Development 136, 3257-3266 (2009) doi:10.1242/dev.037242

dbx mediates neuronal specification and differentiation through cross-repressive, lineage-specific interactions with *eve* and *hb9*

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Individual neurons adopt and maintain defined morphological and physiological phenotypes as a result of the expression of specific combinations of transcription factors. In particular, homeodomain-containing transcription factors play key roles in determining neuronal subtype identity in flies and vertebrates. *dbx* belongs to the highly divergent H2.0 family of homeobox genes. In vertebrates, *Dbx1* and *Dbx2* promote the development of a subset of interneurons, some of which help mediate left-right coordination of locomotor activity. Here, we identify and show that the single *Drosophila* ortholog of *Dbx1/2* contributes to the development of specific subsets of interneurons via cross-repressive, lineage-specific interactions with the motoneuron-promoting factors *eve* and *hb9* (*exex*). *dbx* is expressed primarily in interneurons of the embryonic, larval and adult central nervous system, and these interneurons tend to extend short axons and be GABAergic. Interestingly, many Dbx$^+$ interneurons share a sibling relationship with Eve$^+$ or Hb9$^+$ motoneurons. The non-overlapping expression of *dbx* and *eve*, or *dbx* and *hb9*, within pairs of sibling neurons is initially established as a result of Notch/Numb-mediated asymmetric divisions. Cross-repressive interactions between *dbx* and *eve*, and *dbx* and *hb9*, then help maintain the distinct expression profiles of these genes in their respective pairs of sibling neurons. Strict maintenance of the mutually exclusive expression of *dbx* relative to that of *eve* and *hb9* in sibling neurons is crucial for proper neuronal specification, as misexpression of *dbx* in motoneurons dramatically hinders motor axon outgrowth.

KEY WORDS: *dbx*, Neuronal specification, *Drosophila*, *eve*, *hb9*, CNS

INTRODUCTION

As beautifully illustrated by Ramon y Cajal (Cajal, 1911), the nervous system is remarkable for its diversity of cellular phenotypes. In fact, recent physiological and expression studies suggest the presence of thousands of distinct neuronal subtypes in the mammalian brain (Masland, 2004; Nelson et al., 2006). The genetic and molecular basis through which individual or small groups of neurons adopt and maintain specific, often unique, morphological and physiological characteristics (neuronal specification) remains poorly understood.

Studies in mice, *Drosophila* and *Caenorhabditis elegans* highlight the importance of a large and growing number of transcription factors, which act in a combinatorial manner to govern neuronal specification (Thor and Thomas, 2002; Guillemot, 2007). Most of these transcription factors are expressed in complex, partially overlapping patterns of neurons, with the specific differentiated phenotype of a neuron being largely dictated by the precise complement of transcription factors it expresses. As detailed below, much of this work has focused on the specification of distinct motoneuron subtypes due in part to the relative ease of distinguishing individual or groups of motoneurons from each other based on axonal trajectory. Less is known about the factors that govern the specification of interneuron subtypes, even though interneurons outnumber motoneurons and can also be grouped based on morphology. For example, interneurons in the *Drosophila* central nervous system (CNS) outnumber motoneurons by about 10-fold, and can be roughly divided into intersegmental interneurons, which extend projections that span more than one segment, and local interneurons, which terminate their axons within the segment of origin.

Regulatory interactions, often cross-repressive in nature, between transcription factors that govern neuronal specification help ensure that individual neurons adopt the appropriate cellular phenotype. For example, in vertebrates cross-repressive interactions between the LIM-homeodomain (LIM-HD) proteins LIM-1 (Lhx1 – Mouse Genome Informatics) and ISLET1 (Isl1 – Mouse Genome Informatics) establish and maintain the non-overlapping expression of these proteins in lateral and medial neurons of the lateral motor column, respectively (Kania et al., 2000; Kania and Jessell, 2003). Lhx1 and Isl1 direct their respective groups of motoneurons to extend axons dorsally or ventrally into the limb mesenchyme in part by regulating the expression of the repulsive guidance receptor, Epha4 (Kania and Jessell, 2003). Mutually exclusive expression of two sets of transcription factors also defines distinct motoneuron subtypes in the *Drosophila* CNS. Here, all motoneurons that project axons to dorsal muscle targets express the homeodomain protein *even-skipped* (*eve*) (Landgraf et al., 1999; Landgraf et al., 2003; Landgraf and Thor, 2006). By contrast, most motoneurons that project axons to ventral muscles co-express the LIM-HD proteins Lim3 and Islet (Tailup – FlyBase), and the homeodomain proteins Hb9 (Exex – FlyBase) and Nkx6 (HGTX – FlyBase) (Thor and Thomas, 1997; Thor et al., 1999; Odden et al., 2002). Cross-repressive interactions between *eve* and *hb9/nkx6* help maintain these mutually exclusive expression patterns, and these genes in turn help direct the projection patterns of their respective motoneurons along different routes (Broihier and Skeath, 2002; Broihier et al., 2004; Landgraf et al., 1999).

As detailed above, functional studies are beginning to tease apart the transcriptional regulatory networks that govern the specification of postmitotic neurons. However, such analysis is complicated, at least in *Drosophila*, by the context-dependent nature of many of these regulatory interactions. For example, *eve* and *hb9* are each

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Accepted 24 July 2009

expressed in about a handful of distinct groups of neurons per hemisegment, yet *eve* is necessary to repress *hb9* expression in only two of the roughly 20 neurons that normally express *eve* (Broihier and Skeath, 2002). Similarly, *hb9* is required to inhibit *eve* expression in only one or two of its expressing neurons and is sufficient to repress *eve* in only a subset of Eve$^+$ neurons in the CNS.

Context-dependent regulatory interactions may reflect the underlying organization of the *Drosophila* CNS. Essentially all cells in the CNS derive from one of a limited set of stem-cell-like precursors, called neuroblasts (Doe and Goodman, 1993). Thirty neuroblasts develop per hemisegment, with each neuroblast dividing in a stem-cell-like manner to produce a largely invariant family or clone of neurons. Many different transcription factors are expressed within the neurons of any one lineage, with such factors exhibiting overlapping or mutually exclusive expression in the lineage in a transcription-factor-dependent manner. In addition, most transcription factors that govern neuronal specification are expressed in multiple different groups of neurons, with each group of neurons likely to derive from a different neuroblast lineage. Thus, context-dependent regulatory interactions between two such transcription factors may often reflect lineage-specific interactions, with these interactions preferentially occurring in lineages in which both factors are expressed versus lineages in which one or the other is expressed. It is presently difficult to test this model, as in general the individual lineages from which distinct groups of neurons marked by the expression of a given transcription factor arise have not been delineated.

In the vertebrate neural tube, *Dbx1* and *Dbx2*, two paralogous genes that encode homeodomain-containing proteins of the H2.0 class, are expressed within the p0, p1 and pD6 progenitor domains, with *Dbx1* expression nested within that of *Dbx2* (Pierani et al., 2001). The ventral limits of *Dbx1* and *Dbx2* expression are maintained via cross-repressive interactions with Nkx6-2 and Nkx6-1, respectively. Moreover, whereas *Dbx1* and *Dbx2* are expressed in neural progenitors but not postmitotic neurons, Dbx$^+$ progenitors give rise to v0, v1 and dl6 interneurons, with a subset of V0 interneurons expressing *Evx*, the vertebrate ortholog of *eve*, in a *Dbx1*-dependent manner. Functional analysis of V0 interneurons reveals that a subset of Evx-negative commissural interneurons makes inhibitory connections with contralateral motoneurons that innervate hindlimb muscles; *Dbx* function is required in these interneurons to regulate left-right alternation of motoneuron firing, required for proper walking movements (Lanuza et al., 2004).

