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Nab controls the activity of the zinc-finger transcription factors Squeeze and Rotund in *Drosophila* development

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Nab proteins form an evolutionarily conserved family of transcriptional co-regulators implicated in multiple developmental events in various organisms. They lack DNA-binding domains and act by associating with other transcription factors, but their precise roles in development are not known. Here we analyze the role of *nab* in *Drosophila* development. By employing genetic approaches we found that *nab* is required for proximodistal patterning of the wing imaginal disc and also for determining specific neuronal fates in the embryonic CNS. We identified two partners of Nab: the zinc-finger transcription factors Rotund and Squeeze. Nab is co-expressed with *squeeze* in a subset of neurons in the embryonic ventral nerve cord and with *rotund* in a circular domain of the distal-most area of the wing disc. Our results indicate that Nab is a co-activator of Squeeze and is required to limit the number of neurons that express the LIM-homeodomain gene *apterous* and to specify Tv neuronal fate. Conversely, Nab is a co-repressor of Rotund in wing development and is required to limit the expression of *wingless* (*wg*) in the wing hinge, where *wg* plays a mitogenic role. We also showed by pull-down assays that Nab binds directly to Rotund and Squeeze via its conserved C-terminal domain. We propose two mechanisms by which the activation of *wg* expression by Rotund in the wing hinge is repressed in the distal wing.

KEY WORDS: *Drosophila*, *nab*, *squeeze*, *rotund*, Transcriptional co-factors, Proximodistal development, CNS

INTRODUCTION

Precise temporal and spatial control of gene transcription is crucial for development. Sequence-specific DNA-binding factors and their association with a variety of modulator proteins, the co-factors, achieve this control. Co-factors do not bind DNA but act as adaptors between DNA-binding factors and other proteins. A number of transcription factors have been characterized, many of which act by recruiting multiprotein complexes with chromatin-modifying activities (Knoepfler and Eisenman, 1999). By recruiting co-factors, a DNA-binding protein can act as co-activator or as co-repressor depending on the context (Mannervik et al., 1999; Chinnadurai, 2002). An example of a co-repressor is the retinoblastoma protein that converts the E2F transcription factor into a repressor of cell-cycle genes (Weintraub et al., 1995). The identification of co-factors and the determination of their precise roles are crucial for understanding the mechanisms that govern development.

Nab (NGFI-A–binding protein) proteins form an evolutionarily conserved family of transcriptional regulators. Nab was originally identified in mouse as a strong co-repressor by virtue of its capacity to interact directly with the Cys2-His2 zinc-finger transcription factor Egr1 (Krox24; NGFI-A) and inhibit its activity. Two Nab genes, *Nab1* and *Nab2*, have been identified in vertebrates. Nab proteins do not bind DNA but they can repress (Svaren et al., 1998) or activate (Sevetson et al., 2000) gene expression by interacting with Egr transcription factors. Nab proteins have two regions of strong homology: NCD1 and NCD2. The NCD1 domain interacts with the R1 domain of Egr1 (Svaren et al., 1998). The NCD2 domain is required for transcriptional regulation (Swirnoff et al., 1998). Mice harboring targeted deletions of *Nab1* and *Nab2* have phenotypes very similar to *Egr2* (*Krox20*)-deficient mice, suggesting that they act as co-activators of this gene (Le et al., 2005). In zebrafish, *egr2* controls expression of the Nab gene homologs in the r3 and r5 rhombomeres of the developing hindbrain (Mechta-Grigoriou et al., 2000). *Egr2* has been implicated in determining the segmental identities of r3 and r5 by controlling the expression of several target genes as well as cell proliferation. Misexpression experiments suggest that *Nab1*/*Nab2* antagonize *Egr2* transcriptional activity by a negative-feedback regulatory loop. Nevertheless, Nab proteins might have additional functions as these experiments also led to alterations of the neural tube not found in *Egr2*-deficient embryos (Mechta-Grigoriou et al., 2000). Conversely, *Egr2*-deficient mice have a severe hindbrain segmentation defect that is not found in mice deficient in *Nab1* and *Nab2*. Nab might also have Egr-independent functions in mice because, although epidermal hyperplasia has been observed in *Nab1 Nab2* double mutant mice, this phenotype has not been observed in mice lacking any of the Egr proteins (Le et al., 2005).

In *Drosophila*, only one Nab gene has been identified; it is highly homologous to vertebrate Nab genes in the NCD1 and NCD2 domains. *Drosophila nab* mutants are early larval lethal. Detection of *nab* transcripts by in situ hybridization indicates expression in a subset of neuroblasts of the embryonic and larval CNS and weak expression in imaginal discs (Clements et al., 2003). The role of Nab in *Drosophila* development is not known and so far no binding partner has been identified. In this report we show that *nab* is a component of the combinatorial code that determines the number of neurons that express the gene *apterous* (*ap*) in embryonic neural development, and that *nab* specifies the Tv neuronal fate in the *ap* thoracic cluster of neurons.

In early larval development, the wing fate is established in the distal-most region of the wing disc by a combination of two factors: activation of the gene *vestigial* (*vg*) (Williams et al., 1991) and repression of the gene *teashirt* (*tsh*) (Ng et al., 1996). Later, in early third instar larvae, *wingless* (*wg*) is activated in a ring of cells (the inner ring, IR) that borders the *vg* expression domain in the presumptive wing region (Fig. 1A). It has been suggested that activation of the IR involves a signal from the *vg*-expressing cells to the adjacent cells (del Álamo Rodríguez et al., 2002).

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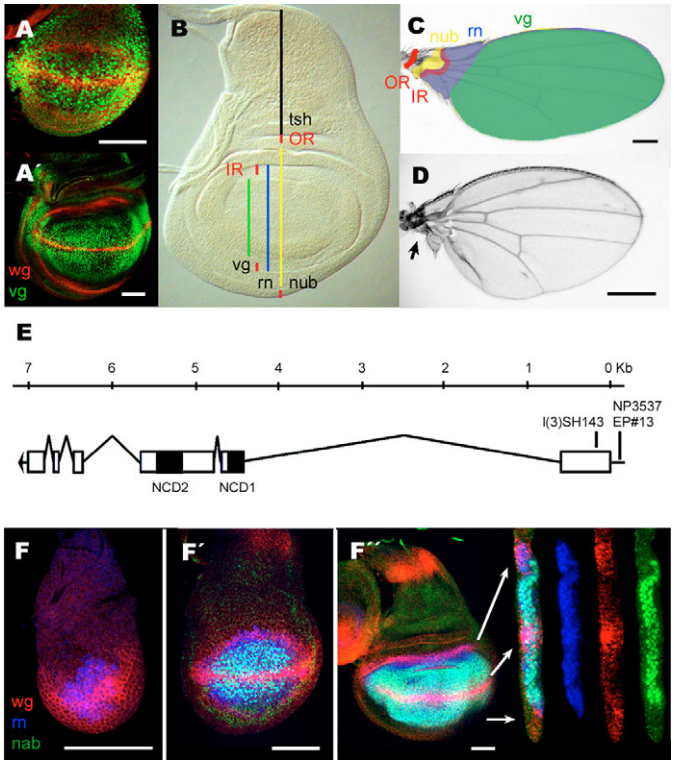

