CA}* is ectopically activated. This result suggests that the signal/s for inducing cell fate changes are activated faster after the ectopic activation of *UAS-hep^{CA}* than with *UAS-rpr*. However, this experiment does not rule out that the levels of JNK activation after 24 hs of *UAS-rpr* overexpression are not strong enough for inducing cell fate changes, either because the number of JNK signalling cells is not enough, as we mentioned before, or because the signal levels of JNK in this condition are too low.

The ectopic activation of JNK signalling promotes the expression of Wingless and Dpp (Fig. S6) (Perez-Garijo et al., 2009). The functions of these factors play a fundamental role in regulating patterning and proliferation during wing disc development. This raised the possibility that Dpp and Wg may be mediating the patterning defects observed after JNK activation. However, we have observed that in *en-Gal4 UAS-hep^{CA} dronc* discs, even though these two factors are ectopically expressed (Perez-Garijo et al., 2009) (Fig. S11) the patterning defects are corrected. In addition, we have shown that the ectopic activation of JNK signalling can produce changes in cell fate specification without affecting *dpp* signalling activity. All together these data imply that the cell fate changes induced by the ectopic activation of JNK signalling are not due to the up-regulation of Wg and Dpp.

Collectively our results lead us to propose that during regeneration the local combined action of JAK/STAT and JNK signalling promotes a signal/s that regulate the fate commitment state of surrounding cells. Changes in the fate state of the cells is likely associated with a re-patterning process that occurs in early stages of regeneration. The signal/s generated in cells where JAK/STAT and JNK signalling are activated also regulates regenerative growth.

5. Conclusion

Our results suggest a cooperative function between JNK and JAK/STAT signalling in the induction of loss of cell fate specification during regeneration. We show that the Caspase initiator Dronc is necessary to promote this function in response to the activation of JNK signalling. The data reported here provide important insights into the mechanisms through which the activities of JNK and JAK/STAT signalling pathways can regulate cell fate decisions in response to tissue damage.

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Conflict of interest

We declare that no competing interest exist.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.05.021.

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