Here, we report the identification and characterization of the *Drosophila dbx* gene. Lineage tracing reveals that many Dbx$^+$ neurons share a sibling relationship with Eve$^+$ or Hb9$^+$ motoneurons. The cellular phenotype of these pairs of sibling neurons is strikingly distinct – Dbx$^+$ interneurons are small and extend short axons; Eve$^+$ or Hb9$^+$ motoneurons are large and extend long axons. Notch/Numb-mediated asymmetric divisions establish the non-overlapping expression of *dbx* and *eve*, or *dbx* and *hb9*, within each pair of sibling neurons. Cross-repressive interactions between *dbx* and *eve*, and *dbx* and *hb9*, then help maintain the complementary expression profiles of these transcription factors in the relevant sibling neurons, a process crucial for the ability of these neurons to adopt and maintain their distinct differentiated phenotypes.

MATERIALS AND METHODS

Drosophila stocks

w^- as wild type, *P{SUPor-P}KG00100*, *Df(3L)R-G7* (Bloomington Stock Center), $hb9^{kk30}$, $hb9^{ij154}$, $Nkx6^{d25}$ (Broihier and Skeath, 2002; Broihier et al., 2004), $numb^2$ (Uemura et al., 1989), $sanpodo^{G104}$ (Skeath and Doe, 1998), *Df(2R)eve ΔRP2A/SM6a; RN2-GAL4; UAS-τlacZ, Df(2R)eve, ΔU-CQ/SM6a; U-CQ-GAL4; UAS-τlacZ, Df(2R)eve, ΔEL/SM6a; EL-GAL4; UAS-τlacZ* (Fujioka et al., 2003), elav-GAL4 (DiAntonio et al., 2001), UAS-*eve*; Chat-GAL4 (Li et al., 2000); Ddc-GAL4 (Yasuyama and Salvaterra, 1999), UAS-*hb9* (Broihier and Skeath, 2002), *y w hsflp,* UAS-tau-myc-GFP, Act5c(FRT.CD2)GAL4.

Antibody production and immunostaining

DNA encoding amino acids 538-741 of Dbx was cloned in-frame into pET29a (Novagen), and standard methods were used to express and purify the resulting protein fragment (Wang et al., 1989). Protein-specific antibody responses were mounted in guinea pigs [Pocono Rabbit Farm and Laboratory (www.prfal.com)], with the resulting anti-serum being specific for Dbx as it fails to detect antigen in the CNS of embryos, larvae and adults homozygous for $dbx^{\Delta 48}$ (Fig. 1H; data not shown).

Immunofluorescent and immunohistochemical stainings were performed as described by Patel (Patel, 1994), using the following antibodies: Guinea pig anti-Dbx (1:1500), rabbit anti-vGlut [1:5000 (Daniels et al., 2004)]; rabbit anti-Eve [1:3000 (Frasch et al., 1987)], rabbit and guinea pig anti-Hb9 (1:1500), rabbit anti-GAD [1:1000 (Kulkarni et al., 1994)], and rabbit anti-GFP (1:1000, Torey Pines). Monoclonal antibodies (obtained from the Developmental Studies Hybridoma Bank, Iowa): 9E10 (Myc 1:10,), 1D4 (FasII; 1:10), BP102 (1:10), 4D9 (engrailed/invected; 1:10), 7E8A10 (ELAV; 1:10). Fluorescent secondary antibodies were obtained from Molecular Probes (Alexa 488, Alexa 594) or Jackson Labs (Cy5) and used at a 1:200 dilution. Immunohistochemical stainings were carried out using the Vectastain ABC Elite kit following manufacturers' protocol (Vector Labs).

Transgenic flies

To generate a full-length *UAS-dbx* transgene, we amplified the entire *dbx* coding region from cDNA clone LP21251 (DGRC) and cloned it into the *Bgl*II-*Xho*I restriction sites of pUAST (Brand and Perrimon, 1993). Construct integrity was verified by sequencing. Standard P element-based transformation methods were used to obtain integrated lines of P[UAS-*dbx*].

Generation of a deletion allele of *dbx*

$dbx^{\Delta 48}$ was generated by standard P element mobilization methods (Salz et al., 1987) using the KG00100 P element, inserted 1.1 kb upstream of the start of *dbx* transcription. From over 70 excision lines, one line ($dbx^{\Delta 48}$) was identified that lacked *dbx* expression in homozygous mutant embryos. PCR and sequence analysis identified a 2.2 kb deletion that removes the *dbx* transcription start site (see Fig. 6A). This 2.2 kb deletion is unlikely to affect nearby genes, as the closest known gene resides 11 kb from *dbx*. Sequences that flank the deletion: 5′, TGCTTCACGGATTAGAT-GAAGCAGTTGAAA; 3′, GAAATATAGAGCAGCCAGCTCCGTCGC-GTT.

$dbx^{\Delta 48}$ behaves as a genetic null allele as $dbx^{\Delta 48}$ homozygous embryos exhibit an essentially identical phenotype with respect to retention of *eve* expression in RP2 sib as embryos homozygous for *Df(3L)R-G7* (a deficiency that removes *dbx* and surrounding genes), or *Df(3L)R-G7*/ $dbx^{\Delta 48}$ trans-heterozygotes (data not shown).

Lineage tracing

We used a modified FLP/FRT system to trace the lineage and projections of Dbx$^+$ neurons (Harrison and Perrimon, 1993). We induced Flp-out events in 3- to 6-hour- or 6- to 9-hour-old embryos heterozygous for the FLP-out GAL4 cassette, UAS-tau-myc-GFP and hs-flp by incubating embryos in a 32°C water bath for 7 minutes. Embryos were aged to the end of embryogenesis, fixed and stained. To identify neuroblast lineages containing Dbx$^+$ neurons we labeled embryos for Dbx, GFP to identify clones, and FasII to label the axon scaffold. To identify sibling relationships between Dbx$^+$, Eve$^+$ and Hb9$^+$ neurons we labeled embryos for Dbx, Eve and GFP, or Dbx, Hb9 and GFP.

RESULTS

Spatial and temporal analysis of *Drosophila* Dbx expression

We carried out a bioinformatics screen of the *Drosophila* genome for uncharacterized orthologs of vertebrate genes known to regulate neuronal-cell-fate specification. Reciprocal Blast analysis identified CG42234 as the *Drosophila* gene with greatest sequence similarity to *Dbx1* and *Dbx2*, the second best hit for both genes being H2.0, the vertebrate ortholog of which is *Hlx* (Bates et al., 2005) (Fig. 1A). In *Drosophila* embryos, CG42234, like *Dbx1* and *Dbx2* in vertebrates, is expressed in the developing CNS, whereas H2.0 expression is restricted to non-neuronal tissues in *Drosophila*, as has been shown for Hlx in vertebrates (Bates et al., 2005; Seo et al., 1999) (and data not shown). Thus, the *Drosophila* genome contains a single *dbx* gene, CG42234, hereafter referred to as *dbx*.

To follow *dbx* expression in the *Drosophila* CNS, we generated Dbx-specific antibodies. The *dbx* mRNA and protein expression profiles mirrored each other, and revealed that *dbx* was expressed in a dynamic pattern of neuroblasts, intermediate precursors termed ganglion mother cells (GMCs), and postmitotic cells (Fig. 1; see Fig. S1 in the supplementary material). *dbx* was first expressed in a few neuroblasts and GMCs in gnathal segments during early stage 11 (not shown). Shortly thereafter, *dbx* expression initiated in multiple cells within each segment as well as in the brain (Fig. 1B); based on size, relative sub-epidermal position and marker gene expression, Dbx$^+$ cells appeared to identify a stereotyped subset of neuroblasts, GMCs and postmitotic cells (Figs 1 and 2; see Fig. S1 in the supplementary material). After stage 12, most Dbx$^+$ cells were postmitotic, with some cells expressing *dbx* transiently and others retaining *dbx* expression throughout embryogenesis. At the end of embryogenesis, approximately 33 Dbx$^+$ cells resided per thoracic hemisegment and about 20 Dbx$^+$ cells per abdominal hemisegment (Fig. 1D,E). Gnathal segments contained even more Dbx$^+$ cells (Fig. 1B,C). Such segment-specific differences in *dbx* expression probably reflect homeotic gene input.