Fig. 1. Proximodistal domains of the *Drosophila* wing imaginal disc. (**A**,**A′**) *vg* (green) and *wg* (red) expression patterns in wing discs of early (A) and late (A′) third instar larvae. In the early third instar the inner ring (IR) of *wg* expression abuts on *vg* expression in the wing pouch, but in the late third instar the two domains are several cells apart. (**B**,**C**) Wing disc (B) and adult wing (C) showing the expression domains of genes involved in proximodistal patterning relevant to this work. *vg* (green), *rn* (blue) and *nub* (yellow) are expressed in concentric domains, and *wg* (red) is expressed in a stripe that defines the wing margin (not shown) and in two rings – the IR and the outer ring (OR). Bars in B represent the extension of the domains on the proximodistal axis. The color scheme in C shows the corresponding expression domains in the adult wing. (**D**) *nubGal4*/+; EP#13/+ wing. Note that the wing hinge (arrow) is deleted. (**E**) Molecular organization of *nab*. The domains NCD1 and NCD2 and the sites of insertion of the alleles used in this work are shown. (**F-F″**) Wing imaginal discs of second instar (F) and early (F′) and late (F″) third instar larvae labeled with Nab (green, here shown as white in combination with blue), Wg (red) and Rn (β-galactosidase, blue). The right-hand panel shows separately the different channels of a view on the *z*-axis. Note how from the initial situation in the early third instar (F′), *nab* expression abuts on *wg* expression in the IR. Scale bars show the relative sizes of the discs (white bars) and wings (black bars). Hereafter, imaginal discs are always shown in the same orientation: dorsal up and anterior to the left.

Interpretation of this signal by the adjacent cells requires the transcription factors encoded by *rotund* (*rn*) and *nubbin* (*nub*) (Ng et al., 1995) (Fig. 1B,C). Expression of *wg* in the IR plays a mitogenic role (Neumann and Cohen, 1996); hence, as a consequence of *wg* expression, cells proliferate and the IR moves away from the *vg* border (Fig. 1A′). At a distance from the source of the signal that drives the initial activation, *wg* IR expression is maintained by an autoregulatory loop that involves *homothorax* (*hth*) (Casares and Mann, 2000). It is thought that an additional mechanism distally represses *wg* IR expression and, in so doing, controls cell proliferation in the wing hinge (del Álamo Rodríguez et al., 2002; Liu et al., 2000). In this report, we show that during imaginal disc development, *nab* is strongly expressed in the wing presumptive domain under the control of *vg*, and that *nab* is required in proximodistal axis development to control the expression of *wg* in the wing hinge.

We have identified two putative partners of Nab: Rn and Squeeze (Sqz). These proteins are members of the Krüppel family of zinc-finger proteins (St Pierre et al., 2002). We show by pull-down assays that Nab interacts with both proteins via a conserved C-terminal domain, and present evidence that Nab acts as co-activator of Sqz in embryo development and as co-repressor of Rn in wing development. Finally, we propose that there are two mechanisms to repress the activation of *wg* expression by Rn in the wing pouch: the first involves Nab as a co-repressor of Rn; the second involves Sqz as a competitor of Rn for binding to specific DNA target sites.

MATERIALS AND METHODS

Fly cultures

Flies were cultured and crossed in fly standard medium at 25°C and 70% humidity.

Fly strains and isolation of the EP#13 line

Fly stocks used were: $nab^{SH143lacZ}$ (Oh et al., 2003), $nabGal4^{NP3537}$ and $nabGal4^{NP1316}$ (Gal4 Enhancer Trap Insertion Database), $sqz^{lacZ02102}$, *sqzGal4* and $UASsqz^{\#7.2}$ (Allan et al., 2005), *Canton-S*, $y\ w^{1118}$, w^{1118}; *Δ2-3 Sb/TM2*, *UASGFP*, $y\ w^{1118}\ hsFLP^{122}$; *UbiGFP FRT80/TM2*, *UASGFP*, (Bloomington *Drosophila* Stock Center), $y\ w^{1118}\ hsFLP^{122}$; *Act5C>y⁺>Gal4 UASGFP* (Ito et al., 1997), $DllGal4^{MD23}$ and $nubGal4^{AC62}$ (Calleja et al., 1996), $dpp^{blk}GAL4$ (Wilder and Perrimon, 1995), rn^{89} (Couso and Bishop, 1998), rn^{5} and rn^{20} (Agnel et al., 1989), *UASrn* and *rnGal4* (St Pierre et al., 2002), *UASvg* (Kim et al., 1996), vg^{83b27r} (Williams et al., 1993), $y\ w^{1118}$; *CyO, EP#720*/dpp^{d12} (Rørth et al., 1998).

EP#720 (inserted in a *CyO* chromosome) was used as a starting line and $nubGal4^{AC62}$ was used as a driver in an F1 screen for dominant phenotypes in adult wings. 69,000 flies were scored and two lines with the same phenotype were selected: EP#13 and EP#29. Both EP lines have insertions in the same gene.

Analysis of genetic mosaics

To induce loss-of-function clones, embryos from crosses: (1) $y\ w\ hsFLP^{122}$; *UbiGFP FRT80* females and either *nab FRT80/TM6, Tb* or *nab FRT80* $sqz^{lacZ02102}$*/TM6, Tb* males; and (2) $y\ w\ hsFLP^{122}$; *FRT82 UbiGFP* females and *FRT82* $sqz^{lacZ02102}$*/TM6* males, were collected over 24 hours and heat shocked at 37°C for 1 hour in a water bath at 36±12 hours of development. To induce clones of ectopic expression, $y\ w^{1118}\ hsFLP^{122}$; *Act5C>y⁺>Gal4 UASGFP* females were crossed either with *UASvg*, *UASnab*, *UASsqz*, *UASrn* or $UASrn^{\Delta 854}$ males. Embryos were collected after 24 hours and heat shocked at 34.5°C for 12 minutes in a water bath at 36±12 hours of development.

