The co-regulator dNAB interacts with Brinker to eliminate cells with reduced Dpp signaling

Oren Ziv[1],*, Yaron Suissa[1],*, Hadar Neuman[1], Tama Dinur[1], Peter Geuking[2], Christa Rhiner[3], Marta Portela[3], Fidel Lolo[3], Eduardo Moreno[3],† and Offer Gerlitz[1],†

The proper development of tissues requires morphogen activity that dictates the appropriate growth and differentiation of each cell according to its position within a developing field. Elimination of underperforming cells that are less efficient in receiving/transducing the morphogenetic signal is thought to provide a general fail-safe mechanism to avoid developmental misspecification. In the developing *Drosophila* wing, the morphogen Dpp provides cells with growth and survival cues. Much of the regulation of transcriptional output by Dpp is mediated through repression of the transcriptional repressor Brinker (Brk), and thus through the activation of target genes. Mutant cells impaired for Dpp reception or transduction are lost from the wing epithelium. At the molecular level, reduced Dpp signaling results in Brk upregulation that triggers apoptosis through activation of the JNK pathway. Here we show that the transcriptional co-regulator dNAB is a Dpp target in the developing wing that interacts with Brk to eliminate cells with reduced Dpp signaling through the JNK pathway. We further show that both dNAB and Brk are required for cell elimination induced by differential dMyc expression, a process that depends on reduced Dpp transduction in outcompeted cells. We propose a novel mechanism whereby the morphogen Dpp regulates the responsiveness to its own survival signal by inversely controlling the expression of a repressor, Brk, and its co-repressor, dNAB.

KEY WORDS: Brinker, Dpp survival signal, Wing development, dMyc (Dm)-induced cell competition, dNAB (*Drosophila* Nab)

INTRODUCTION

A fundamental question in development is how growth, cell fate specification and pattern formation are spatially and temporally coordinated to control the final shape, size and cellular make-up of an organ. Part of the answer resides in the ability of a single morphogenetic molecule to provide simultaneous guiding cues for different developmental processes (Serrano and O'Farrell, 1997). One such signaling molecule is Decapentaplegic (Dpp), a member of the TGFβ superfamily that provides cells of the developing *Drosophila* wing with patterning, growth and survival cues.

Dpp functions in the wing primordium as a long-range morphogen specifying cell fates in a concentration-dependent manner by defining domains of gene expression centered on its restricted expression domain (Lecuit et al., 1996; Nellen et al., 1996). In addition, Dpp plays a key role in promoting cell proliferation and wing growth. Mutant cell clones lacking Dpp receptors [Punt or Thickveins (Tkv)] fail to grow (Burke and Basler, 1996). Conversely, expression of Dpp or its constitutively activated receptor, Tkv^{Q253D}, causes overgrowth (Burke and Basler, 1996; Lecuit et al., 1996; Nellen et al., 1996) due to excess cell proliferation (Martin-Castellanos and Edgar, 2002). Dpp signaling is also crucial for cell survival in the wing disc; thus, impaired Dpp signaling due to Tkv loss-of-function or the disruption of Dpp intracellular signal transduction induces JNK-mediated apoptosis (Adachi-Yamada et al., 1999; Adachi-Yamada and O'Connor, 2002; Burke and Basler, 1996; Moreno et al., 2002). Dpp acts through a

well-characterized transduction pathway. The binding of Dpp to its serine/threonine kinase receptor complex triggers the phosphorylation of the transcription factor Mad, which together with associated factors translocates to the nucleus and regulates the expression of target genes.

The *brinker* (*brk*) gene is a key target of the Dpp pathway that is negatively regulated by Dpp signaling throughout embryonic and larval development. *brk* encodes a transcriptional repressor. Loss of Brk function leads to ectopic expression of Dpp target genes, tissue overgrowth and cell fate transformations corresponding to elevated levels of Dpp signaling (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). Moreover, removal of Brk leads to overgrowth and ectopic expression of Dpp targets even in the absence of Dpp or other essential components of the pathway, such as Tkv or Mad (Jazwinska et al., 1999; Marty et al., 2000), indicating that to a large extent Dpp signaling acts through negative regulation of *brk* expression. Interestingly, although a Dpp gradient controls differential gene expression by gradually downregulating *brk*, the slope of the Dpp gradient is not itself important for proliferation (Muller et al., 2003; Schwank et al., 2008). Brk is a sequence-specific transcriptional repressor that alternatively requires the co-repressors Groucho (Gro) and CtBP for repressing some Dpp-responsive genes, but not for others (Hasson et al., 2001; Zhang et al., 2001).

In *Drosophila* imaginal discs, a phenomenon of cell competition has been described in which normal cells overproliferate at the expense of neighboring Minute cells that have reduced ribosomal protein gene dose, eliminating them via apoptosis from developing tissues (Morata and Ripoll, 1975; Simpson and Morata, 1981). Similar competitive interactions occur when cells that express more dMyc [Diminutive (Dm) – FlyBase] or cells that are mutant for components of the Hippo/Warts pathway, behave as super-competitors that both outgrow adjacent wild-type cells and induce their death (de la Cova et al., 2004; Moreno and Basler, 2004; Tyler et al., 2007). Competition for the Dpp survival signal appears to be

[1]Developmental Biology and Cancer Research, IMRIC, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel. [2]Institut fur Molekularbiologie, Universitat Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. [3]Spanish National Cancer Institute (CNIO), Melchor Fernandez Almagro 3, E-28029 Madrid, Spain.

*These authors contributed equally to this work
†Authors for correspondence (e-mails: emoreno@cnio.es; offerg@ekmd.huji.ac.il)

the driving force behind cell competition. This notion is based on the finding that outcompeted cells exhibit reduced Dpp signaling (Moreno and Basler, 2004; Moreno et al., 2002; Tyler et al., 2007) and their elimination can be prevented by forced expression of either Dpp or its activated receptors (Moreno and Basler, 2004; Moreno et al., 2002). At the molecular level, reduced Dpp signaling activity results in failure to repress the expression of Brk, the upregulation of which triggers apoptosis through activation of the JNK pathway.

Elimination of underperforming cells from a developing field may be a general feature of morphogen gradients that circumvents misspecification and the accumulation of detrimental developmental mistakes that would otherwise lead to embryonic malformation. Here, we find that the transcriptional co-regulator dNAB (Nab – FlyBase) is a target of Dpp in the wing primordium that interacts with Brk to promote JNK-mediated elimination of cells with impaired Dpp signaling. We further demonstrate that both dNAB and Brk are required for dMyc-induced cell competition. In contrast to Gro, a known co-repressor of Brk, dNAB is not required for Dpp-dependent patterning, whereas Gro does not promote JNK-mediated cell death.

