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

Javier Terriente Félix1, Marta Magariños2 and Fernando J. Díaz-Benjumea1,*

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- regulator 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 neuronal development, and that nab specifies the Tv neuronal fate in the 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).
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: nabSH143lacZ (Oh et al., 2003), nabGal4NP3537 and nabGal4NP1310 (Gal4 Enhancer Trap Insertion Database), sqαact20102, sqGα4 and UASsqz67.2 (Allan et al., 2005), Canton-S, y w118, w119; Δ2-3, Sb/TM2, UASGFP, y w118 hsFLP122; UbiGFP FRT80/TM2, UASGFP, (Bloomington Drosophila Stock Center), y w118 hsFLP122; Act5C>y > Gal4 UASGFP (Ito et al., 1997), DllGal4M022 and nabGal4M022 (Calleja et al., 1996), dpp190Gal4 (Wildler and Perrimon, 1995), rnt1 (Cousio and Bishop, 1998), rnt2 (Angel et al., 1989), UASrnr and rnrGal4 (St Pierre et al., 2002), UASvg (Kim et al., 1996), vgAC82 (Williams et al., 1993), y w118; CyO, EP#720; dpp122 (Roth et al., 1998).

EP#720 (inserted in a CyO chromosome) was used as a starting line and nabGal4M022 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 hsFLP122; UbiGFP FRT80 females and either nab FRT80/TM6, Tb or nab FRT80 sqαact20102/TM6, Tb males; and (2) y w hsFLP122; FRT82 UbiGFP females and FRT82 sqαact20102/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 w118 hsFLP122; Act5C>y > Gal4 UASGFP females were crossed either with UASvg, UASnab, UASSqz, UASrnr or UASnabrnt54 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-Fúené 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 nabSH143lacZ and nabGal4 lines NP3136 and NP3537.
Mapping of EP insertion lines
The EP element contains 14 Gal4 target sites and is described by Rothe (Rothe, 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 UASrn894
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. UASrn894 was generated by cloning the rr894 fragment into the puAST vector.

In vitro GST pull-down assays
For protein interaction assays we used the following procedure: [35S]-labeled Rn, Sqz and Rn894 were cloned into pCDNA3 tagged with Flag (Invitrogen) and transcribed/translated with the TNT Coupled Reticulocyte Lysate System (Promega) and [35S]-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. Rn894 was generated by in vitro site-directed mutagenesis (QuickChange 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 first detected at early third 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 nabSH143 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 nabSH143 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 larval lethality. We were able to rescue the lethality of the heterozygous combination nabSH143/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). 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 vg894 (vg) wing discs (Fig. 2A). However, nab was ectopically expressed in clones of vg-expressing cells (Fig. 2B). 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 vg and nab, 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.

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 nabGal4 driver, deletes the wing hinge (Fig. 1D). This phenotype is similar to that of wg894 alleles, which affect wg expression in the IR (Cousso 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)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
residual function. In order to isolate new mutant alleles of \( \text{nab} \) 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 \( \text{nab}^{b32} \). As we were not able to detect any Nab protein in clones of \( \text{nab}^{SH143} \) or \( \text{nab}^{b32} \) (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 \( \text{wg} \) in the wing: the wing margin enhancer, which is activated by the Notch signaling pathway (Díaz-Benjumea and Cohen, 1995), and the \( \text{spd} \) enhancer, which drives \( \text{wg} \) expression in the IR (Neumann and Cohen, 1996). Previous results suggest that activation by the latter depends on a nonautonomous signal coming from the \( \text{wg} \)-expressing cells (del Álamo Rodríguez et al., 2002; Liu et al., 2000). \( \text{nab} \) co-expresses with \( \text{wg} \) in the wing margin and abuts on \( \text{wg} \) expression in the IR (Fig. 1F). We therefore assumed that Nab should repress activation of the IR enhancer derepressed in \( \text{nab} \) clones. To obtain independent evidence that the IR enhancer is being activated, we tested whether other genes activated in the wing margin were also misexpressed in the \( \text{nab} \) clones. To this end, we analyzed cut (ct) and detected no ectopic expression (data not shown). It has been reported that \( \text{wg} \) expression can be detected in the wing after induction of cell death (del Álamo Rodríguez et al., 2004; Pérez-Garrio et al., 2004). To detect cell death in the \( \text{nab} \) 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 \( \text{nab} \) clones and, therefore, that in normal development Nab acts as a repressor of the \( \text{wg} \) IR enhancer in the distal wing. To confirm this hypothesis we expressed \( \text{nab} \) ectopically in the IR domain using the \( \text{nubGAL4} \) driver, which is expressed in a circular domain that includes the IR (Fig. 3B). In \( \text{nubGAL4} > \text{UASnab} \) larvae, expression of \( \text{wg} \) in the IR was lost, whereas its expression in the wing margin was not affected (Fig. 3C). We also generated clones of \( \text{nab} \)-expressing cells and found that \( \text{wg} \) expression was cell-autonomously lost in these clones, whereas \( \text{wg} \) expression in the wing margin was not affected (Fig. 3D, D’). In the light of these results, we propose that the function of \( \text{nab} \) in wing development is to delimit, distally, the domain of \( \text{wg} \) expression in the IR by inhibiting the mechanism of IR activation.