In situ hybridization and antibody staining

Standard in situ protocols were used to examine *nab* and *sqz* expression (Tautz and Pfeifle, 1989). Imaginal discs were fixed and stained for confocal microscopy following standard protocols. Primary antibodies used were: rat anti-Ap (1:200) (Fernández-Fúnez et al., 1998); mouse anti-β-galactosidase (1:2000; Promega Z3781); guinea pig anti-Dimm (1:500) (Allan et al., 2005); rabbit anti-FMRFa (1:200; Biotrend); rabbit anti-Nab (1:500; described below); mouse anti-Wg (1:25; Developmental Studies Hybridoma Bank).

Antibody production

To generate the Nab antibody, two rabbits were immunized with a 6xHis fusion of the complete Nab protein. After three immunizations, the rabbits were bled and sera tested on imaginal discs. The two sera gave rise to the same expression pattern. We confirmed that the antibody recognized Nab by immunolabeling *dppGal4/UASnab* wing discs. The expression patterns revealed by the antibodies were identical to those obtained with the $nab^{SH143lacZ}$ and *nabGal4* lines *NP1316* and *NP3537*.

Mapping of EP insertion lines

The EP element contains 14 Gal4 target sites and is described by Rørth (Rørth, 1996). A molecular map of the EP#13 insertion site was constructed by inverse PCR using primers Pry1 and Pry4, as described at the Berkeley *Drosophila* Genome Project website (http://www.fruitfly.org/about/methods/index.html). Sequencing of the flanking DNA indicated that the P element was inserted at position 4144528, 86 bp upstream of the *nab* transcription initiation site.

Generation of novel *nab* alleles

New *nab* mutant alleles were generated by imprecise excision of either *P(lacW)SH143* (22 new alleles) or EP#13 (three new alleles). All were homozygous lethal and belonged to a unique lethal complementation group. After characterizing several alleles, *R52* was selected. This allele corresponds to a deletion of 2.89 kb from the EP#13 insertion site that removes the first exon (Fig. 1E). Neither *R52* nor the original *P(lacW)SH143* showed any expression when homozygous clones induced by mitotic recombination were probed with the Nab antibody. We consider that these two alleles are genetic nulls.

Generation of *UASnab* and *UASrn$^{\Delta 894}$*

A complete cDNA from the EST LP22227 sequence was cloned into the pUAST vector and transgenic lines were generated by P-element transformation (Spradling and Rubin, 1982). Insertions were tested both by *nab* RNA in situ hybridization and Nab antibody staining using *dppGal4* as driver. Nab expression was stronger in the *UASnab* lines than in the EP#13 insertion. *UASrn$^{\Delta 894}$* was generated by cloning the *rn$^{\Delta 894}$* fragment into the pUAST vector.

In vitro GST pull-down assays

For protein interaction assays we used the following procedure: [^{35}S]-labeled Rn, Sqz and Rn$^{\Delta 894}$ were cloned into pCDNA3 tagged with Flag (Invitrogen) and transcribed/translated with the TNT Coupled Reticulocyte Lysate System (Promega) and [^{35}S]-L-Met (Amersham Pharmacia Biotech). *nab* was cloned into pGEX-4T-2 (GST) (Amersham Bioscience Research) and purified from bacterial cells that had been induced by IPTG and incubated with glutathione resin. The resin-binding/washing buffer contained 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% NP40, 2 mM DTT. Rn$^{\Delta 894}$ was generated by in vitro site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene #200518) by the exchange of Asn894 and Lys895 for two stop codons (TAG). The interaction assay between Nab-GST and a synthetic peptide containing the 32 amino acids of the C-terminal domain of Rn was run in a Tricine-SDS-PAGE gel for small proteins as previously described (Schägger and von Jagow, 1987).

RESULTS

Genetic and molecular identification of *nab*

We performed a gain-of-function screen looking for genes involved in proximodistal wing patterning. We identified a new viable EP insertion (EP#13) which, when expressed in the wing disc under the control of the *nubGal4* driver, deletes the wing hinge (Fig. 1D). This phenotype is similar to that of *wg^{spd}* alleles, which affect *wg* expression in the IR (Couso et al., 1994; Neumann and Cohen, 1996). By inverse PCR, we identified a unique insertion 86 bp upstream of the transcription initiation site of *nab* (Fig. 1E). We confirmed by RNA in situ hybridization that this gene (CG33545/LP22227) is misexpressed in *dppGal4*>EP#13 wing discs (data not shown). *nab* was originally identified in a screen of an embryonic cDNA library using cDNA fragments from domains NCD1 and NCD2 as probes. Northern analysis revealed a single transcript encoding a predicted protein of 569 amino acids (Clements et al., 2003). Several P-element insertions have been identified in *nab*, including the larval lethal insertion *P(lacW)l(3)SH143* in the first exon (Oh et al., 2003), and several *P(GawB)* insertions a few nucleotides upstream of the transcription initiation site (GETDB) (Fig. 1E), one of which, *NP3537Gal4*, is larval lethal. We were able to rescue the lethality of the heterozygous combination *nab$^{l(3)SH143}$/NP3537Gal4* with EP#13, which drives the gene controlled by the EP#13 insertion in the *nab* expression pattern. This result confirms that *nab* is misexpressed in EP#13.

We next analyzed the expression pattern of Nab (Fig. 1F,F″). Antibody against Nab (see Materials and methods) revealed a low level of expression in all imaginal discs. In late third instar wing discs, Nab was strongly expressed in a circular domain that delimits the expression of *wg* in the IR. Nab expression was first detected in early third instar larvae, in a group of cells of the distal-most wing, and was maintained throughout the remainder of the larval and pupal stages. There was a low level of expression in the rest of the wing disc, except in the hinge where there was no detectable expression. In the eye disc, Nab was detected in a stripe corresponding to the morphogenetic furrow (data not shown).