Many CNS neurons retained *dbx* expression into larval stages, during which time additional Dbx$^+$ neurons arose in the nerve cord (Fig. 1F; see Fig. S1 in the supplementary material). Dbx$^+$ neurons were also found in the CNS of adults (Fig. 1G; see Fig. S1 in the supplementary material). We also observed a few Dbx$^+$ neuroblasts in thoracic segments of third instar larvae. These Dbx$^+$ neuroblasts appeared to bud off Dbx$^+$ GMCs and neurons, as small clusters of Dbx$^+$ neurons resided immediately adjacent to these neuroblasts (see Fig. S1 in the supplementary material). Thus, many neurons maintained *dbx* expression for extended periods of time in larvae, pupae and adults, consistent with *dbx* helping to maintain the specific differentiated phenotype of these neurons.

dbx identifies a novel subset of interneurons many of which are GABAergic

Double-label studies with molecular markers that label all neurons [ELAV (Robinow et al., 1988)], all glia [Repo (Xiong et al., 1994)] or most motoneurons [Eve/Hb9 (Landgraf et al., 1999; Broihier and Skeath, 2002)] revealed that essentially all postmitotic Dbx$^+$ cells were interneurons (Fig. 2). For example, by the end of embryogenesis all postmitotic Dbx$^+$ cells expressed ELAV but not Repo (Fig. 2; see Fig. S1 in the supplementary material). In addition, no Dbx$^+$ neurons expressed *eve* or *hb9* (Fig. 2), which together marked most embryonic motoneurons, identifying Dbx$^+$ neurons as interneurons. Together with the data detailed above, these results indicate that as in vertebrates, *dbx* expression labels progenitor cells of interneurons, and in contrast to vertebrates many interneurons maintain *dbx* expression in flies.

Dbx$^+$ neurons identified a largely uncharacterized population of interneurons, as we observed little if any co-expression between *dbx* and markers of different subsets of interneurons, such as *engrailed*, *dachshund*, *nmr-1* (*H15* – FlyBase), *nmr-2* (*mid* – FlyBase) and *eagle* (not shown). However, many Dbx$^+$ interneurons are GABAergic, as significant co-expression occurred between Dbx and glutamic acid decarboxylase (GAD), a marker of GABAergic

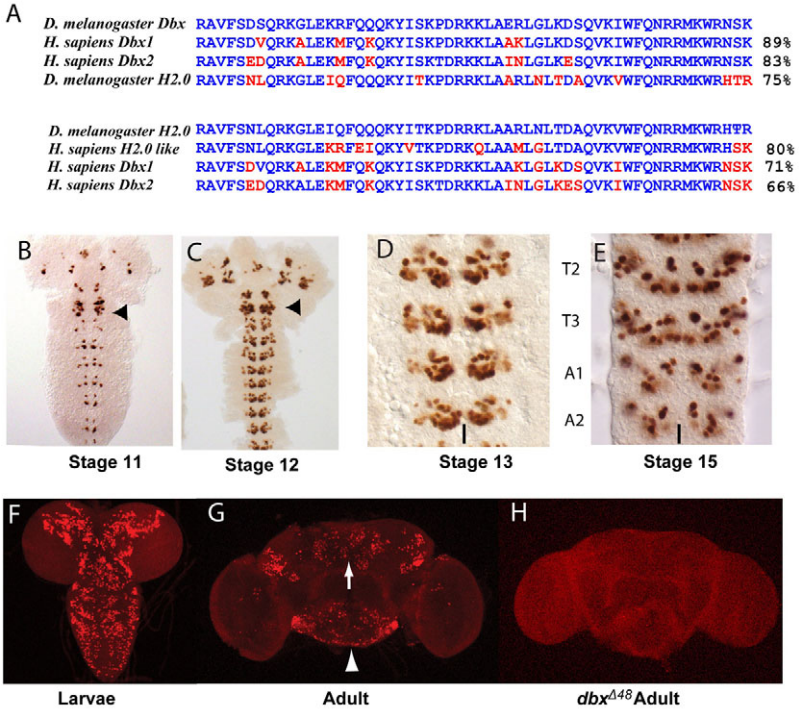

Fig. 1. Characterization of *dbx* expression in the *Drosophila* CNS. (**A**) Alignment of the amino acid sequence of the homeodomains from *Drosophila* and human Dbx and H2.0 proteins; the red letters identify amino-acid differences between *Drosophila Dbx* and the other proteins. (**B-H**) Dbx expression in the embryonic, late third instar and adult CNS. (B,C) Dbx is expressed in progressively more cells in the CNS from stage 11 (B) to stage 12 (C); gnathal segments contain the most Dbx$^+$ cells in the CNS (arrowheads). (D,E) High-magnification views of the embryonic CNS indicate that *dbx* expression is largely restricted to postmitotic cells during stages 13-15, with more Dbx$^+$ neurons found in thoracic versus abdominal segments (compare T2-T3 with A1-A2). (F) Additional Dbx$^+$ neurons arise in the larval CNS relative to the embryo. (G) Dbx is expressed in the sub-esophageal ganglia (arrow) and central part (arrowhead) of adult brains. (H) *dbx*$^{\Delta 48}$ mutant adult flies lack Dbx protein. Anterior: up.

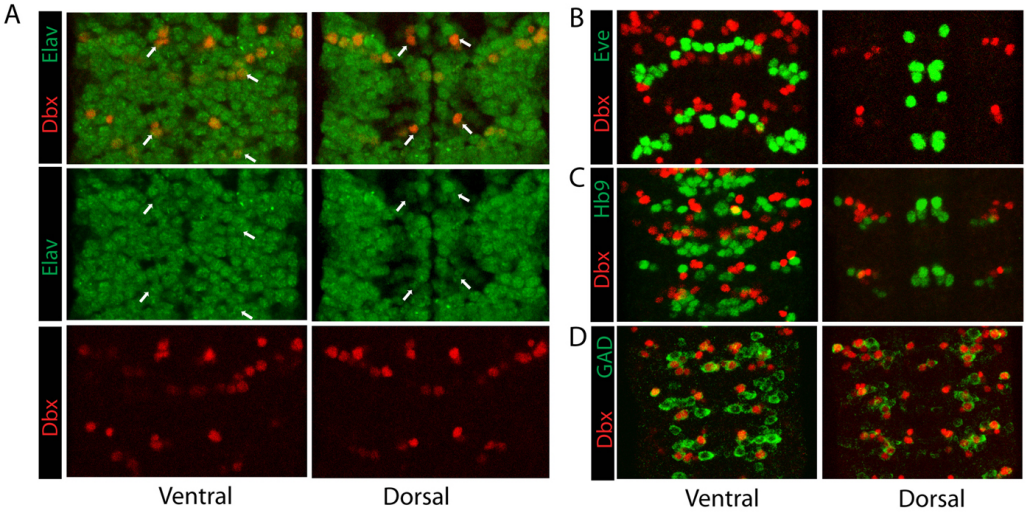

Fig. 2. A subset of interneurons expresses *dbx*. Wild-type stage 15 *Drosophila* nerve cords co-labeled for indicated proteins. (**A**) All Dbx$^+$ neurons express ELAV, identifying them as neurons. (**B**,**C**) Dbx is expressed in a mutually exclusive set of neurons relative to Eve (B) and Hb9 (C). (**D**) Many Dbx$^+$ neurons express GAD, a marker of GABAergic neurons (see also Fig. S2 in the supplementary material). Anterior: up.

neurons (Fig. 2; see Fig. S2 in the supplementary material) (Jackson et al., 1990). By contrast, no Dbx$^+$ interneurons appeared to be seratonergic, dopaminergic or glutamatergic by the first larval instar stage, and only one Dbx$^+$ interneuron was cholinergic (see Fig. S2 in the supplementary material). As GABAergic interneurons are inhibitory in *Drosophila* as in vertebrates (Lee et al., 2003), we infer that many embryonic Dbx$^+$ cells are inhibitory GABAergic interneurons.