MATERIALS AND METHODS
Fly strains and transgenes
We used the following fly strains: brk^{M68}, $dnab^{R6H8}$, $dnab$-$lacZ^{SH143}$, omb-$lacZ$, vg-$lacZ$, puc-$lacZ^{E69}$, arm-$lacZ$, brk-$lacZ^{X47}$, UAS-tkv^{Q235D}, UAS-gro, UAS-Dad, UAS-brk, UAS-puc and EP-$dnab$. P-element S149, which maps 23 bp upstream of the 5′ end of the $dnab$ transcription unit, was replaced with the y^+-marked EP element DA530inv (Gerlitz and Basler, 2002) essentially as described by Sepp and Auld (Sepp and Auld, 1999). Transgenes were expressed using the Gal4/UAS binary system with the following drivers: hh-$Gal4$, sd-$Gal4$ and C765-$Gal4$.

Generation of Flp-out and loss-of-function clones
We generated overexpressing Flp-out clones using either the abx-$ubx>forked>Gal4$-$IRES$-$lacZ$ or the $act>CD2>Gal4$ cassette, recombined to a UAS-GFP construct for the detection of the clones. Larvae were subjected to a 37°C heat shock for 10 minutes. Genotypes of dissected larvae were as follows. Tkv^{Q235D}-overexpressing clones: yw $hsp70$-$flp;;$ $act>CD2>Gal4$ UAS-$GFP/dnab$-$lacZ$ UAS-tkv^{Q235D}. Brk-overexpressing clones: yw $hsp70$-$flp;$ UAS-brk/abx-$ubx>forked>Gal4$-$IRES$-$lacZ;$ UAS-GFP. dNAB-overexpressing clones: yw $hsp70$-$flp;;$ $act>CD2>Gal4$ UAS-GFP/EP-$dnab$ puc-$lacZ^{E69}$ or yw $hsp70$-flp brk-$lacZ^{X47};;$ $act>CD2>Gal4$ UAS-GFP/EP-$dnab$. dNAB- and Puc-overexpressing clones: yw $hsp70$-$flp;;$ $act>CD2>Gal4$ UAS-GFP/EP-$dnab$ UAS-puc. Gro-overexpressing clones: yw $hsp70$-$flp;;$ $act>CD2>Gal4$ UAS-GFP/UAS-gro.

We generated mutant clones using Flp-mediated mitotic recombination and identified them by the loss of the GFP or β-galactosidase (β-gal) markers. Clones were induced either with hh-$Gal4/UAS$-flp or by heat shock (60 minutes at 37°C). Genotypes of dissected larvae were as follows. $dnab$ loss-of-function clones: yw $hsp70$-flp/omb-$lacZ;;$ $dnab^{R6H8}$ $FRT80/ubi$-GFP $FRT80$. $hsp70$-$flp;$ vg-$lacZ;$ $dnab^{R6H8}$ $FRT80/ubi$-GFP $FRT80$. $dnab$ overexpression and brk loss-of-function clones: arm-$lacZ$ $FRT18A/yw$ brk^{M68} $FRT 18A;$ UAS-flp/UAS-$GFP;$ hh-$Gal4/EP$-$dnab$.

For MARCM experiments, genotypes of dissected larvae were as follows: sd-$Gal4/hs$-$flp;$ UAS-Dad/UAS-$GFP;$ $tubP$-$Gal80$ $FRT80/dnab^{R6H8}$ $FRT80$ and sd-$Gal4/hs$-$flp;$ UAS-Dad/UAS-$GFP;$ $tubP$-$Gal80$ $FRT80/FRT80$. Clones were induced by heat shock for 60 minutes at 37°C. Larvae were dissected at 48, 72 and 96 hours after clone induction.

Wild-type clones in a $tub>dmyc$ background
Larvae of genotype yw $hsp70$-$flp;$ $tub>dmyc>Gal4;$ UAS-GFP with or without UAS-$RNAi$ constructs (Dietzl et al., 2007) to knock down $dnab$ (#6273) or brk (#2919), were heat shocked for 15 minutes at 37°C and dissected after 24, 48 or 72 hours. UAS-$RNAi$ constructs against other $Drosophila$ genes (more than 50 different genes) were also used and most of them (>90%) did not cause the rescue that was observed when knocking down either brk or $dnab$.

Immunohistochemistry
Imaginal discs from third instar larvae were fixed and stained by standard techniques. The specific primary antibodies used were: mouse anti-β-gal (1:1000; Promega), rabbit anti-Spalt [1:1000; a gift from A. Salzberg (Halachmi et al., 2007)], rabbit anti-dNAB [1:500; a gift from J. Díaz-Benjumea (Terriente Felix et al., 2007)], rat anti-Brk (1:1000; a gift from F. A. Martín and G. Morata, CDBM University Autonoma De Madrid, Madrid, Spain) and rabbit anti-cleaved Caspase 3 (1:40; Cell Signaling). Images were taken on a TE2000-E confocal microscope (Nikon) using a 203 objective.

Plasmid construction
Molecular manipulations were conducted according to standard protocols. Constructs containing full-length $dnab$ cDNA and its derivatives were prepared by standard PCR amplification. Following sequencing, these were inserted in-frame into the pGEX-2T vector. A PCR-amplified full-length brk was cloned into the pET17b vector.

A 9.5 kb genomic rescue construct containing the $dnab$ transcription unit and flanking endogenous regulatory sequences was prepared by PCR amplification and subsequent cloning into a pCaSpeR4 vector (details provided on request).

Cleaning of the R6H8 chromosome and rescue experiment
In order to clean the chromosomal region distal and proximal to the $dnab^{R6H8}$ mutation (Nairz et al., 2004), two y^+-marked P-insertions located in close proximity to the $dnab$ gene ($W158$ at 63B and $W55$ at 65D, our unpublished results) were sequentially first recombined on to the $dnab^{R6H8}$ mutant chromosome and then removed by recombination.

Two independent P[genomic $dnab$] insertions on the third chromosome rescued the lethality of homozygous $dnab$ mutant flies (S149 and $dnab^{R6H8}$). Flies carrying the P[genomic $dnab$] and the $dnab$ mutations on the third chromosome lost the Tm6B balancer chromosome.

RNA in situ hybridizations
RNA in situ hybridizations were carried out according to standard protocols. DIG (Roche) RNA probes were synthesized from a template derived by PCR from genomic DNA using the following primers: dNAB_fw, 5′-AGACCATCTGGCTGCTGACC-3′ and dNAB_rev, 5′-AATTAACC-CTCACTAAAGGTCTGGTGAAGCAGCACTCC-3′.

GST pull-down experiments
GST pull-down experiments were carried out according to standard protocols, essentially as described by Hasson et al. (Hasson et al., 2001).