Vestigial controls *nab* expression in the wing

We next asked whether, as with other genes involved in proximodistal patterning, *nab* expression in the wing was dependent upon *vg*. No expression of *nab* was detected in the distal wing of *vg^{83b27r}* wing discs (Fig. 2A). However, *nab* was ectopically expressed in clones of *vg*-expressing cells (Fig. 2B,B′). Together, these results indicate that the expression of *nab* in the wing depends on *vg*. In wild-type discs and *vg* ectopic-expressing clones, the domain of *nab* expression is broader than that of *vg*, pointing to the nonautonomous control of *nab* expression. A similar mechanism has been proposed for other genes, such as *rn* and *nub*, whose expression depends on *vg* (del Álamo Rodríguez et al., 2002). Expression of *vg* in the wing starts in second instar larvae, whereas *nab* expression is first detected at early third instar. This suggests that some other mechanism controls the initiation of *nab* expression.

nab delimits the expression of *wg* in the wing inner ring

The *nab^{SH143}* allele is a *P(lacW)* insertion in the first exon. Most larvae homozygous for this allele die in first instar. Thus, to analyze the role of *nab* in development of the wing we generated *nab^{SH143}* homozygous mutant clones by mitotic recombination using the FLP/FRT mitotic recombination system (Xu and Rubin, 1993). In the wing, these clones activated *wg* ectopically (Fig. 3A). However, we noted that not all the clones activated *wg* (29%; total number of clones scored=78). It is therefore possible that this allele has some

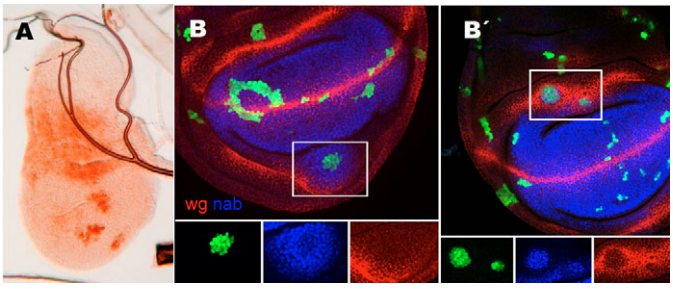

Fig. 2. The *Drosophila* gene *vestigial* is necessary and sufficient to activate *nab* expression. (**A**) *nab* expression in a *vg^{283b27r}* wing disc. Expression in the wing pouch is lost; only basal expression remains in the notum. (**B**,**B′**) Clones of *vg*-expressing cells (green) showing expression of *wg* (red) and *nab* (blue): *y w hsFLP^{122}; Act5C>y^{+}>Gal4 UASGFP/UASvg*. Insets at the bottom show the individual channels of the selected areas in B and B′. (B) *vg* misexpression activates *nab* nonautonomously in a broader domain.

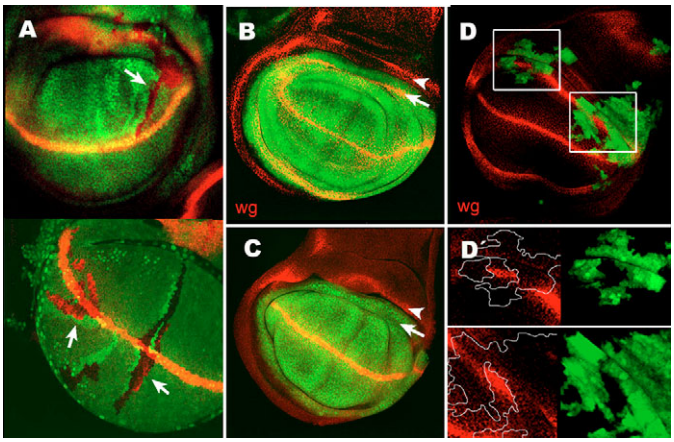

Fig. 3. *nab* limits distal expression of *wg* in the inner ring. (**A**) nab^{SH143} *FRT80* clones in the *Drosophila* wing pouch labeled by the loss of GFP (green) and stained with Wg antibody (red). *wg* is misexpressed in these clones (arrows). (**B**,**C**) *nubGal4/UASGFP* (B) and *nubGal4/UASGFP*; *UASnab/+* (C) wing discs stained with Wg antibody (red). *nab* misexpression represses activation of *wg* expression by the IR enhancer. Arrows, IR; arrowheads, OR. (**D**,**D′**) Clones of *nab* misexpression (green): *y w* $hsFLP^{122}$; *Act5C>*y^{+}*>Gal4 UASGFP/+*; *UASnab/+*. *wg* expression (red) in the IR is cell-autonomously repressed. D′ shows the individual channels for the selected areas in D.

residual function. In order to isolate new mutant alleles of *nab* we looked for imprecise excisions of the EP#13 insertion and identified several new lethal alleles. We obtained the same results as before with homozygous mutant clones of the new allele nab^{R52}. As we were not able to detect any Nab protein in clones of nab^{SH143} or nab^{R52} (data not shown), we conclude that these are very strong or null alleles. The possibility of functional redundancy between Nab and other proteins is analyzed below.

Two enhancers drive the expression of *wg* in the wing: the wing margin enhancer, which is activated by the Notch signaling pathway (Díaz-Benjumea and Cohen, 1995), and the *spd* enhancer, which drives *wg* expression in the IR (Neumann and Cohen, 1996). Previous results suggest that activation by the latter depends on a nonautonomous signal coming from the *vg*-expressing cells (del Álamo Rodríguez et al., 2002; Liu et al., 2000). *nab* co-expresses with *wg* in the wing margin and abuts on *wg* expression in the IR (Fig. 1F″). We therefore assumed that Nab should repress activation of the IR enhancer derepressed in *nab* clones. To obtain independent evidence that the IR enhancer is being activated, we tested whether other genes activated in the wing margin were activated in the *nab* clones. To this end, we analyzed *cut* (*ct*) and detected no ectopic expression (data not shown). It has been reported that *wg* expression can be detected in the wing after induction of cell death (del Álamo Rodríguez et al., 2004; Pérez-Garijo et al., 2004). To detect cell death in the *nab* clones we made use of an antibody that recognizes the activated form of Caspase 3 (Decay – Flybase) (Thornberry and Lazebnik, 1998), but detected no cell death (data not shown). These results, together with the pattern of expression (Fig. 1F′,F″), strongly suggest that the IR enhancer is being activated in the *nab* clones and, therefore, that in normal development Nab acts as a repressor of the *wg* IR enhancer in the distal wing. To confirm this hypothesis we expressed *nab* ectopically in the IR domain using the *nubGAL4* driver, which is expressed in a circular domain that includes the IR (Fig. 3B). In *nubGAL4>UASnab* larvae, expression of *wg* in the IR was lost, whereas its expression in the wing margin was not affected (Fig. 3C). We also generated clones of *nab*-expressing cells and found that *wg* expression was cell-autonomously lost in these clones, whereas *wg* expression in the wing margin was not affected (Fig. 3D,D′). In the light of these results, we propose that the function of *nab* in wing development is to delimit, distally, the domain of *wg* expression in the IR by inhibiting the mechanism of IR activation.