Dbx$^+$ interneurons derive from five neuroblast lineages

dbx expression thus marks a poorly defined population of interneurons. To characterize Dbx$^+$ interneurons in greater detail we used a modified version of the FLP/FRT system to map their lineage (Harrison and Perrimon, 1993) (see Materials and methods). We generated random clones of tau-myc-GFP$^+$ cells in otherwise wild-type embryos, and then screened for GFP$^+$ lineage clones that contain Dbx$^+$ neurons (see Table S1 in the supplementary material). Comparison of the morphology and location of such clones relative to the location and morphology of identified neuroblast lineages as determined by Dil-labeling (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999) enabled mapping of essentially all Dbx$^+$ neurons in abdominal segments to five neuroblast lineages (Fig. 3). This approach also revealed that at least two additional neuroblasts, preliminarily identified as NBs 2-4 and 6-4 (not shown), produced Dbx$^+$ neurons in thoracic segments, and that many Dbx$^+$ neurons exhibited at most short axonal projections.

The NB4-2 lineage produced four small Dbx$^+$ interneurons in abdominal segments and seven Dbx$^+$ neurons in thoracic segments. This lineage contained the CoR motoneurons, which projected axons out of the segmental nerve (SN), and the Eve$^+$ RP2 motoneuron, which projected its axon out of the inter-segmental nerve (ISN) (Fig. 3A; see Fig. S4 in the supplementary material) (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). The four Dbx$^+$ neurons included RP2 sib and at least two CoR sibs (see below, Fig. 4). These Dbx$^+$ neurons extended at most short axons, consistent with previous observations that all interneurons in this lineage are local interneurons (Schmid et al., 1999).

The NB5-2 lineage produced two medial Dbx$^+$ neurons within a large family of over 20 cells. Axons from this clone crossed the midline via the anterior and posterior commissures (Fig. 3B), with a single motoneuron, the AC motoneuron, projecting its axon across the midline and out of the SNb nerve branch (not shown: the motoneuron axon is not visible in Fig. 3B due to removal of confocal sections that complicated the merged image).

The NB6-1 lineage produced two Dbx$^+$ neurons in abdominal segments and four Dbx$^+$ neurons in thoracic segments at the end of embryogenesis (Fig. 3C). Abdominal NB6-1 clones analyzed at stage 13 included three additional Dbx$^+$ neurons as well as *dbx* expression in NB6-1 (data not shown). Thus, some cells expressed *dbx* transiently in this lineage. The neurons that retained *dbx* expression appeared to be late-born neurons, as they resided at the extreme ventral surface of the NB6-1 family of neurons.

The NB6-2 lineage contained three Dbx$^+$ neurons in abdominal segments and five Dbx$^+$ neurons in thoracic segments. Axonal projections from these clones crossed the midline as two bundles through the posterior commissure; these bundles arched anteriorly, mirroring the morphology of NB6-2 clones (Fig. 3D) (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). These neurons expressed *dbx* at levels below those observed in other lineages.

The NB7-1 lineage produced eight small Dbx$^+$ neurons in thoracic and abdominal segments, with many of these neurons residing next to the well-characterized and lineally related Eve$^+$ U motoneurons (Fig. 3E) (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). As detailed below (Fig. 4), the close proximity of U motoneurons and Dbx$^+$ neurons reflect sibling relationships in multiple cases. In accord with the previous observation that all interneurons in this lineage are local interneurons (Schmid et al., 1999), all Dbx$^+$ neurons in this lineage extended at most short axons.

Many Dbx$^+$ interneurons share a sibling relationship with motoneurons

Expression and additional lineage-tracing studies confirmed that the juxtaposition of Dbx$^+$ interneurons next to Eve$^+$ or Hb9$^+$ motoneurons reflects sibling relationships in many cases. For example, double-labeling studies in wild-type embryos revealed transient co-expression of *eve* and *dbx* in RP2 sib and in the siblings of the U1-U3 motoneurons immediately after their birth from Eve$^+$ GMCs (Fig. 4A,B). Whereas *eve* expression was quickly extinguished in these cells, *dbx* expression was maintained in RP2 sib until the end of embryogenesis, and in the U1-U3 sibs for an extended period of time (the presence of eight Dbx$^+$ neurons in the 7-1 lineage rendered it difficult to follow individual Dbx$^+$ neurons throughout embryogenesis).

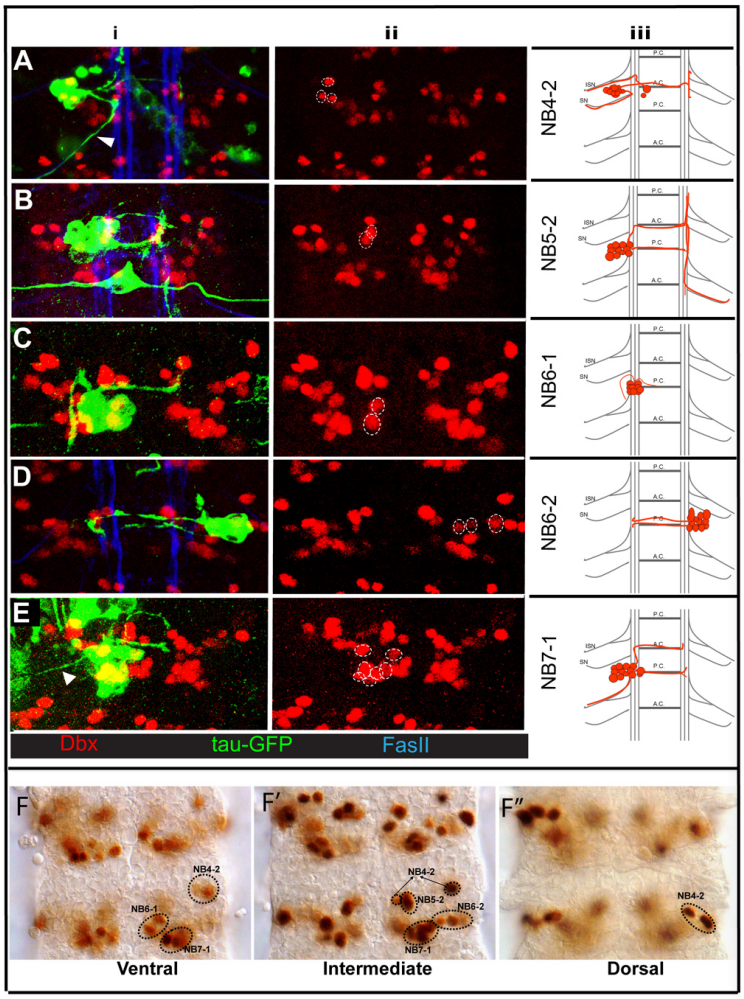

Fig. 3. Dbx$^+$ neurons derive from five neuroblast lineages in abdominal segments. (**A-E**) Abdominal segments of late-stage wild-type embryos containing lineage clones labeled for Dbx (red), FasII (blue) and GFP (green) (i). (ii) Dbx$^+$ neurons contained within clones are marked by white dotted circles. (iii) Schematic representations of entire clones of neurons produced from individually identified neuroblasts, modeled on results from DiI labeling studies (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). (A) NB4-2 lineage clone that contains three Dbx$^+$ neurons [RP2-RP2 sib neurons are not labeled in this clone (see Fig. S1 in the supplementary material)]; the arrowhead indicates motor axon projections of CoR motoneurons. (B) A NB5-2 clone contains two Dbx$^+$ neurons; neurons in this clone project axons via the anterior and posterior commissures. (C) An abdominal NB6-1 clone that contains two Dbx$^+$ neurons; neurons in this clone project axons ipsilaterally and contralaterally via the posterior commissure. Dbx$^+$ neurons reside at ventral surface of the clone. (D) A NB6-2 clone contains three Dbx$^+$ neurons; neurons in this clone project axons contralaterally as two bundles through the posterior commissure. (E) A NB7-1 clone with seven Dbx$^+$ neurons and the U motoneurons, which project axons ipsilaterally out the ISN root (arrowhead). (**F-F″**) Ventral, intermediate and dorsal views of Dbx$^+$ neurons in abdominal segments with the cell lineage of each set of neurons indicated.