RESULTS
dnab is a Dpp target in the wing disc
The Dpp signaling pathway controls growth, survival and patterning during *Drosophila* appendage development. To identify novel putative Dpp targets, we screened a collection of 2000 wing disc Gal4 enhancer-trap insertion lines (Gerlitz et al., 2002) with a UAS-GFP reporter for lines that exhibit expression patterns centered on the stripe of dpp expression at the anterior-posterior compartment boundary. The responsiveness of each of the selected enhancer-trap lines to changes in Dpp signaling was then assessed using UAS transgenic lines of either a constitutively active form of the type-I Dpp receptor $thickveins$ (tkv^{Q235D}) (Nellen et al., 1996), or of $Daughters$ $against$ dpp (Dad) (Tsuneizumi et al., 1997), an inhibitor of the Dpp pathway. Using this selection process, we identified S149, a lethal Gal4 insertion, the expression of which is confined to the presumptive wing blade (Fig. 1A). Through plasmid rescue of the mutagenic P-element and RNA in situ hybridization (Fig. 1B), we identified this insertion as a hypomorphic mutant allele of *Drosophila nab* ($dnab$) (Clements et al., 2003), a member of the NAB family of transcriptional co-repressors (Russo et al., 1995; Svaren et al., 1996). Complementation crosses with a known lethal deletion allele ($dnab^{R6H8}$) (Nairz et al., 2004) and introduction of a
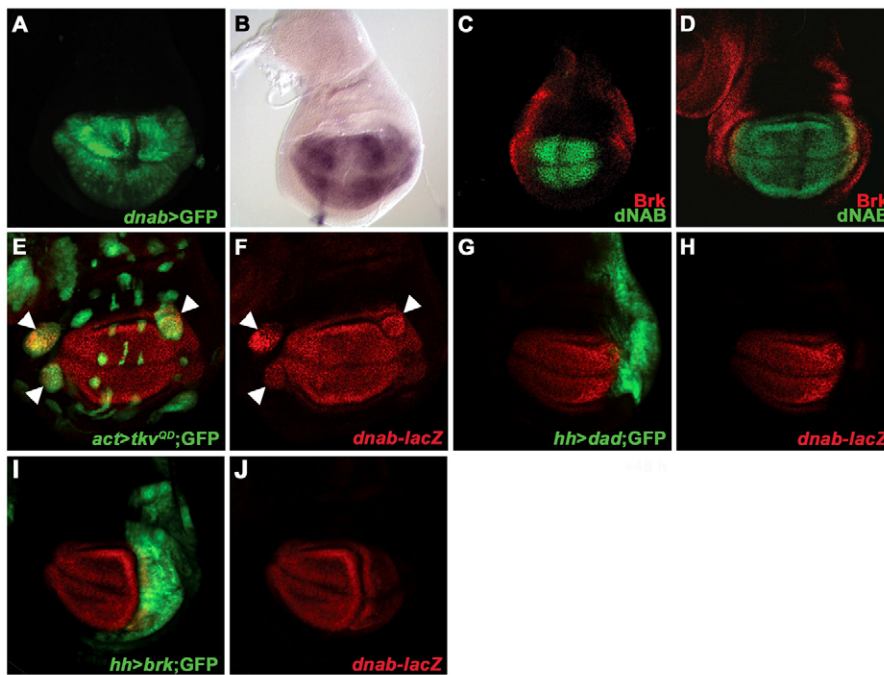
Fig. 1. *dnab* is a Dpp target gene expressed in the *Drosophila* wing pouch. (**A**) Wing imaginal disc (anterior to the left, dorsal up, in all figures) showing the activity of the S149 Gal4 enhancer-trap insertion as revealed by *UAS-GFP* (green). (**B**) In situ hybridization with an RNA probe derived from the *dnab* ORF. (**C,D**) dNAB and Brk expression pattern in second and third instar larvae. Double staining of wing discs with antibodies directed against Brk (red) and dNAB (green) shows no overlap in second instar (C) and a slight overlap in third instar (D) larvae. (**E-J**) Dpp signaling positively regulates *dnab* expression. (E,F) *dnab-lacZ* (red) is ectopically expressed in clones overexpressing Tkv^{Q235D} (green, arrowheads); in all figures, overexpression clones (marked with GFP, green) are derived from activation of a Flp-able actin promoter (unless stated otherwise). Dad (G,H) and Brk (I,J) overexpression (using the *hh-Gal4* driver, green) downregulates the expression of the *dnab-lacZ* reporter (red).

9.5 kb genomic rescue construct of *dnab* confirmed that the observed lethality is due to disruption of *dnab*. We further found that the expression of a *dnab-lacZ* reporter gene (SH143) (Oh et al., 2003) is ectopically induced by misexpression of Tkv^{Q235D} and is abolished by overexpression of either *Dad* or *brk* (Fig. 1E-J), establishing that *dnab* expression is positively regulated by Dpp signaling in the developing wing. We have no indication as to whether this regulation is direct or indirect. Interestingly, the reduction of *dnab-lacZ* expression resulting from Brk overexpression was not as robust as that resulting from Dad overexpression (compare Fig. 1G,H with 1I,J), suggesting that *dnab* expression is regulated by Dpp/Mad signaling and not only through the removal of *brk* repression. It is important to note that, as with other wing-specific genes, *dnab* expression has been shown to depend on Vestigial (Vg) (Terriente Felix et al., 2007). Since *vg* is a Dpp target, it is possible that Dpp regulates *dnab* expression at least in part through regulation of *vg*. The fact that not every Tkv^{Q235D}-expressing clone was able to induce *dnab* expression (Fig. 1E) might indicate that *dnab* expression requires inputs from both Vg and Dpp signaling, as is the case for other Dpp target genes such as *spalt* (*sal*; *salm* – FlyBase) (Halder et al., 1998).

dNAB is not required for Dpp-dependent patterning

Motivated by the idea that the global patterning and growth activities of the Dpp pathway are executed by its downstream targets, we first analyzed the effect of dNAB overexpression on wing development. We found that dNAB overexpression in the wing disc represses the expression of the Dpp/Brk target genes *sal* and *vg* (Fig. 2A-F). To analyze the role of dNAB in wing development, we generated homozygous clones mutant for *dnab* in heterozygous wing discs. However, we found that loss of *dnab* function affects neither the size of the clones, which were similar to their sibling twin spots, nor the expression of known Dpp target genes in the wing disc, such as *optomotor-blind* (*omb*; *bifid* – FlyBase), *sal* and *vg* (Fig. 2G-O), indicating that dNAB function is not required for Dpp-dependent growth and patterning in this tissue.

dNAB induces JNK-mediated cell death

Dpp is essential for cell survival in the wing disc, and mutant cells impaired for Dpp reception or transduction are lost from the wing epithelium (Adachi-Yamada and O'Connor, 2002; Burke and Basler, 1996; Moreno et al., 2002). To test the possibility that dNAB is involved in processes that regulate cell viability, we generated cell clones overexpressing dNAB in a wild-type background and followed their descendents at various time points after induction. We found that these cells undergo apoptosis, as shown by the dramatic increase in the levels of activated Caspase 3, and that they are gradually eliminated first from the periphery of the wing disc where *brk* levels are high, and subsequently from the medial region (Fig. 3A-D).