The Rn zinc-finger transcription factor is a potential partner of Nab in wing development

The mammalian Nab partner Egr1 contains an inhibitory domain called R1. When this domain is deleted the transcriptional activity of Egr1 increases 15-fold (Gashler et al., 1993; Russo et al., 1993). It has been shown that the R1 domain mediates a functional interaction between Nab and Egr1. Since no R1 domain has been identified in the fly genome and all the previously identified partners of Nab are Krüppel-type zinc-finger transcription factors, we examined, as potential Nab partners in the fly, transcription factors of the Krüppel family expressed in the wing. The gene *rn* encodes a Krüppel-like zinc-finger protein (St Pierre et al., 2002) that in the wing is expressed in a circular domain slightly broader than the *nab* domain (Fig. 1F″). The *wg* IR enhancer is only active in the cells that express *rn* and that do not express *nab*. Previous studies have shown that Rn is required for activation of the *spd* enhancer (del Álamo Rodríguez et al., 2002). Our results so far suggest that Rn could be a partner of Nab in the wing: first, *nab* is expressed in the *rn*-expressing cells that do not express *wg*; second, *nab* loss-of-function clones contain ectopic Wg; and third, *nab* misexpression represses the *wg* IR enhancer.

rn was also expressed in leg discs in a broad ring that corresponded to three tarsal segments (T2-4) (Fig. 4A). In *rn* mutant legs, the T2-4 tarsal segments were deleted (Fig. 4B). We would therefore expect that if Rn were a partner of Nab, ectopic expression of *nab* in the leg would generate the same phenotype as the lack of Rn. This proved to be the case when *nab* was misexpressed in the *rn* expression domain under the control of the *rnGal4* driver (Fig. 4C). The phenotype of these flies was indistinguishable from the *rn* mutant phenotype in both legs and wings (compare Fig. 4B with C). We examined the specificity of this interaction by rescuing the phenotype caused by *nab* misexpression by co-expressing *rn* (*rnGal4>UASrn+UASnab*), as well as by misexpressing *nab* in a broader domain using *Distal-less Gal4 (DllGal4)*, which is expressed from mid-tibia to distal leg (*DllGal4>UASrn*). In the first experiment, the phenotype was markedly reduced in both wing and leg (compare Fig. 4B with D), indicating that adding more *rn* antagonizes the inhibitory effect of *nab* misexpression. In the second experiment, although *nab* was misexpressed in a broader domain of the leg, the phenotype was unaltered and was restricted to the area where *rn* was expressed (compare Fig. 4B with E). Taken together, these results support a role for Rn as a potential partner of Nab and that Nab acts as co-repressor of Rn function in the cells where both are expressed. The *rn* mutant phenotype in the wing is caused by the loss of *wg* expression in the IR (del Álamo Rodríguez et al., 2002). We wanted to check whether *wg* expression was affected in *rnGal4 UASnab* and *rnGal4 UASnab UASrn* wings. In the first case, the IR was found to be absent (Fig. 4F), whereas in the second it was partially restored (Fig. 4G). In summary, the results presented here indicate that Nab functions in wing development by antagonizing the transcriptional activation function of Rn.

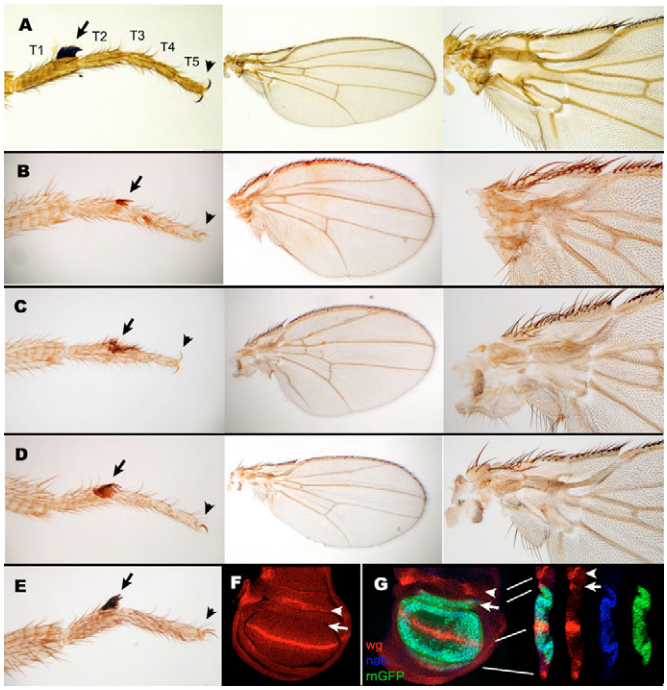

Fig. 4. Nab represses the transcriptional activity of Rn. (**A-E**) Leg, wing and a high magnification of the wing hinge area of wild-type (A), rn^{5}/rn^{20} (B), *rnGal4*/EP#13 (C), *UASrn*/+; *rnGal4*/EP#13 (D) and *DllGal4*/+; EP#13/+ (E) *Drosophila*. T1-5, tarsal segments. Arrows point to the sex combs at the distal end of the first tarsal segment (T1), and arrowheads point to the claw organs at the distal end of the leg. Note that the *rn* lack-of-function phenotypes (B) are identical to the *nab* misexpression phenotypes (C): tarsal segments (T2-T4) are lost or much reduced and the wing hinge is deleted. In D, the leg and wing phenotypes shown in C are partially rescued by overexpression of *UASrn*. Note that although *nab* is expressed in a broader domain in E than in C (from mid-tibia to the distal end of the leg) the phenotype is the same. (**F**,**G**) Wg antibody staining (red) in *rnGal4*/EP#13 (F) and *UASrn*/+; *rnGal4 UASGFP*/EP#13 (G) wing discs. Nab antibody staining is shown in blue and *rn* expression in green (GFP). Arrows, IR; arrowheads, OR. Note that in F, *wg* expression in the IR is lost. The simultaneous misexpression of Rn and Nab (G) partially rescues *wg* expression in the IR. In the right-hand panel, individual channels of a view in the *z*-axis are shown to indicate that in flies with this genotype, *nab* is co-expressed with *wg* in the IR.