To ascertain if all Eve$^+$ U motoneurons share a sibling relationship with Dbx$^+$ neurons, we generated two-cell clones marking each U motoneuron and its sibling (Fig. 4B,C). Each U motoneuron can be unambiguously identified based on its relative position (Pearson and Doe, 2003). Thus, this approached revealed that, like RP2, the U1 and U5 sibs expressed *dbx* from shortly after their birth until the end of embryogenesis. Similarly, the U2 and U3 sibs expressed *dbx* from their birth until stage 14, at which point they begin to downregulate *dbx* expression. Although the U4 sib did not express *dbx* after stage 15, we did not obtain two-cell U4 clones before stage 15. Thus, the U4 sib may transiently express *dbx* (Fig. 4C). We conclude that most of the sibling interneurons of the RP2 and U motoneurons express *dbx* transiently or continuously during embryogenesis.

The generation of many two-cell clones within the NB4-2 lineage that contained one Hb9$^+$ CoR motoneuron and one Dbx$^+$ interneuron confirmed sibling relationships between Dbx$^+$ interneurons and Hb9$^+$ motoneurons (Fig. 4D). In contrast to U motoneurons, individual CoR motoneurons could not be identified unambiguously by position. Thus, our clones may not have marked all CoR motoneurons. However, we did identify four five-cell clones that contain three Hb9$^+$ CoR motoneurons and two Dbx$^+$ interneurons (Fig. 4E), pointing to a one-to-one sib relationship between at least two CoR motoneurons and Dbx$^+$ interneurons. The 5-2 lineage is the only other lineage in abdominal segments that contained Dbx$^+$ interneurons and an Hb9$^+$ motoneuron. Although we were unable to create two-cell clones containing this motoneuron, sublineage clones are consistent with a sibling relationship between this motoneuron and a Dbx$^+$ interneuron. Thus, our lineage and expression studies reveal that *dbx* expression labels the sibling interneuron of at least seven motoneurons in each abdominal segment; all of these interneurons are local interneurons that extend short axons or no axons.

Analysis of *dbx*, *eve* and *hb9* expression in embryos homozygous mutant for *numb* or *spdo* – two genes that exert opposite effects on Notch/Numb-mediated asymmetric divisions – confirmed the observed sibling relationships between Dbx$^+$ neurons and Eve$^+$ or Hb9$^+$ motoneurons. For example, loss of *numb* function led to reciprocal effects on *eve* and *dbx* expression in the 4-2 and 7-1 lineages, with duplication of Eve$^+$ U motoneurons occurring at the expense of Dbx$^+$ interneurons in the 7-1 lineage, whereas RP2 sib (Dbx$^+$) was duplicated at the expense of the Eve$^+$ RP2 in the 4-2 lineage (Fig. 5). Removal of *spdo* function elicited the reciprocal effect on *dbx* and *eve* expression in these lineages. Similarly, loss of *numb* (or *spdo*) function led to reciprocal effects on *dbx* and *hb9* expression in the 4-2 and 5-2 lineages (Fig. 5). The *spdo* and *numb* mutant phenotypes each displayed essentially 100% penetrance and expressivity with respect to the groups of neurons assayed (Table 1). We conclude that Notch/Numb-mediated asymmetric divisions direct the expression of *dbx* and *eve* (or *dbx* and *hb9*) to opposite siblings within multiple sib pairs, and in so doing establish the non-overlapping nature of *dbx* and *eve*, and *dbx* and *hb9* expression in different pairs of sibling neurons.

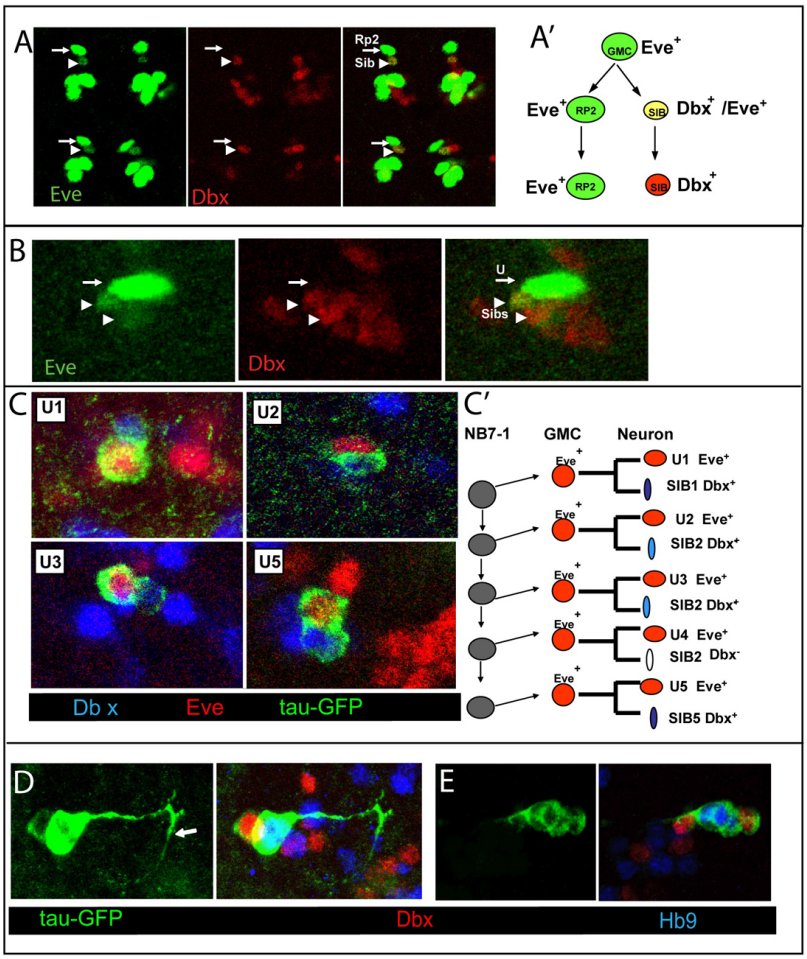

Fig. 4. Dbx$^+$ neurons share sibling relationships with the RP2, U and CoR motoneurons. Wild-type embryos labeled for the indicated proteins and GFP (to mark lineally related cells). (**A**) At stage 12, RP2 expresses *eve* (arrows), and RP2 sib expresses *eve* and *dbx* (arrowheads). (**A′**) Schematic of *eve* and *dbx* expression in the RP2 and RP2 sib neurons. (**B**) During stage 11 *eve* is expressed in U motoneurons (arrow) and transiently in the U sib neurons (arrowheads). (**C**) Two-cell GFP$^+$ sibling clones in stage 13-15 embryos show that U1, U2, U3 and U5 share sibling relationships with Dbx$^+$ neurons; note Dbx is only transiently expressed in U2 and U3 sibling interneurons. (**C′**) Schematic of *eve* and *dbx* expression in U/Usib neurons. (**D**) Two-cell GFP clone that contains one Hb9$^+$ CoR motoneuron and one Dbx$^+$ interneuron; arrow indicates motor axon of CoR motoneuron, note absence of axon extension from Dbx$^+$ interneuron. (**E**) Five-cell GFP$^+$ clone that contains three Hb9$^+$ CoRs motoneurons and two Dbx$^+$ interneurons. For the individual channels for the proteins shown in panels C-E, see Fig. S3 in the supplementary material.

dbx exhibits cross-repressive regulatory interactions with *eve* and *hb9*

The transient co-expression of *dbx* and *eve* in RP2 sib and U sib interneurons raises the possibility that negative regulatory interactions between *dbx* and *eve* help maintain the non-overlapping nature of the expression profiles of these factors in distinct sets of sibling neurons. To assay for regulatory interactions between *dbx*, *eve* and *hb9*, we created a null allele of *dbx* via imprecise P element excision and a full-length UAS-*dbx* transgene (see Materials and methods). As detailed below, systematic loss of function and misexpression studies uncovered cross-repressive interactions between *dbx* and *eve*, and *dbx* and *hb9*, that were largely specific to those lineages that produce sib pairs of Dbx$^+$/Eve$^+$ or Eve$^+$/Hb9$^+$ neurons (Fig. 6; Table 2 provides penetrance and expressivity data for each misexpression background).