The JNK pathway mediates apoptosis in various developmental contexts (Adachi-Yamada et al., 1999; McEwen and Peifer, 2005), including the elimination of cells with impaired Dpp signaling (Adachi-Yamada et al., 1999; Adachi-Yamada and O'Connor, 2002; Moreno et al., 2002). We asked whether JNK activation is involved in dNAB-induced cell death. The extent of activation of the JNK pathway can be monitored through the expression of the target gene *puckered* (*puc*), which encodes a protein phosphatase that negatively regulates the pathway (Martin-Blanco et al., 1998). We found that overexpression of dNAB induces the expression of *puc* (Fig. 3E,F), indicating that JNK signaling is activated in the dying cells. Furthermore, when JNK signaling was experimentally downregulated in clones of cells overexpressing dNAB by co-expression of *puc*, Caspase 3 activation was to a large extent inhibited (Fig. 3G,H), and the clones were distributed randomly throughout the wing disc. These results demonstrate that dNAB induces cell death through induction of the JNK pathway, which in turn triggers Caspase-3-mediated apoptosis.

dNAB-induced apoptosis is Brk-dependent

In order to elucidate whether dNAB promotes cell death through the Dpp signaling pathway, dNAB overexpression was combined with Brk loss-of-function, a dedicated downstream effector of the Dpp pathway. To this end, clones mutant for *brk* were generated in the
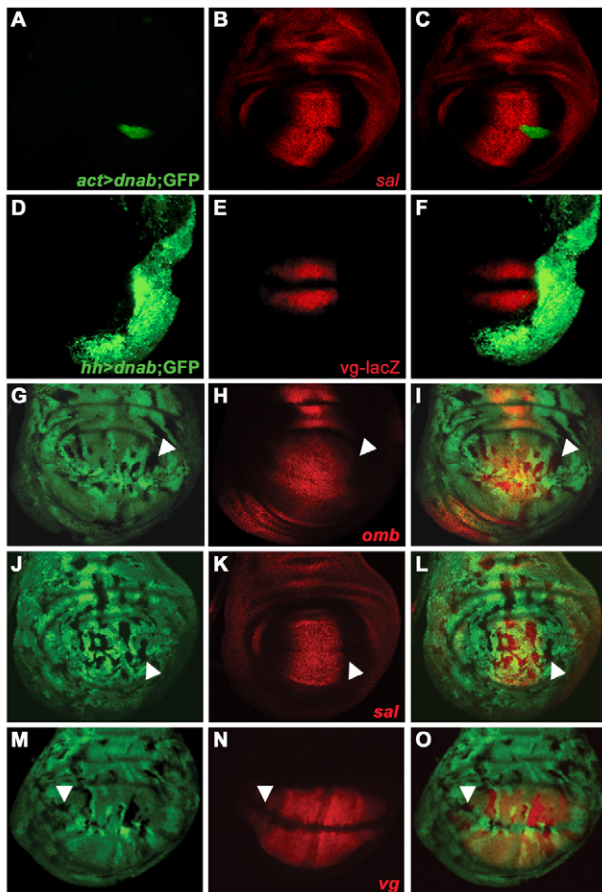
Fig. 2. dNAB overexpression represses Dpp target genes, but is not required for Dpp-dependent growth and patterning in the *Drosophila* wing disc. (**A-C**) dNAB-overexpressing clone (A, green) represses Sal expression as revealed by immunostaining (B, red). (C) Merge of A and B. (**D-F**) *hh-Gal4* driver was used to overexpress *dnab* in the posterior compartment (D, green). *vg* expression, as visualized by means of the *vg-lacZ* reporter, is abolished by *dnab* overexpression (E, red). (F) Merge of D and E. (**G-O**) In dNAB loss-of-function clones (G,J,M), marked by the loss of GFP (arrowheads), the expression of the Dpp target genes *omb* (H, red), *sal* (K, red) and *vg* (N, red) is unaffected. (I,L,O) Merges.

posterior compartment where dNAB was overexpressed using a *hedgehog* (*hh*)-*Gal4* driver (Fig. 4A-C). We found that dNAB-induced apoptosis is completely nullified by loss of Brk function, as evidenced by the reduction of activated Caspase 3 to normal levels (Fig. 4B). Thus, the cell death-promoting activity of dNAB is Brk-dependent, and therefore functions through the Dpp signaling pathway.

Epistatic positioning of dNAB within the Dpp signaling pathway

To determine the epistatic position of dNAB within the Dpp pathway, we co-expressed dNAB with Tkv^{Q235D}. We found that excessive activation of the Dpp signal transduction pathway by Tkv^{Q235D} does not rescue dNAB-induced apoptosis (Fig. 4G-I). Furthermore, expression of Tkv^{Q235D} is known to produce abnormally large discs, whereas co-expression of dNAB with Tkv^{Q235D} partially reversed this effect of overgrowth such that wing discs varied in size, ranging from small through normal to large (Fig.

4D-F; data not shown). Thus, dNAB appears to affect cell survival by impinging on the Dpp signaling pathway downstream of the receptor complex.

We next tested whether dNAB acts through transcriptional repression of *brk* (Marty et al., 2000; Muller et al., 2003) at the level of Mad, similar to the mode of action of the inhibitory Smad, Dad (Tsuneizumi et al., 1997). According to this possibility, excess dNAB should interfere at the level of *brk* transcriptional repression and lead to its accumulation. On the contrary, we found that dNAB overexpression, while inducing apoptosis, had no effect on the expression levels of a *brk-lacZ* reporter (Fig. 4J-L). Thus, dNAB acts downstream of *brk* transcriptional regulation.

dNAB and Brk physically interact in vitro

The fact that overexpression of dNAB represses Dpp/Brk target genes and that dNAB has been shown to act as a transcriptional co-regulator in *Drosophila* (Terriente Felix et al., 2007; Tsuji et al., 2008) prompted us to assess the possibility that dNAB physically interacts with Brk. Using a GST pull-down assay, we found that like Gro, a known Brk co-repressor, dNAB binds directly to the Brk protein (Fig. 5A). We then used sequential fragments of the dNAB protein to narrow down the Brk-binding region of dNAB to the NAB conserved domain 2 (NCD2) (Fig. 5B,C), a region found in the C-terminal half of all NAB proteins that contains a bipartite-like nuclear localization sequence and the transcriptional repression function (Swirnoff et al., 1998). Taken together, our results demonstrate that dNAB acts together with the Brk repressor, apparently through direct protein-protein interactions.

