Development 125, 1037-1048 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV8492

dorsotonals/*homothorax*, the Drosophila homologue of *meis1*, interacts with *extradenticle* in patterning of the embryonic PNS

Estee Kurant[1], Chi-yun Pai[2], Rakefet Sharf[1], Naomi Halachmi[1], Y. Henry Sun[2] and Adi Salzberg[1,*]

[1]Unit of Genetics, Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel
[2]Institute of Molecular Biology, Academica Sinica, Nankang, Taipei 11529, Taiwan, Republic of China
*Corresponding author (e-mail: adis@tx.technion.ac.il)

Accepted 7 January 1998: published on WWW 17 February 1998

SUMMARY

The homeotic genes of the bithorax complex are required, among other things, for establishing the patterns of sensory organs in the embryonic peripheral nervous system (PNS). However, the molecular mechanisms by which these genes affect pattern formation in the PNS are not understood and other genes that function in this pathway are not characterized. Here we report the phenotypic and molecular analysis of one such gene, *homothorax* (*hth;* also named *dorsotonals*). Mutations in the *hth* gene seem to alter the identity of the abdominal chordotonal neurons, which depend on Abd-A for their normal development. However, these mutations do not alter the expression of the *abd-A* gene, suggesting that *hth* may be involved in modulating *abd-A* activity. We have generated multiple mutations in the *hth* locus and cloned the *hth* gene. *hth* encodes a homeodomain-containing protein that is most similar to the murine proto-oncogene *meis1*. The *hth* gene is expressed throughout embryonic development in a spatially restricted pattern, which is modulated in abdominal segments by *abd-A* and *Ubx*.

The spatial distribution of the HTH protein during embryonic development is very similar to the distribution of the Extradenticle (EXD) protein, a known modulator of homeotic gene activity. Here we show that the PNS phenotype of *exd* mutant embryos is virtually indistinguishable from that of *hth* mutant embryos and does not simply follow the homeotic transformations observed in the epidermis. We also show that the HTH protein is present in extremely low levels in embryos lacking *exd* activity as compared to wild-type embryos. In contrast, the EXD protein is present in fairly normal levels in *hth* mutant embryos, but fails to accumulate in nuclei and remains cytoplasmic. Ectopic expression of *hth* can drive ectopic nuclear localization of EXD. Based on our observations we propose that the genetic interactions between *hth* and *exd* serve as a novel mechanism for regulating homeotic protein activity in embryonic PNS development.

Key words: *dtl*, *hth*, Homeobox, *exd*, Nuclear localization, Pattern formation

INTRODUCTION

The embryonic peripheral nervous system (PNS) of Drosophila comprises approximately 600 neurons and 1200 associated cells, organized in a highly stereotyped segment-specific pattern (Campos-Ortega and Hartenstein, 1985; Ghysen et al., 1986). Five distinct patterns of sensory organs are present in different thoracic and abdominal segments of the embryo, seen in segments T1, T2-T3, A1-A7, A8 and A9. Each of the abdominal hemisegments (A1-A7) contains 44 PNS neurons organized in four clusters along the dorsoventral axis: 12 neurons in the dorsal cluster, 12 neurons in the lateral cluster and 20 neurons in the two ventral clusters (Fig. 2A). Three main types of neurons are present in the PNS: external sensory (ES) neurons that innervate mechanoreceptors and chemoreceptors in the cuticle, chordotonal (Ch) neurons that innervate stretch receptors located subepidermally, and multiple dendritic neurons.

Mutations in many genes that affect pattern formation in the embryonic PNS have been identified (for a review see Jan and Jan, 1993 and references therein; Salzberg et al., 1994, 1997; Kania et al., 1995). Phenotypic analysis of such mutations and molecular characterization of some of the corresponding genes shed light on specific aspects of pattern formation in the PNS, mainly the regulation of cell number and cell fate within the PNS lineages (for reviews see Posakony, 1994; Campos-Ortega, 1995; Hassan and Vaessin, 1996; Doe, 1996); however, many questions remained open. For example, what are the mechanisms that govern the localization of neurons to specific positions within a segment? What are the mechanisms that generate the diversity among PNS patterns of different segments?

To address these questions we focused on a specific group of sensory neurons, the five lateral Ch (LCh5) neurons of abdominal segments A1-A7. These neurons and the three dorsal chordotonal neurons of thoracic segment T2-T3 (DCh3) are the only sensory neurons originating in the posterior compartment of the corresponding segments (Hartenstein, 1987). In addition to the difference in their number (three versus five) and position (dorsal versus lateral), the DCh3 and LCh5 neurons differ from

each other in their orientation. Whereas the DCh3 neurons are oriented such that their dendrites point ventrally, the LCh5 neurons are oriented such that their dendrites point dorsally (Fig. 2B). The precursors of the LCh5 and DCh3 neurons are born in a similar dorso-lateral position. Later in development the abdominal precursors, or their descendants, are thought to move ventrally and rotate to assume a lateral position and proper orientation (Salzberg et al., 1994). The three Ch neurons of the eighth abdominal segment (LCh3 in A8, Fig. 2A) also originate in a dorso-lateral position similar to that of the LCh5 and DCh3 neurons, but exhibit a dorso-lateral position in the mature PNS and a DCh3/LCh5 intermediate orientation.