In embryos homozygous mutant for $dbx^{\Delta 48}$ we observed inappropriate retention of *eve* expression in the normally Dbx$^+$ RP2 sib (Fig. 6B,C). A majority of thoracic (58%; *n*=90), but a minority of abdominal (21%; *n*=300), segments exhibited the RP2 sib phenotype. Conversely, *elav-Gal4*-mediated misexpression of *dbx* in all postmitotic neurons was sufficient to repress *eve* expression in the RP2 and U motoneurons but not in aCC/pCC or the EL neurons, the

Table 1. *numb* and *spdo* exert opposite effects on Dbx$^+$ and Eve$^+$ and on Dbx$^+$ and Hb9$^+$ sibling neurons

	Genotype			
	numb^2		*sanpodo*^{G104}	
Specific set of neurons assayed	Effect	% Expressivity (*n*)	Effect	% Expressivity (*n*)
NB 4-2 lineage				
Eve$^+$ RP2 motoneurons	Loss	99.1 (234)	Duplication	95.1 (183)
Hb9$^+$ CoR motoneurons	Loss	96.1 (180)	Duplication	97.7 (132)
Dbx$^+$ RP2 sib and coR sibs	Duplication	99.0 (103)	Loss	98.9 (176)
NB 7-1 lineage				
Eve$^+$ U motoneurons	Duplication	100 (216)	Loss	100 (192)
Dbx$^+$ U sib neurons	Loss	100 (110)	Duplication	100 (176)

Percentage expressivity refers to the percentage of hemisegments that exhibit a complete or near complete loss or duplication of the indicated neurons. The number of hemisegments scored (*n*) is indicated in parentheses.

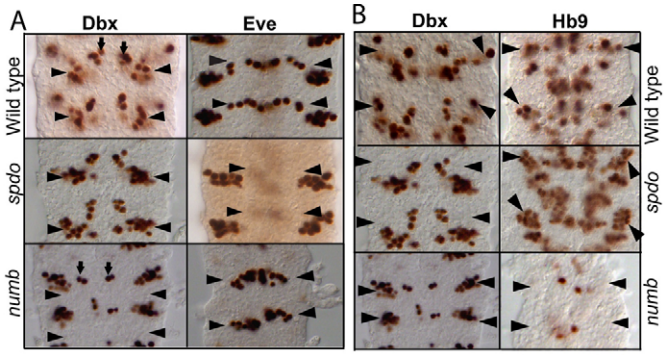

Fig. 5. Notch/Numb-mediated asymmetric divisions exert opposite effects on the expression of *dbx* and *eve*, and *dbx* and *hb9*, in sibling neurons. (**A**) The arrowheads mark Eve$^+$ U neurons and Dbx$^+$ neurons in the 7-1 lineage; the arrows mark RP2 sib. In the 7-1 lineage Dbx$^+$ interneurons are duplicated at the expense of Eve$^+$ U motoneurons in *spdo*^{G104} mutant embryos; conversely, in *numb*^{2} germline clones, Eve$^+$ U motoneurons are duplicated at the expense of their Dbx$^+$ siblings. RP2 sib is also duplicated at the expense of RP2. (**B**) The arrowheads point to Dbx$^+$ CoR sib interneurons or Hb9$^+$ CoR motoneurons. In *spdo* mutant embryos Hb9$^+$ CoRs are duplicated at the expense of Dbx$^+$ interneurons; in *numb*^{2} germline clones Dbx$^+$ interneurons are duplicated at the expense of Hb9$^+$ CoR motoneurons.

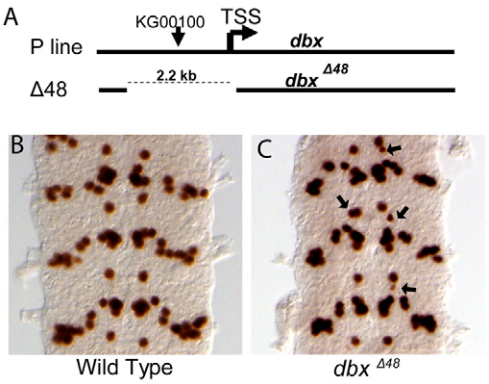

Fig. 6. Characterization of the *dbx*$^{\Delta 48}$ mutant allele. (**A**) *dbx*$^{\Delta 48}$ contains a 2.2 kb deletion that removes the *dbx* transcription start site (TSS). (**B**,**C**) Stage 15 wild-type and *dbx*$^{\Delta 48}$ mutant embryos are stained for Eve. The arrows in C identify RP2 sib cells inappropriately expressing *eve*.

other Eve$^+$ neurons in the CNS (Fig. 7A,B; Table 2). Thus, *dbx* is necessary and sufficient to repress *eve* in the RP2/RP2 sib pair of sibling neurons, and sufficient but not necessary to repress *eve* expression in U motoneurons, revealing that the ability of *dbx* to repress *eve* is restricted to those lineages that produce Eve$^+$ and Dbx$^+$ sibling neurons.

In reciprocal experiments, embryos that lacked *eve* function specifically in the CNS (Fujioka et al., 2003) displayed normal *dbx* expression (not shown). By contrast, *eve* misexpression throughout the CNS specifically repressed *dbx* expression in RP2 sib (and other neurons in the 4-2 lineage) and the U sib neurons (and other neurons of the 7-1 lineage) (Fig. 7C,D; Table 2); *dbx* expression in all other lineages was grossly normal. Thus, the ability of *eve* to repress *dbx* also appears restricted to those lineages that normally produce Dbx$^+$ and Eve$^+$ sibling neurons.