dNAB promotes cell elimination induced by impaired Dpp signaling

It is well documented that cells impaired for Dpp signaling, due to removal of the Dpp receptor or to forced expression of the Dpp pathway inhibitor Dad, or of Brk, are first eliminated from the center of the wing disc and subsequently from lateral regions, where normally *brk* is expressed and *dnab* is not (Adachi-Yamada and O'Connor, 2002; Burke and Basler, 1996; Moreno et al., 2002). Thus, dNAB is not essential for cell elimination induced by very high levels of Brk. We investigated whether loss of dNAB function affects cell removal from the wing pouch region induced by reduced Dpp signaling. Using the MARCM system (Lee and Luo, 2001) in combination with a wing Gal4 driver [*scalloped* (*sd*)-*Gal4*], we generated *dnab* loss-of-function clones that overexpressed the Dpp pathway inhibitor Dad in the wing disc. We favored this experimental set-up because activation of the UAS transgene is dependent on Gal80 perdurance, and therefore should allow dNAB protein to dissipate in the loss-of-function clones prior to Dad accumulation and the subsequent upregulation of *brk*. Seventy-two hours after clone induction, we found a greater than 2-fold increase (two-tailed test, $P<0.005$) in the number of *dnab* loss-of-function clones that survived in the wing pouch region compared with control clones (Fig. 6A-E). In addition, in many cases we observed higher levels of active Caspase 3 in control clones as compared with *dnab* loss-of-function clones (Fig. 6B,D). We conclude that dNAB promotes the elimination of cells with reduced Dpp signaling.

The fact that dNAB expression is regulated by Dpp/Brk signaling raises the question of how clones impaired for Dpp signaling, such as Dad-overexpressing clones, die in a dNAB-dependent manner, for one might expect that in such clones *dnab* expression would be lost when *brk* expression is gained. The simplest explanation is that the dNAB protein is stable and has a high perdurance, so that under conditions in which Brk expression is gained there is enough dNAB
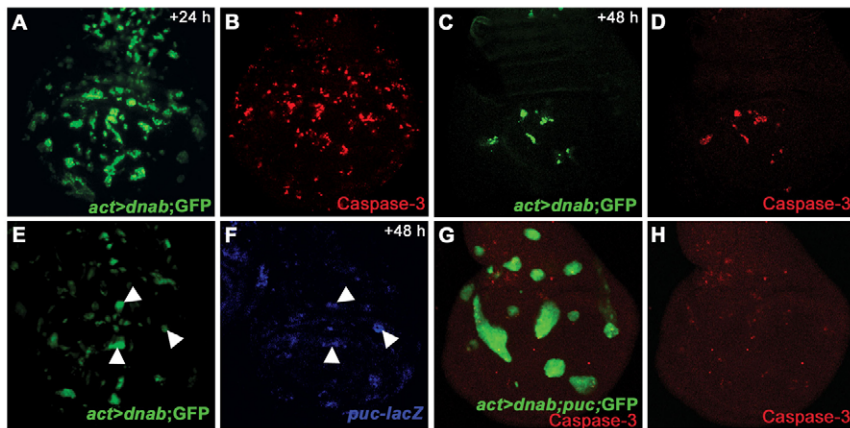
Fig. 3. dNAB overexpression induces apoptosis through the JNK signaling pathway.
(**A-D**) dNAB-overexpressing clones are apoptotically eliminated from the *Drosophila* wing disc in a lateral-to-medial pattern. Clones overexpressing dNAB (green) 24 (A) and 48 (C) hours after induction. (**B,D**) Immunodetection of activated Caspase 3 (red) as a marker of apoptosis. (**E-H**) dNAB-dependent cell elimination is associated with, and caused by, activation of the JNK pathway. Clones overexpressing dNAB (E, green, arrowheads) upregulate *puc-lacZ* reporter expression (F, blue, arrowheads). Non-autonomous JNK and Caspase 3 activation is occasionally seen around the border of dNAB-overexpressing clones (B,D,F). Co-expression of Puc with dNAB (G, green) abolishes Caspase 3 activation (H, red).

protein to allow for interaction. Consistently, we found that dNAB protein was present in Brk-overexpressing clones that survived in the wing pouch region 30 hours after induction (Fig. 6I,J). Significantly, the expression of the Dpp/Brk target gene *sal* was completely lost in such clones (Fig. 6K,L), indicating that Brk is upregulated and active when dNAB is still present.

dNAB sensitizes cells to the cell death-promoting activity of Brk

We tested whether dNAB enhances the cell death-promoting activity of Brk. Expression of Brk in the wing disc using a mild ubiquitous Gal4 driver (C765) resulted in low Caspase 3 activation mainly in the wing pouch region (Fig. 6F) where *dnab* is normally expressed, whereas driving dNAB expression with the same Gal4 driver resulted in an almost complementary pattern of enhanced Caspase 3 activation in the wing periphery (Fig. 6G). Importantly, co-expression of dNAB and Brk resulted in significantly smaller wing discs and a dramatic increase in Caspase 3 activation in the wing pouch (Fig. 6H). Thus, in the presence of dNAB, lower levels of Brk induce cell death, implying that dNAB expression in the wing pouch region sensitizes cells to the killing activity of Brk. Altogether, these results indicate that dNAB acts as a co-repressor that interacts with Brk to promote apoptotic elimination of cells with reduced Dpp signaling.

dNAB and Brk are both required for dMyc-induced cell competition

Recent studies, in which apposing cell populations with different levels of dMyc or of a *Minute* gene product were generated in the wing disc, established Dpp as a crucial survival factor for which cells continuously compete to prevent apoptosis (Moreno and Basler, 2004; Moreno et al., 2002). Reduced Dpp signaling activity in outcompeted cells results in the upregulation of Brk, which in turn triggers apoptosis through activation of the JNK pathway. The results presented so far prompted us to investigate whether dNAB could also play a positive role in cell elimination driven by different levels of dMyc. Using the transgene *tub>dmyc>Gal4*, we generated wild-type cells surrounded by cells expressing extra dMyc and found, in accordance with previous results (de la Cova et al., 2004; Moreno and Basler, 2004), that they were rapidly lost from the wing primordium (Fig. 6M; see Fig. S1 in the supplementary material). However, knocking down the expression of either *dnab* or *brk* specifically in the wild-type cells [using the appropriate UAS-*RNAi*s (Dietzl et al., 2007)] led, remarkably, to their rescue and reversed

their proliferation deficit (Fig. 6N-P). Notably, *dnab* RNAi appeared to result in ragged-edge clones, whereas *brk* knockdown led to round clones, indicating that unlike Brk, dNAB has no apparent role in cell affinity. From these results, we concluded that both dNAB and Brk play a crucial role in mediating dMyc-induced apoptotic cell competition.

dNAB and Gro qualitatively differ in their ability to induce JNK-mediated cell killing

The results presented above raised the possibility that the previously identified co-repressor of Brk, Gro (Hasson et al., 2001; Zhang et al., 2001), which has been implicated in patterning, could play a role similar to that of dNAB in promoting Brk-dependent cell elimination. However, several lines of evidence appear to contradict this idea. First, in contrast to the situation in which overexpression of dNAB leads to rapid cell loss (Fig. 3A-D), clones of cells overexpressing Gro appear large in size, do not show Caspase 3 activation and are not readily eliminated, but rather are distributed randomly throughout the wing disc, including the lateral regions where *brk* is highly expressed (Fig. 7A-C). Notably, these Gro clones readily repress the Brk target genes *sal*, *omb* and *vg* (Hasson et al., 2001; Zhang et al., 2001). Second, whereas excess dNAB leads to induction of both JNK signaling and Caspase 3 activation (Fig. 7D-F), overexpression of Gro throughout the entire posterior compartment shows neither of these effects (Fig. 7G-I). Thus, dNAB and Gro qualitatively differ with respect to their ability to induce JNK-mediated cell killing and Dpp-mediated patterning.