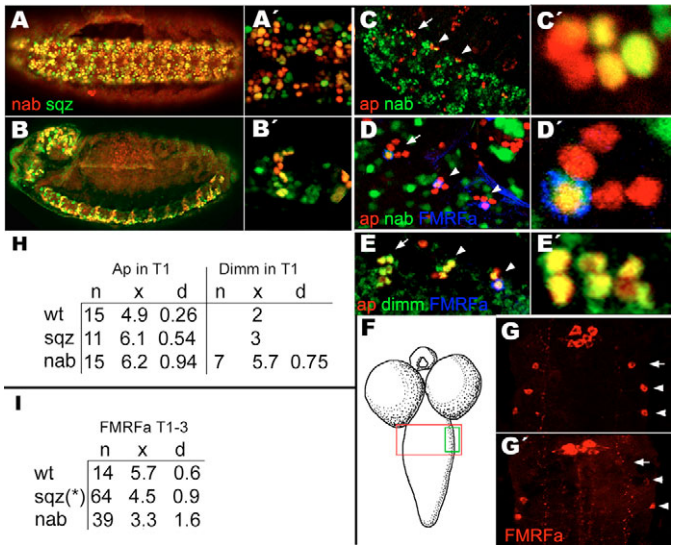

Fig. 5. Nab is a co-activator of Sqz in embryonic CNS development. (**A**,**B**) Ventral (A) and lateral (B) views of stage-17 *Drosophila* embryos showing *nab* (red) and *sqz* (green) expression patterns. (**A′**,**B′**) Magnified views of the segmental pattern showed in A,B. (**C**,**C′**) Stage-17 embryo stained with antibodies to Ap (red) and Nab (green). Three cells in the *ap* cluster express *nab* (C′, a magnified view of the T1 segments), one of them showing stronger expression. (**D**,**D′**) First instar larvae stained for Ap (red), Nab (green) and FMRFa (blue). Only the Tv neuron, detected by anti-FMRFa staining, expresses *nab* in the *ap* cluster (D′, a magnified view of the T1 segments). (**E**,**E′**) *nab* first instar larvae showing expression of *ap* (red), *dimm* (green) and *FMRFa* (blue). More cells occupy the *ap* cluster (T3 on the right is out of focus), *FMRFa* expression is mostly lost and is present only in T3, and additional cells express *dimm* (E′, a magnified view of the T1 segments). (**F**) The larval CNS. The green square indicates the area shown in D and E, the red square the area shown in G and G′. (**G**,**G′**) Wild-type (G) and *nab* mutant (G′) second instar CNS showing *FMRFa* expression. In all panels, arrows indicate the first (T1) thoracic segment and arrowheads the second (T2) and third (T3) thoracic segments. (**H**,**I**) Averages (x) and s.d. (d) of the number of cells that express *ap* and *dimm* (H) and *FMRFa* (I) in the T1 segments of wild-type, sqz^{lacZ} and nab^{SH143} embryos; n, number of larvae scored; *, data from Allan et al. (Allan et al., 2005).

The Sqz zinc-finger transcription factor is a potential partner of Nab in neuronal fate specification

Although *nab* loss-of-function alleles are larval lethal, the *rn*-null condition is homozygous viable. This suggests that Nab may have at least one other partner in embryonic development. Rn belongs to a conserved subfamily of zinc-finger proteins that include *Drosophila* Sqz (St Pierre et al., 2002), *C. elegans* LIN-28 (Rougvie and Ambros, 1995) and rat Ciz (Nakamoto et al., 2000). Sqz and Rn have two highly homologous domains: the zinc-finger domain (90% identity) and a 32 amino acid C-terminal domain (over 80% identity). *sqz* mutant alleles are larval lethal and have a motility defect. *sqz* is first required in embryonic CNS development to define the number of cells that express the LIM-homeodomain gene *ap* in the *ap* thoracic cluster of interneurons. Later on, it is also involved in the combinatorial code of transcription factors that specifies the fate of the Tv neuron in the *ap* cluster. The Tv neuron is distinguished from the rest of the neurons in the cluster by the fact that it contains the neuropeptide FMRFa [FMRFamide-related (Fmrf) – Flybase]. In *sqz* mutant embryos, additional *ap*-expressing neurons are generated and the Tv neuron is not specified as no *FMRFa* expression is found (Allan et al., 2003). To determine whether Nab is a co-factor of Sqz, we first analyzed the expression of *nab* and *sqz* in stage-17 embryos. We found that a subset of the CNS neurons that expressed *sqz* also expressed *nab*, whereas other neurons expressed either *sqz* or *nab* (Fig. 5A,B). Two or three neurons in the *ap* cluster of stage-17 embryos expressed *nab*, one typically at a relatively high level of expression (Fig. 5C,C′). By the first instar larval stage only one neuron in the *ap* cluster expressed *nab*. By double staining with anti-FMRFa and anti-Nab we were able to identify this as the Tv neuron (Fig. 5D,D′). At this stage, *sqz* was expressed at high levels in the Tv neuron and at low levels in two other neurons of the *ap* cluster. We next analyzed the expression of *ap* and *FMRFa* in *nab* mutant larvae. In first instar nab^{SH143} larvae, we found additional *ap*-expressing

neurons in the *ap* cluster (Fig. 5E,E′,H). In *nab*^{SH143} embryos, additional cells expressed the bHLH gene *dimmed* (*dimm*), as shown for *sqz* mutants (Hewes et al., 2003) (Fig. 5E,E′,H). We also examined the expression of *FMRFa* in the *ap* clusters of first instar larvae and found that FMRFa staining was lost or reduced in all the Tv neurons, mainly in the T1 cluster (Fig. 5G,G′,I). We conclude that lack-of-function alleles of *nab* and *sqz* generate the same embryonic phenotypes: the number of *ap*-expressing cells in the *ap* thoracic clusters is increased, additional *dimm*-expressing neurons are detected in the clusters, and Tv neuronal fate is absent. These results strongly suggest that, unlike the situation in imaginal disc development where Nab acts as a co-repressor of Rn, in CNS development Nab is required as a co-activator of Sqz.

Nab binds directly to Rn and Sqz via a conserved C-terminal domain

In order to analyze the molecular role of Nab as a co-factor of Sqz and Rn we performed GST pull-down assays. The complete *nab* cDNA was cloned in a glutathione S-transferase (GST) vector and incubated with radioactively labeled Rn or Sqz. Nab-GST, but not GST alone, readily retained [^{35}S]methionine-labeled Rn or Sqz (Fig. 6A). Rn and Sqz share a C-terminal domain of 32 amino acids with a homology greater than 80% (Fig. 6B,C). To further test whether this domain mediates the interaction with Nab, we repeated the pull-down assays with an [^{35}S]Rn in which the C-terminal domain was deleted. This deletion removes the region from amino acid 894 to the C-terminus (943) of the protein (Rn$^{\Delta 894}$). The ability of Nab-GST to retain the [^{35}S]Rn$^{\Delta 894}$ was notably reduced. We conclude that this conserved domain mediates the direct interaction of Nab with Rn and Sqz. To further test whether the C-terminal domain is sufficient to mediate this interaction, we incubated the Nab-GST with a 32 amino acid peptide containing just the sequence of the C-terminal domain. Nab-GST did not retain the peptide, indicating that the C-terminal domain is not sufficient to mediate Nab-Rn interaction (data not shown). As we have not identified other conserved domains between Rn and Sqz than the zinc-finger and C-terminal domains, we consider that either secondary structure or an additional modification of the protein is required for binding Nab. In order to provide an in vivo functional test of this hypothesis, the *rn*$^{\Delta 894}$ fragment was cloned into the pUAST vector and clones of cells misexpressing *UASrn*$^{\Delta 894}$ were generated (*Act>Gal4>UASrn*$^{\Delta 894}$). These clones activated the expression of *wg* throughout the wing pouch (Fig. 6D). As a control experiment, we misexpressed the wild-type version of *rn* (*Act>Gal4>UASrn*). These clones only activated *wg* expression in the wing hinge, outside of the *nab* expression domain (Fig. 6E).