Analogous tests revealed similar cross-repressive interactions between *dbx* and *hb9*. Loss of *dbx* or *hb9* function had no effect on *hb9* or *dbx* expression, respectively. However, *dbx* misexpression repressed *hb9* expression in the CoR motoneurons of the 4-2 lineage as well as in the Hb9$^+$ neurons of the 5-2 lineage (Fig. 7E,F; Table 2). We also observed reduced *hb9* expression in other neurons; however, the lineages to which these cells belong are unknown. Conversely, generalized *hb9* misexpression repressed *dbx* expression in the RP2 sib and CoR sibs of the 4-2 lineage as well as the Dbx$^+$ neurons of the 5-2 and 7-1 lineages (Fig. 7G,H; Table 2). Thus, *dbx* and *hb9* also exhibit cross-repressive interactions that are largely restricted to those lineages that produce Dbx$^+$ and Hb9$^+$ sibling neurons. Together our functional studies suggest that negative regulatory interactions between *dbx* and *eve*, and *dbx* and *hb9*, help maintain the mutually exclusive expression patterns of these factors in different pairs of sibling neurons.

dbx expression is sufficient to inhibit axonal growth

As many Dbx$^+$ neurons extend short axons, we asked whether *dbx* regulates axonal growth. Although axonal projections were grossly normal in embryos homozygous mutant for *dbx*, *dbx* misexpression in all postmitotic neurons led to a decrease in the ability of many neurons to extend axons (Fig. 8). For example, 93% of hemisegments exhibited a significant decrease in motor axon projection into the periphery (n=139), with thinning or loss of the ISN nerve (within which U and RP2 motoneurons project axons), the SNc nerve (CoR motoneurons) and the SNb nerve (U and AC motoneurons). Within the nerve cord we observed – at 100% penetrance (n>20 embryos) – thin and broken longitudinal connectives between neuromeres as well as an occasional increase in the apparent size of the axonal scaffold within neuromeres (Fig. 8), raising the possibility that *dbx* misexpression induces neurons that normally project axons into the periphery or between segments to project axons locally. We conclude that *dbx* expression must be strictly repressed in Hb9$^+$ and Eve$^+$

Table 2. *dbx* exhibits cross-repressive regulatory interactions with *eve* and *hb9* in the 4-2 and 7-1 lineages

Genotype	Affected neurons	% Penetrance* (n)	% Expressivity† (n)
ElavGAL4::UAS-Dbx	Eve$^+$ U motoneurons	100 (>20)	93.5 (108)
	Eve$^+$ RP2 motoneuron	100 (>20)	38.0 (100)
	Hb9$^+$ CoR motoneurons	100 (>20)	75.0 (120)
ElavGAL4::UAS-Eve	Dbx$^+$ Usib neurons	100 (>20)	69.7 (238)
	Dbx$^+$ RP2 sib neuron	100 (10)	45.3 (106)
ElavGAL4::UAS-Hb9	Dbx$^+$ CoR sib neurons	100 (>20)	85.5 (200)
	Dbx$^+$ neurons of the 7-1 lineage	100 (>20)	80.5 (200)

*Percentage of embryos that contain at least one hemisegment in which the expression of *eve*, *hb9* or *dbx* is lost or clearly reduced in at least half of the relevant neurons.
†Percentage of hemisegments that exhibit a loss or clear reduction of *eve*, *hb9* or *dbx* expression in at least half of the relevant neurons.
The number (n) of embryos scored for penetrance and the number of hemisegments scored for expressivity is indicated in parentheses.

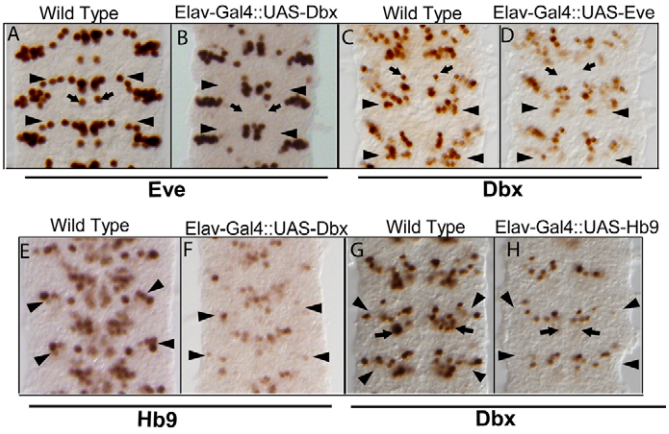

Fig. 7. *dbx* exhibits cross-repressive interactions with *eve* and *hb9*. Stage 15 embryos of indicated genotypes stained for *eve* (A,B), *dbx* (C,D,G,H) and *hb9* (E,F). (**A**,**B**) Generalized *dbx* expression in the CNS represses *eve* expression in the RP2 (arrows) and U motoneurons (arrowheads). (**C**,**D**) *eve* misexpression represses *dbx* expression in the NB4-2 (arrows) and NB7-1 (arrowheads) lineages. (**E**,**F**) *dbx* misexpression inhibits *hb9* expression in the CoR motoneurons (arrowheads), and reduces *hb9* expression in most neurons. (**G**,**H**) *hb9* misexpression represses *dbx* expression in the NB4-2 (arrowheads) and NB7-1 (arrows) lineages.

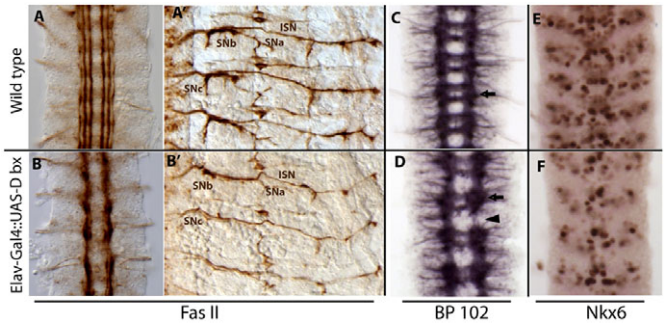

Fig. 8. *dbx* misexpression impairs axonal projections. (**A**,**A′**) FasII expression in stage 16 wild-type embryos marks (A) the longitudinal fascicles and (A′) motor axons. (**B**,**B′**) *dbx* misexpression (B) disrupts the organization of FasII$^+$ longitudinal fascicules and (B′) decreases the number of motor axons that project in SNb and SNc. (**C**) Stage 15 wild-type embryo stained for BP102. (**D**) *dbx* misexpression causes breaks in longitudinal connectives (arrrowhead) and an increased size of the axonal scaffold within neuromeres (arrow, compare to C). (**E**,**F**) *dbx* misexpression inhibits Nkx6 expression in many neurons.

motoneurons for these neurons to extend axons to their appropriate targets. Note *dbx* misexpression is unlikely to convert motoneurons into interneurons, as expression of the vesicular-Glutamate receptor, a relatively specific motoneuron marker, is grossly normal in the face of generalized *dbx* expression (not shown).

The ability of *dbx* misexpression to inhibit motor axon outgrowth in the ISN, SNb and SNc branches generally correlates with its ability to inhibit *eve* or *hb9* expression in specific motoneurons that extend axons in these branches. However, loss of *hb9* or *eve* function elicits significantly weaker motor axon phenotypes than that observed upon generalized *dbx* misexpression. *nkx6* has been shown to promote axon outgrowth in flies (Broihier et al., 2004), and *dbx* and *nkx6* exhibit cross-repressive interactions in the vertebrate neural tube (Gribble et al., 2007). Thus, we assayed the effect of *dbx* misexpression on *nkx6* expression, and found that *dbx* was sufficient to reduce *nkx6* expression in all, and to eliminate expression in some, Nkx6$^+$ neurons (Fig. 8), supporting the model that *dbx* limits axon growth at least in part by repressing *nkx6*. However, in contrast to vertebrates, loss of *dbx* function had no obvious effect on *nkx6* expression, and neither loss of *nkx6* function nor generalized Nkx6 misexpression grossly disrupted *dbx* expression (not shown).

dbx mutant flies exhibit locomotor and behavioral defects

Homozygous mutant $dbx^{\Delta 48}$ adult flies are viable, fertile and exhibit locomotor phenotypes. For example, *dbx* mutant flies were sessile and uncoordinated, and performed poorly in simple climbing assays, and although they could initiate the flight response, they could not maintain flight (see Fig. S5 in the supplementary material). We infer that Dbx$^+$ neurons function within neural circuits crucial for specific locomotor functions. Of note, in vertebrates, Dbx$^+$ neurons are known to regulate proper left-right alternation of motoneuron firing required for proper walking movements (Lanuza et al., 2004).