DISCUSSION

Morphogens are secreted signaling molecules that organize a developing field by determining the growth and fate of responding cells according to the level of the morphogen they perceive. Elimination of underperforming cells with improper reduced morphogenetic signaling that would otherwise assume an inappropriate positional identity is thought to provide a corrective mechanism to circumvent aberrant development. Here we report on the identification of the transcriptional co-regulator dNAB as a target and an effector of the Dpp morphogen in the developing wing and demonstrate that dNAB is required for the elimination of cells with impaired Dpp signaling.

NAB proteins comprise a family of transcriptional co-regulators implicated in various developmental processes in different organisms. *Drosophila* NAB was found to be required for determining specific neuronal fates in the embryonic CNS and for
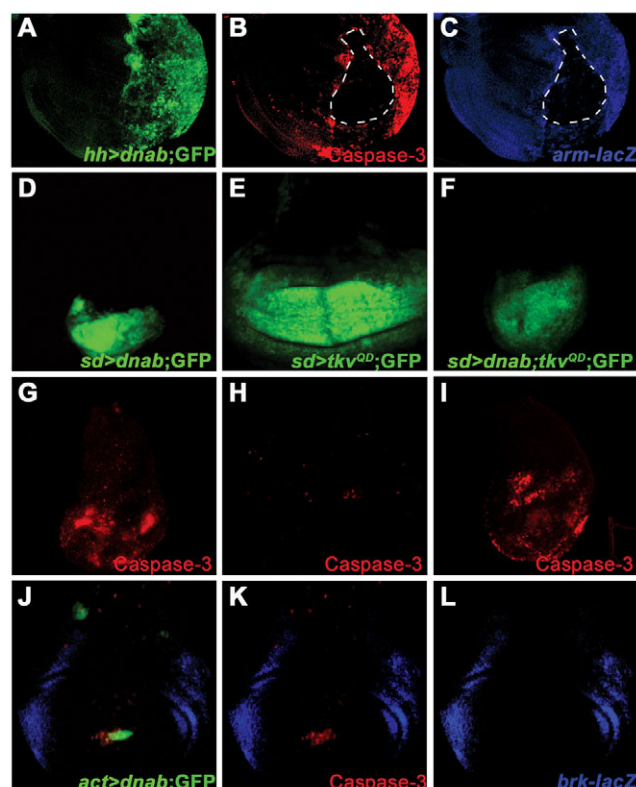
Fig. 4. The cell death-promoting activity of dNAB is Brk-dependent and positioned downstream of *brk* transcriptional regulation. (**A-C**) dNAB requires Brk to induce cell death. Overexpression of dNAB in the posterior compartment using the *hh-Gal4* driver (marked by GFP, green, A) leads to apoptosis as visualized by activated Caspase 3 (red, B). Caspase 3 activation induced by dNAB is abolished in the large *brk* loss-of-function clone marked by the lack of *arm-lacZ* expression (blue, C, outlined by the dashed line). (**D-I**) dNAB acts downstream of the Dpp receptor complex. *Drosophila* wing imaginal discs overexpressing *dnab* (D,G), *tkv*^{Q235D} (E,H) or both (F,I) using the *sd-Gal4* wing pouch driver (marked by GFP, green, D-F). Apoptosis is detected by staining for active Caspase 3 (red, G-I). (**J-L**) dNAB activates Caspase 3 without inducing *brk* expression. A dNAB-overexpressing clone (J, green) showing Caspase 3 activation (J,K, red). *brk* expression is visualized by *brk-lacZ* staining (J-L, blue).

Fig. 5. dNAB binds Brk via its NCD2 domain. Brk binds to dNAB derivatives that contain the NCD2 domain. (**A,B**) dNAB binds Brk via its NCD2 domain in a GST pull-down assay. (A) GST-Brk full-length (GST-Brk FL) fusion protein was incubated with either Gro or with dNAB derivative proteins (^{35}S-labeled, in vitro translated). Following washing, retained proteins were separated by SDS-PAGE and exposed for autoradiography. (B) GST-dNAB fusion proteins were incubated with ^{35}S-labeled full-length Brk and processed as above. (**C**) Schematic representation of the various dNAB peptides fused to GST.

wing hinge patterning (Clements et al., 2003; Tsuji et al., 2008). Our work shows that dNAB induces cell elimination through induction of the JNK pathway, which in turn triggers Caspase-3-mediated apoptosis. We show that dNAB acts as a co-repressor that interacts with Brk to induce apoptotic cell elimination. This conclusion is based on several lines of evidence. First, dNAB-induced apoptosis is completely nullified by removal of Brk. Second, our epistatic analysis placed dNAB in the Dpp signaling pathway downstream of the receptor complex and of *brk* transcriptional repression and upstream of Brk. Third, dNAB physically associates with Brk through its NCD2 domain in vitro. Fourth, dNAB enhances the killing activity of Brk in the presumptive wing blade region and is required for elimination of Dad-overexpressing cells, a process that is completely dependent upon Brk function. Finally, ectopic expression of dNAB represses the expression of Dpp/Brk target genes.

Competitive interactions occur between cells differing in their levels of dMyc, such that cells expressing more dMyc both outgrow neighboring cells and induce their death (de la Cova et al., 2004;
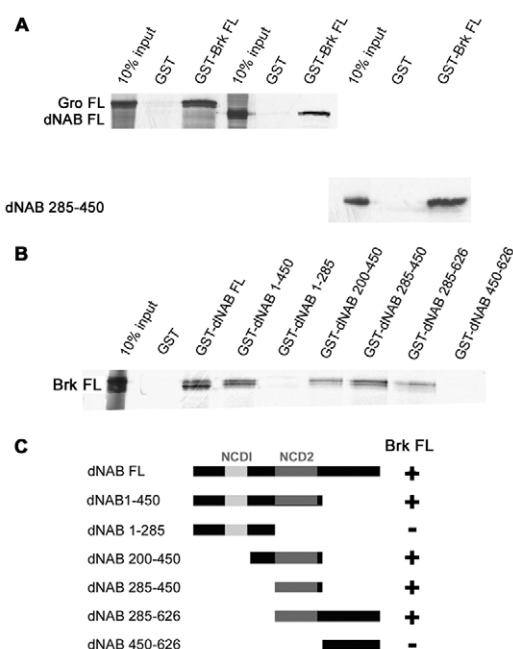
Moreno and Basler, 2004). This competitive behavior correlates with, and can be modulated by, the activation of the Dpp survival signaling pathway, showing that dMyc-induced cell competition relies on Dpp signaling. The fact that dNAB, similar to Brk, is crucial for dMyc-induced cell competition strongly supports a role for dNAB as an effector of cell elimination of underperforming cells with reduced Dpp signaling.