Sqz competes with Rn in wing disc development

We wished to ascertain whether *sqz* is expressed in the wing disc. Because of the high degree of sequence homology between *rn* and *sqz* and to avoid interference with the *rn* mRNA present in the wing, we performed an in situ hybridization assay in *rn* mutant discs. *sqz* expression was detected by in situ hybridization in *rn*^{20} wing discs in a circular pattern that faded off laterally and whose proximal limit coincided with the limit of *vg* expression; this corresponded to the distal-most wing fold (Fig. 7A; compare with Fig. 1B). To determine whether *sqz* plays a role in wing development we analyzed the phenotype of *sqz* mutant clones induced by mitotic recombination. These clones had no adult phenotype, nor did they alter the expression of *wg*. Since Sqz and Rn share zinc-finger and the C-terminal domains and differ in their N-terminal domains, we wondered whether the roles of Sqz and Nab might be functionally redundant, both repressing Rn activity but by different mechanisms: Nab would repress Rn activity by direct binding to Rn protein as a co-repressor, whereas Sqz would compete for binding to the same DNA targets. To test this hypothesis, we analyzed the effect of misexpressing *sqz* in the *rn* expression domain. *rnGal4/UASsqz UASGFP* flies had small deletions of the wing hinge and shortened legs (Fig. 7B,C), a phenotype that resembles the *nab* misexpression and *rn* mutant phenotypes (compare with Fig. 4B,C). In agreement with these results, *wg* expression in the IR was downregulated in *rnGal4/UASsqz* wing discs (Fig. 7D). An alternative explanation for these results is that *sqz* activates *nab* expression, but we did not detect *nab* misexpression in this experiment. We suggest that there must be some functional redundancy, irrespective of whether Nab and Sqz play similar roles in the wing by repressing Rn activity, and this would account for the low penetrance of the *nab* mutant clones. Because *nab* and *sqz* map on different chromosome arms it was not possible to generate double-mutant clones. We therefore generated *nab*^{SH143} homozygous clones in a *sqz*^{lacZ}/+ background. In this situation, the frequency of clones misexpressing *wg* increased (38%;

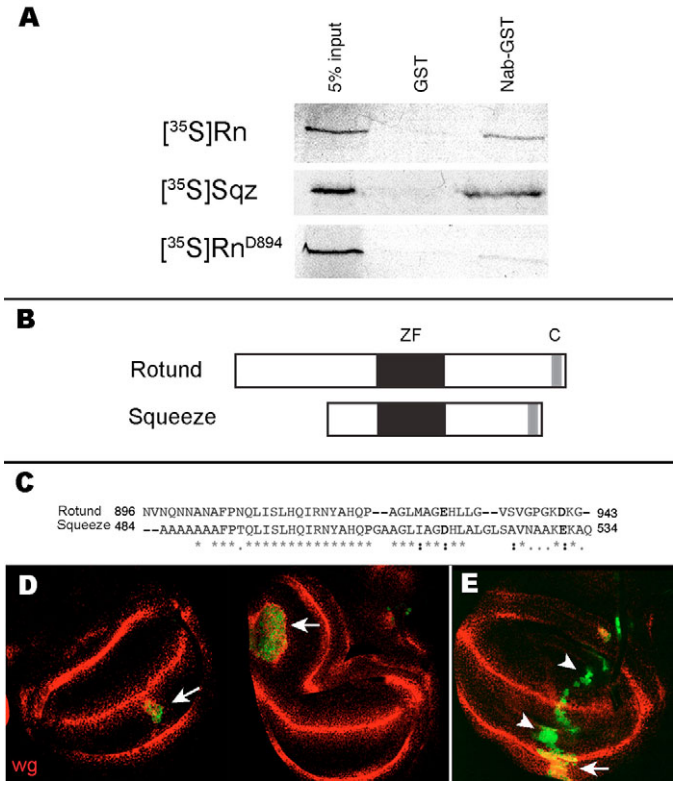

Fig. 6. *Drosophila* Nab physically interacts with Rn and Sqz via their conserved C-terminal domains. (**A**) Results of the GST-Nab pull-down assays. GST-Nab pulls down intact [^{35}S]Rn and [^{35}S]Sqz proteins, indicating direct binding. Binding was greatly reduced when the Rn protein lacked its C-terminal domain ([^{35}S]Rn$^{\Delta 854}$). Each experiment was repeated three times with identical results. (**B**) Comparison of Rn and Sqz proteins showing the two conserved domains: the zinc-finger domain (black box) with 90% homology, and the C-terminal domain (gray box). (**C**) Amino acid alignment of the conserved C-terminal domains of Rn and Sqz. *, identical; (:) conserved substitution; (.) semi-conserved substitution. (**D**) Clones of *rn*$^{\Delta 854}$ misexpression (green, *Act5C>Gal4>UASGFP UASrn*$^{\Delta 854}$). *wg* expression (red) is activated throughout in the wing pouch (arrows). (**E**) Clones of *rn* misexpression (green, *Act5C>Gal4>UASGFP UASrn*) only activate *wg* expression (red) in the wing hinge (arrows) where Nab is not expressed, but not in the wing pouch (arrowheads).

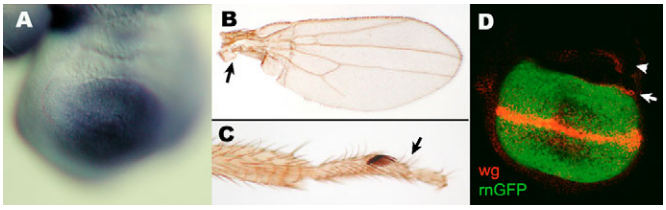

Fig. 7. Sqz represses Rn function in *Drosophila* wing and leg. (**A**) *sqz* expression in rn^{20} wing disc as monitored by in situ hybridization. The expression is strong in the center and fades off laterally. The proximal limit coincides with the distal-most fold that is the limit of *vg* expression. (**B-D**) Wing (B), leg (C) and wing disc (D) showing *wg* expression (red, D) in *rnGal4/UASsqz UASGFP*. The wing hinge is reduced and tarsal segments are deleted (B,C, black arrows). *wg* expression in the IR is much reduced (D, arrow) but expression in the OR is not affected (D, arrowhead; here partially out of focus).

total number of clones scored=55). We also noted that the clones that showed *wg* misexpression were preferentially located in the lateral-most regions of the wing, which correspond to the regions with the lowest levels of *sqz* expression. Taken together, these observations support the hypothesis that Nab and Sqz play similar roles in wing development: Nab as a co-repressor of Rn via its conserved C-terminal domain, and Sqz by competing with Rn for binding to its DNA targets. This function of Sqz would differ from its above-proposed role as a transcriptional activator in CNS development, and would not require Nab.