DISCUSSION

Many Dbx$^+$ interneurons share a sibling relationship with Eve$^+$ or Hb9$^+$ motoneurons, and the cellular phenotypes of these sibling neurons are highly disparate: Dbx$^+$ interneurons extend short axons; Eve$^+$ or Hb9$^+$ motoneurons extend long axons. Our work suggests a model for the establishment and maintenance of distinct cellular phenotypes between sibling neurons. Initially, Notch-mediated asymmetric divisions establish distinct transcription factor expression profiles in sibling neurons, here demonstrated by the ability of such asymmetric divisions to direct *dbx* expression to one neuron and *eve* (or *hb9*) expression to its sibling in multiple pairs of sibling neurons. Once sibling neurons establish distinct transcription factor expression profiles, cross-repressive interactions between these factors help maintain gene expression differences between sibling neurons, here, implied by the lineage-specific, cross-repressive regulatory relationships observed between *dbx* and *eve*, and *dbx* and *hb9*. Such cross-repressive interactions are crucial for proper neuronal differentiation, as inappropriate *dbx* expression in motoneurons impairs motor-axon projections. Transcription factors, such as *dbx*, *eve* and *hb9*, which partake in cross-repressive interactions, also contribute more directly to neuronal differentiation via regulation of downstream effector genes. For example, *eve* upregulates the netrin receptor, Unc-5, in RP2 and other dorsal motoneurons, which in turn helps guide the motor-axons of these neurons to their appropriate targets (Labrador et al., 2005).

Genetic redundancy is a common theme between transcription factors that exhibit cross-repressive regulatory interactions during neuronal specification, and may in fact increase the robustness of this process. For example, loss of function in *dbx*, *eve* or *hb9* yields subtle effects on the expression of the other two genes, whereas generalized misexpression of each gene leads to clear changes in the expression of the other two (this paper) (Broihier and Skeath, 2002). In addition, as loss of *dbx* function induces inappropriate retention of *eve* expression in about 50% of the normally Dbx$^+$ RP2 sib

neurons, other transcription factors must partially compensate for loss of *dbx* within RP2. Such factors might also compensate for loss of *dbx* function during axon growth. Moreover, *hb9* and *nkx6*, which are expressed in nearly identical patterns of CNS neurons, also act redundantly to repress *eve* in a specific set of neurons (Broihier et al., 2004). Within the bristle lineage, the Su(H) and Sox15 transcription factors act redundantly in the socket cell to repress the expression of Shaven, a Pax-family transcription factor, thus restricting *shaven* expression to the sibling shaft cell (Miller et al., 2009). This event is crucial for socket cell differentiation, as de-repression of *shaven* in the socket cell transforms socket cells towards shaft cells.

Our work also highlights the lineage-specific nature of regulatory relationships between transcription factors that govern neuronal specification. For example, *dbx* is competent to inhibit *eve* expression only in the two lineages that produce Dbx^+ and Eve^+ sib neurons. Similarly, *eve* can inhibit *dbx* expression in the same two lineages, but not in the three other lineages that produce Dbx^+ but not Eve^+ neurons. Similar lineage-specific, cross-repressive regulatory interactions occur between transcription factors expressed in neurons born at different times within the same lineage. The $Hb9^+$ CoR and Eve^+ RP2 motoneurons probably arise from sequentially born GMCs within the 4-2 lineage, with loss-of-function studies indicating that *eve* represses *hb9* in RP2, consistent with *hb9* repressing *eve* in the CoR motoneurons (Broihier and Skeath, 2002; Fujioka et al., 2003). Thus, cross-repressive interactions between individual transcription factors during neuronal specification often reflect lineage-specific, rather than CNS-wide, regulatory relationships.

Notch/Numb-mediated asymmetric divisions also exhibit lineage-specific effects on *dbx* and *eve* expression. During these asymmetric divisions, high-level Notch signaling in one daughter cell induces it to adopt Notch-dependent 'A' fate, whereas the absence of Notch signaling in the other daughter permits it to adopt the 'B' fate. In the 4-2 lineage Notch signaling promotes the development of Dbx^+ interneurons and inhibits the formation of the Eve^+. By contrast, in the 7-1 lineage Notch signaling inhibits the formation of Dbx^+ neurons and promotes the development Eve^+ U motoneurons. Thus, the presence/absence of Notch signaling exerts opposite effects on *dbx* and *eve* expression in a lineage-specific manner.

In contrast to *dbx* and *eve*, Notch signaling inhibits the formation of nearly all $Hb9^+$ motoneurons (Fig. 5). Notch signaling also inhibits the motoneuron fate during the asymmetric divisions that produce the RP2, aCC and three VUM motoneurons (Skeath and Doe, 1998; Wheeler et al., 2008). To our knowledge the U motoneurons, which require Notch activity to develop, are the only exception to Notch-mediated inhibition of the motoneuron fate in flies. These observations are interesting in light of a previous model that speculated that vertebrate motoneurons share a common evolutionary ancestry with *Drosophila* $Hb9^+$ motoneurons, based on the common expression of *hb9*, *lim3* and *islet* in most vertebrate motoneurons and all fly motoneurons that project to ventral body wall muscles (Thor and Thomas, 2002). Might Notch signaling generally inhibit the motoneuron fate in vertebrates? Recent work in zebrafish reveals that Notch inhibits the motoneuron fate during the asymmetric divisions of some pMN progenitors that yield sibling motoneuron and interneurons (Park et al., 2004; Shin et al., 2007). However, the fraction of pMN progenitors that divide asymmetrically in this manner remains unclear. Thus, whether Notch signaling strictly inhibits the motoneuron fate during asymmetric divisions in vertebrates awaits further investigation.

Our work on *dbx*, *eve* and *hb9* indicates that the transcriptional networks that control neuronal specification are organized in a lineage-specific manner. Support for this model comes from the identification of three largely lineage-specific enhancers in the *eve* regulatory region: one enhancer drives expression in the U neurons, one drives expression in the lineally related EL neurons and a third drives expression in the RP2 and a/pCC neurons (Fujioka et al., 2003). Might Dbx, Hb9 and the Notch pathway exert their lineage-specific effects on *eve* directly via the appropriate cis-regulatory module? Preliminary data support this notion, as consensus and evolutionarily conserved Dbx-binding sites (Noyes et al., 2008) reside in the RP2 and U motoneurons enhancers (not shown). More generally, are the regulatory regions of *dbx*, *hb9* and other similarly functioning genes also organized in a lineage-specific manner? And, do the regulatory regions of the effector genes through which transcription factors such as Dbx, Hb9 and Eve regulate neuronal differentiation reflect a similar organization? Future work that addresses these questions should help clarify the cis-regulatory and transcriptional logic that governs neuronal specification and differentiation in *Drosophila*.

In contrast to most transcription factors that govern neuronal subtype identity, *dbx* is not an essential gene. Adult flies that lack *dbx* function exhibit defects in flight and ambulatory movement. In vertebrates, interneurons derived from Dbx^+ progenitors coordinate left-right alternation of motoneuron firing required for proper walking movements via direct, probably inhibitory synaptic input to motoneurons (Lanuza et al., 2004). The sibling relationship between Dbx^+ interneurons and many motoneurons suggests that Dbx^+ interneurons perform similar functions in *Drosophila*. Such speculation is supported by work in other insects, where small axonless interneurons modulate motoneuron function (Pearson and Fourtner, 1975; Burrows, 1996). In this light, the non-essential nature of *dbx* in flies may facilitate charting of the neural circuits through which Dbx^+ neurons regulate distinct locomotor functions in *Drosophila*.

Acknowledgements

We are indebted to Heather Broihier for initiating this line of research. We thank: Manfred Frasch, Rob Jackson, Paul Taghert, Ross Cagan, Miki Fujioka, Aaron DiAntonio, Kathy Matthews, Kevin Cook, the Bloomington Stock Center and the Developmental Studies Hybridoma Bank for strains/reagents; Dongkook Park for help with larval stainings; and Swathi Arur, Heather Broihier and Martyn Goulding for critical reading of the manuscript and helpful discussions. This research was funded by an NIH NINDS grant (RO1 NS036570) to J.B.S. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/19/3257/DC1

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