Elimination of underperforming cells takes place only during early larval stages. Clones generated later, during the third instar larval stage, persist to adulthood (e.g. Burke and Basler, 1996; Morata and Ripoll, 1975; Simpson, 1979). Consistently, using double staining of wing discs with antibodies directed against Brk and dNAB, we have found that the two do not overlap in the second instar larval stage [60 hours after egg laying (AEL)] (Fig. 1C) and only slightly overlap during the third instar (80 hours AEL) (Fig. 1D). These findings suggest that the Brk-dNAB complex is active in cell elimination only during early development. This might indicate that either another factor required for complex activity is present only during early development, or that a factor is present during later stages that inhibits the complex. Alternatively, intensive growth/proliferation might be required for the execution of the killing activity of the complex.

The morphogen Dpp acts through a well-characterized transduction pathway to simultaneously regulate growth, survival and patterning. To a large extent, Dpp signaling acts through negative regulation of *brk* expression. This implies that a complete answer to how the Dpp signal directs different cellular and developmental processes requires an understanding of how Brk

Fig. 6. dNAB interacts with Brk to eliminate cells with reduced Dpp signaling and is required for *dmyc*-induced cell competition. (**A-E**) dNAB loss-of-function decreases the elimination of Dad-overexpressing clones. (**A-D**) dNAB loss-of-function clones overexpressing Dad (A, green) visualized 72 hours after induction are significantly more abundant in the pouch region compared with control clones (C, green). Activated Caspase 3 levels in control clones (D, red) are higher than in dNAB loss-of-function clones (B, red). (E) Bar chart showing a 2-fold increase (*P*<0.005) in the average number of dNAB loss-of-function clones overexpressing Dad (left column, blue, *n*=51) that survived in the wing pouch region compared with wild-type control clones (right column, red, *n*=54). (**F-H**) dNAB enhances the killing activity of Brk in the wing pouch region (outlined by dashed line). The *C765-Gal4* driver was used to drive ubiquitous (mild) expression of *brk* and *dnab* in the wing disc. (F) Wing disc overexpressing Brk shows mild Caspase 3 activation (red) in the wing pouch region. (G) Wing disc overexpressing dNAB shows strong Caspase 3 activation (red) at the periphery around the wing pouch. (H) Wing disc overexpressing both Brk and dNAB shows dramatic enhancement of Caspase 3 activation (red) in the pouch region. The delineation of the pouch is based on the natural folds of the disc seen in bright-field

microscopy. All discs were photographed under the same magnification (×200). Discs that co-express *dnab* and *brk* (H) are much smaller than those that express either gene alone (F,G). (**I-L**) Stability of dNAB protein in *brk*-overexpressing clones (generated by Flp-able *abx-ubx* promoter). (I,J) dNAB protein levels in the wing pouch, as revealed by immunostaining (red), are not affected in GFP-marked *brk*-overexpressing clones (arrowhead, green) 30 hours after induction. (K,L) Sal protein levels, as revealed by immunostaining (red), are diminished in *brk*-overexpressing clones (arrowhead, green) 30 hours after induction. (**M-P**) Brk and dNAB are required for dMyc-induced cell competition. (M-O) GFP-marked clones of wild-type cells (green) generated in a *tub>dmyc* genetic background and monitored for survival 72 hours after induction. (M) Most clones have disappeared from the wing pouch, and only a few persist in the periphery (*n*=50). (N,O) When expressing a *UAS-RNAi* construct that knocks down the expression of either *brk* (N) or *dnab* (O), many clones persist in the wing pouch (*n*=50). (P) Bar chart showing the percentage of the wing pouch area occupied by GFP-marked wild-type cells recovered in M-O. To measure the percentage of wild-type cells that survived, each wing pouch was subdivided into four quadrants and the surviving GFP-marked clones were fitted into the quadrants. Discs were analyzed either by computer imaging or by hand and were assigned to one of four groups according to the percentage of quadrants occupied: 0-10, 25, 50 or 75%.
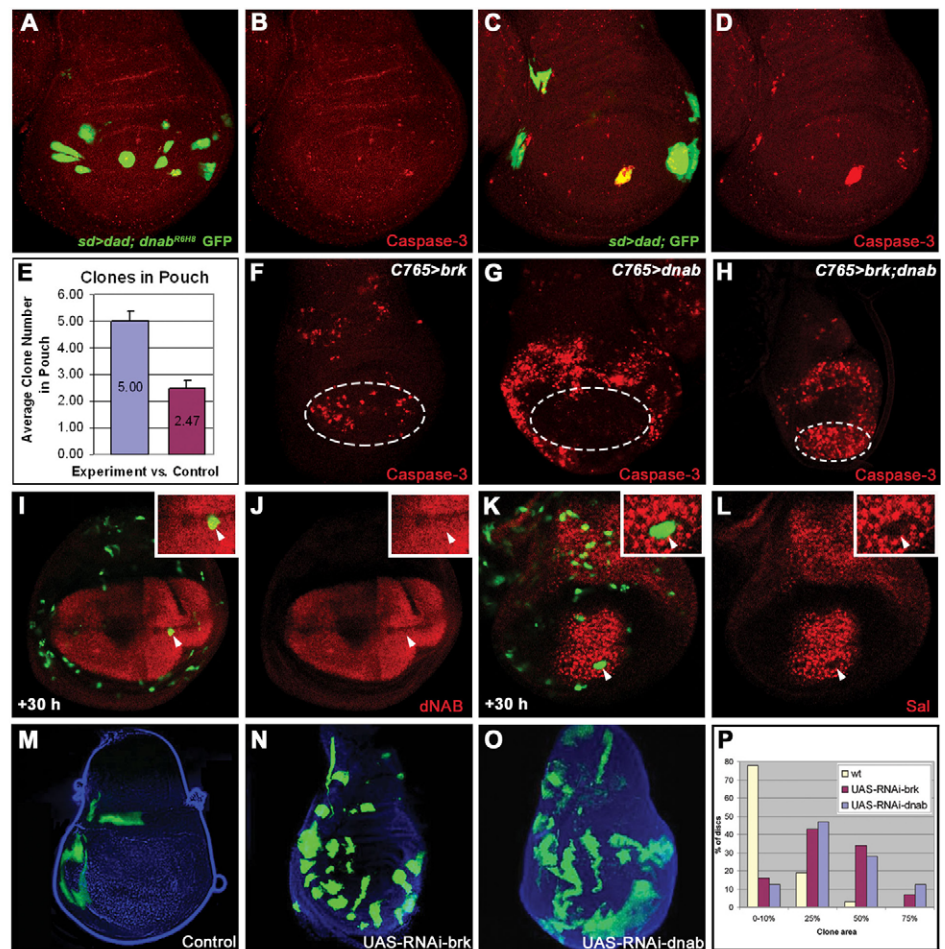
executes its transcriptional repression functions. Our finding that dNAB is a Brk co-repressor is in accordance with recent results showing that overexpression of Brk forms that cannot bind either Gro or CtBP results in repression of *sal*, *omb* and *vg*, and that Brk contains additional co-repressor-binding domains (Winter and Campbell, 2004). We found that in contrast to Gro, a known co-repressor of Brk, the function of dNAB is not required for Dpp-dependent patterning. However, Gro does not play a similar role to that of dNAB in promoting JNK-mediated cell killing. These findings imply that the choice of Brk co-repressor determines the specificity of target gene repression, thereby modulating different Dpp outputs. Mechanistically, this could be achieved in a number of ways: for example, dNAB or Gro association might alter the DNA-binding specificity of Brk, or the promoters of Brk target genes might be differentially responsive to dNAB and Gro. In addition, the fact that Gro is ubiquitously expressed throughout the developing