DISCUSSION

During development, regulatory genes are reiteratively activated at different times and in different tissues. The final outcome of their activation is strongly dependent on the developmental context, in particular on a set of factors that cooperate with or modulate the activity of other gene products. In this report we have functionally characterized one of these factors, the product of the *Drosophila* gene *nab*. This gene had previously been identified in other organisms, but its role in vertebrate development has only been recently elucidated. Nab proteins are involved in many different developmental events, most of them related to modulation of transcription by members of the Krüppel-like family of zinc-finger transcription factors. We have presented findings implicating *nab* in two important developmental events: proximodistal patterning of the wing disc and specification of the fate of a neuron in the ventral nerve cord of the embryo. Interestingly, these two developmental events are also mediated by Krüppel-like zinc-finger transcription factors encoded by the genes *rn* and *sqz*.

Genetic evidence suggests that Nab is a repressor of Rn function. Rn has previously been implicated in two aspects of imaginal disc development: activation of the *wg* IR enhancer in the wing hinge, and development of the tarsal segments of the leg. *nab* misexpression phenocopies *rn* loss-of-function in both instances. Moreover, misexpression of *nab* outside the normal *rn* expression domains produces no phenotype, and the phenotype of *nab* misexpression is substantially reduced by simultaneous overexpression of *rn*. Since *nab* is not expressed in the leg, we think that the only function of *nab* in imaginal disc development is in the distal wing. In this tissue, *rn* and *nab* are expressed in concentric circles under the control of the gene *vg*. Nevertheless, the expression domain of *rn* is slightly broader than that of *nab*. As a result, these two circles define a ring of cells that express *rn* but not *nab*, and it is precisely in these cells that the *wg* IR enhancer is activated. *nab* misexpression in these cells represses *wg*, whereas *nab* loss-of-function in its expression domain causes misexpression of *wg*. These findings, together with the misexpression experiments and the *nab* expression pattern, strongly support the proposed role of Nab as a co-repressor of Rn. Interestingly, *wg* is not misexpressed in all the *nab* loss-of-function clones. There are two possible reasons for this: first, Sqz might act as a competitor of Rn for DNA binding (see below); and second, *nab* loss-of-function might not be sufficient to produce a complete transformation of the hinge region and full activation of the IR enhancer. It is important to observe that both *rn* and *nab* are targets of *vg*, but they are expressed in circular domains of different sizes. This is probably due to *rn* being expressed earlier than *nab*. This difference in the expression domains permits and delimits the activation of *wg* to a narrow ring of cells, which is crucial for the correct development of the wing. Other genes that play important roles in wing development, such as *nub* (Ng et al., 1995), *dve* (Koelzer et al., 2003; Nakagoshi et al., 2002) and the *vg* quadrant enhancer (Williams et al., 1993), are also expressed at different times in late second and early third instar larvae. The mechanisms by which the expression of these genes is temporally controlled are not known.

We have also presented evidence that Nab is a co-activator of Sqz. This protein has been implicated in two aspects of embryonic ventral nerve cord development: first, in a Notch-dependent lateral inhibition mechanism that specifies the number of cells that express *ap* in the *ap* thoracic neuronal cluster; and second, in the specification of the Tv neuronal fate. *nab* and *sqz* are co-expressed in a subset of neurons, including several of the *ap* cluster, as well as the Tv neuron. *nab* loss-of-function embryos reproduce all the phenotypes of *sqz* loss-of-function embryos: additional cells express *ap* in the cluster and the Tv neuronal fate is lost. In addition, in both *nab* and *sqz* mutants an increased number of cells in the clusters express *dimm*. These findings indicate that Nab is required for all identified Sqz functions in embryonic development. Although we have focused our analysis on the *ap* thoracic cluster of neurons, both *sqz* and *nab* are co-expressed in many cells in the ventral nerve cord and others expressed either *sqz* or *nab*. But no other functions have been identified for *sqz* and it is not known how the expression of *sqz* is controlled. It has been reported that the expression of *nab* in the ventral nerve cord depends on the gene *castor* (Clements et al., 2003). Thus, the results presented here reveal greater complexity in the mechanisms of neuronal fate specification. The combined expression of genes, whose expression is individually activated by different mechanisms, is required to determine specific neuronal fates.

Sqz and Rn share two regions of strong homology: the zinc finger and a stretch of 32 amino acids in the C-terminal domain. By contrast, only *rn* has a long N-terminal domain. Our results indicate that the C-terminal domain mediates the interaction with Nab. By GST pull-down assays, we have shown that Nab binds to the full-length Rn protein but not to the $Rn^{\Delta 894}$ version, and clones of cells misexpressing $rn^{\Delta 894}$ activate *wg* expression in the *nab* expression domain. The similarity between *sqz* misexpression and *rn* loss-of-function phenotypes in leg and wing suggests that Sqz acts like a dominant-negative form of Rn in the *rn* domain: both proteins would bind to the same target sites but have opposite effects, and our results indicate that this role of Sqz would not require interaction with Nab. It is possible that the long N-terminal region of Rn is involved in interaction with other partners specifically required for Rn function.

Thus, our results indicate that Nab has a dual role as co-repressor of Rn and co-activator of Sqz. Previous studies in vertebrates also suggest that Nab is involved in both repression and activation of transcription. Co-repressors are proteins that bridge the interaction of the repressor with its target. Two main co-repressors have been

identified in *Drosophila*: Groucho and CtBP. CtBP binds to a specific sequence motif (P-DLS-K) that has been found in the sequence of three repressors present in the early embryo: Snail, Knirps and Krüppel. All three are zinc-finger transcription factors, and genetic evidence suggests that they all require CtBP to repress their targets (reviewed by Chinnadurai, 2002). Neither Rn nor Sqz have a CtBP-binding motif but we have identified one in Nab (P-DLS--K). Although the functional significance of this motif remains to be confirmed, we suggest that Nab is acting as a bridge between Rn and CtBP.

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