**The \( \text{Rn} \) 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 \( \text{rn} \) 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 \( \text{nab} \) domain (Fig. 1F). The \( \text{wg} \) IR enhancer is only active in the cells that express \( \text{rn} \) and that do not express \( \text{nab} \). Previous studies have shown that \( \text{rn} \) is required for activation of the \( \text{spd} \) enhancer (del Álamo Rodríguez et al., 2002). Our results so far suggest that \( \text{rn} \) and Nab could be a partner of Nab in the wing: first, \( \text{rn} \) is expressed in the \( \text{rn} \)-expressing cells that do not express \( \text{wg} \); second, \( \text{nab} \) loss-of-function clones contain ectopic \( \text{Wg} \); and third, \( \text{nab} \) misexpression represses the \( \text{wg} \) IR enhancer.

\( \text{rn} \) was also expressed in leg discs in a broad ring that corresponded to three tarsal segments (T2–4) (Fig. 4A). In \( \text{rn} \) mutant legs, the T2-4 tarsal segments were deleted (Fig. 4B). We would therefore expect that if \( \text{rn} \) were a partner of Nab, ectopic expression of \( \text{nab} \) in the leg would generate the same phenotype as the lack of \( \text{Rn} \). This proved to be the case when \( \text{nab} \) was misexpressed in the \( \text{rn} \) expression domain under the control of the \( \text{rnGal4} \) driver (Fig. 4C). The phenotype of these flies was indistinguishable from the \( \text{rn} \) 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 \( \text{nab} \) misexpression by co-expressing \( \text{rn} \) (\( \text{rnGal4} > \text{UASrn} + \text{UASnab} \)), as well as by misexpressing \( \text{nab} \) in a broader domain using Distal-less \( \text{Gal4} \) (\( \text{DilGal4} \)), which is expressed from mid-tibia to distal leg (\( \text{DilGal4} > \text{UASrn} \)). In the first experiment, the phenotype was markedly reduced in both wing and leg (compare Fig. 4B with D), indicating that adding more \( \text{rn} \) antagonizes the inhibitory effect of \( \text{nab} \) misexpression. In the second experiment, although \( \text{nab} \) was misexpressed in a broader domain of the leg, the phenotype was unaltered and was restricted to the area where \( \text{rn} \) was expressed (compare Fig. 4B with E). Taken together, these results support a role for \( \text{Rn} \) as a potential partner of Nab and that Nab acts as co-repressor of \( \text{Rn} \) function in the cells where both are expressed. The \( \text{rn} \) mutant phenotype in the wing is caused by the loss of \( \text{wg} \) expression in the IR (del Álamo Rodríguez et al., 2002). We wanted to check whether \( \text{wg} \) expression was affected in \( \text{rnGal4} \) \( \text{UASnab} \) and \( \text{rnGal4} \) \( \text{UASnab} \) \( \text{UASrn} \) 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 \( \text{Rn} \).
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 LIN-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 mutant larvae, we found additional ap-expressing
neurons in the ap cluster (Fig. 5E–E’). In nabSH143 embryos, additional cells expressed the bHLH gene dimmed (dimm), as shown for sqz mutants (Hewes et al., 2003) (Fig. 5E–E’). 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’). 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 [35S]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 [35S]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 (Rn894). The ability of Nab-GST to retain the [35S]Rn894 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 rn894 fragment was cloned into the pUAST vector and clones of cells misexpressing UASrn894 were generated (Act>Gal4>UASrn894). 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 rn850 wing discs in a circular pattern that faded off laterally and whose proximal limit coincided with the limit of wg 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 nabSH143 homozygous clones in a sqz854 Background. In this situation, the frequency of clones misexpressing wg increased (38%;
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 nab (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 RnA894 version, and clones of cells misexpressing RnA894 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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References