wing, and that Dpp induces dNAB expression in the center of the wing disc while restricting Brk expression to lateral regions, provide another means for differentially modulating Dpp outputs.

Based on our findings, we propose a molecular model to explain how the morphogen Dpp regulates the cellular response to its own survival signal in the developing wing by inversely controlling the expression of two key factors, Brk and dNAB. In the center of the wing disc, Dpp represses *brk* and induces *dnab* expression, so that in situations in which Dpp signaling activity is abnormally reduced, the resulting local increase in the levels of Brk, which complexes with dNAB, activates the apoptotic pathway. Thus, the Dpp signal sensitizes cells in the center of the wing disc to the apoptotic effect associated with reduced Dpp signaling by maintaining dNAB expression. In lateral regions of the wing disc, where Brk expression is normally higher, apoptotic cell elimination is attenuated, at least in part owing to a lack of dNAB. Thus, by invoking dNAB as a Dpp
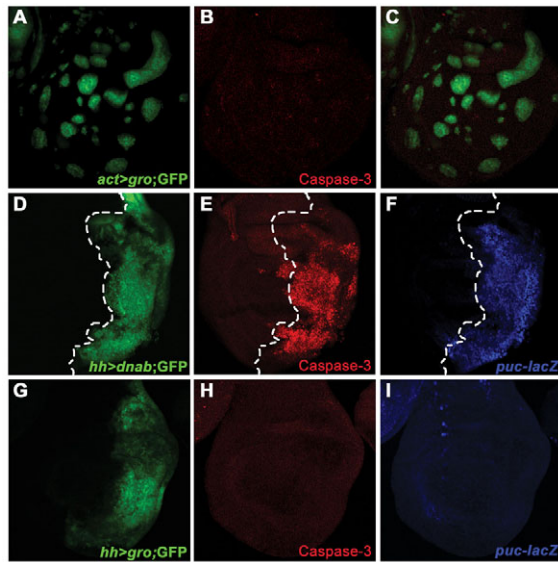
Fig. 7. Differential effects of the Brk co-repressors dNAB and Gro on JNK activation and cell survival. (**A-C**) Clones overexpressing Gro (marked with GFP, green, A) are not eliminated from the *Drosophila* wing disc, and do not show Caspase 3 activation (red, B). (C) Merge of A and B. (**D-I**) *hh-Gal4* driver was used to overexpress *dnab* (D-F) or *gro* (G-I) in the posterior wing compartment (marked by GFP, green, D,G). Overexpression of dNAB activates both Caspase 3 (red, E) and the JNK pathway (*puc-lacZ*, blue, F). Gro overexpression activates neither Caspase 3 (red, H) nor the JNK pathway (*puc-lacZ*, blue, I).

effector molecule that sensitizes cells to the levels of Brk, we can at least in part explain why cells in the center of the wing disc, near the Dpp source, are more susceptible to cell elimination induced by reduced Dpp signaling, and why high levels of Brk in the periphery do not necessarily bring about apoptosis.

Given that dNAB appears to play no role in Dpp-mediated patterning, we propose that dNAB functions in the wing to prevent developmental errors and discontinuities along the Dpp signaling gradient. This mechanism might be a general feature of morphogen gradients that functions to avoid the accumulation of detrimental developmental mistakes that would otherwise lead to embryonic malformation, and is potentially important in cancer, where tumor cells overexpressing oncogenes such as *Myc* may act as super-competitors. Thus, the molecular principles underlying such developmental fail-safe mechanisms are clearly of biomedical interest.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/7/1137/DC1

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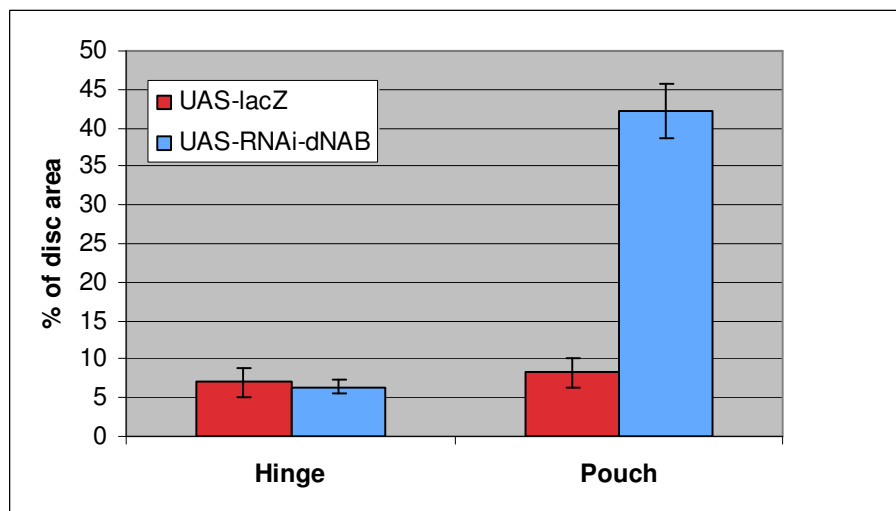
UAS-lacZ	Hinge	Pouch	UAS-RNAi-dNAB	Hinge	Pouch
Disc 1	15	15	Disc 1	5	45
Disc 2	10	5	Disc 2	10	50
Disc 3	10	15	Disc 3	5	40
Disc 4	5	10	Disc 4	5	30
Disc 5	1	5	Disc 5	5	45
Disc 6	1	5	Disc 6	10	30
Disc 7	7	3	Disc 7	5	55
Average	7.0	8.3	Average	6.4	42.1
Error	1.9	1.9	Error	0.9	3.6