The mechanisms controlling the migration of Ch neurons from their dorsal place of origin to a lateral position are not known. However, several assumptions can be made based on phenotypic analysis of various mutations that disrupt this process. First, one may assume that the 'homeotic identity' of the segment in which the neurons originate is crucial. The DCh3 neurons that originate in a similar position to LCh5, but in thoracic segments, remain associated with the dorsal PNS cluster and do not migrate. In the absence of *abd-A* activity, the LCh5 neurons are transformed into DCh3 neurons and remain associated with the dorsal PNS cluster (Heuer and Kaufman, 1992; Salzberg et al., 1994). In *cut* mutant embryos, in which ES organs are transformed into Ch organs, the ectopic Ch neurons remain in their dorsal position and do not move ventrally (Bodmer et al., 1987; Blochlinger et al., 1988). In embryos that overexpress the *cut* gene under the regulation of the heat shock promoter (Blochlinger et al., 1991) the LCh5 neurons are transformed into ES neurons and often fail to migrate ventrally, even though they are born in a proper posterior position. Altogether, these observations suggest that the identity of the neuronal cells and the place in which they are born within the segment (anterior versus posterior compartment) play an important role in determining their ability to acquire a lateral position.

Here we describe the cloning and phenotypic analysis of the *homothorax* (*hth*) gene, which is allelic to *dorsotonals* (*dtl*). Loss-of-function alleles of *hth* lead to a 'dorsal chordotonals' phenotype that is somewhat similar to the *abd-A* phenotype, but do not affect *abd-A* expression. This observation suggests that *hth* may function as a modulator of *abd-A* activity. The *hth* gene encodes a homeobox-containing protein of the *meis1* family that is required for the nuclear localization of Extradenticle (EXD), a known modulator of homeotic protein activity. The EXD protein itself is required for maintaining normal levels of the HTH protein. Phenotypic analyses revealed that loss-of-function mutations in *exd* lead to an *hth*-like phenotype in the PNS. Based on our observations we propose that *hth*, *exd* and *abd-A* interact genetically in patterning of the embryonic PNS.

MATERIALS AND METHODS

Fly strains, excision mutagenesis and lethal phase determination

For excision mutagenesis in the *dtl*/*hth* locus, males of the genotype *yw*; *P{lacZ, w+}1422-4*/*TM6* were crossed to *yw; Ki P{ry+, Δ2-3}99B* females. Individual *P{lacZ, w+}1422-4*/*Ki P{ry+, Δ2-3}99B* male progeny were mated to *yw; Ki H/TM6B,D*^{3} virgins. Male progeny with white eyes were tested for complementation with *P{lacZ, w+}1422-4*/*TM6B* females. Approximately 150 excisions were isolated. Genomic DNA of the excision strains was analyzed by PCR to determine the molecular nature of the excision event. The primers used were the 31 bp of the *P*-element inverted repeat 5′-CGACGGGACCACCTTATGTTATTTCATCATG-3′ and two genomic primers located 180 bp upstream and 270 bp downstream of the *P{lacZ, w+}1422-4* insertion: 5′-TTGTTGCATTCTGAACAG-AGCGGGGCAG-3′ and 5′-GCGCGCGATTTCCAAAGTGTTT-

Table 1. Available *hth* alleles

Allele	Type of aberration	Originally described	Lethal phase*	Comments
hth^{H321}	EMS induced allele[a]	Salzberg et al., 1994	E	Weak hypomorph
hth^{J186}	EMS induced allele[a]	Salzberg et al., 1994	E	Weak hypomorph
hth^{P1422}	Lethal P[lacZ, w^{+}] insertion (65 bp upstream to the 5′ of the longest hth cDNA)[a]	Salzberg et al., 1997	L2	Weak hypomorph
hth^{P1323}	Lethal P[lacZ, w^{+}] insertion (approx. 300 bp upstream to the 5′ of the longest cDNA)[a]	Salzberg et al., 1997	E	Moderate
P1	Viable P[lacZ,w^{+}] insertion	Sun et al., 1995	V	
P2	Lethal P[lacZ, w^{+}] insertion	Sun et al., 1995	E	Strong
P-1Δ15	Lethal excision of *P1*	Pai et al., 1998	E	Weak to moderate hypomorph
P-1K6-1	Lethal excision of *P1*	Pai et al., 1998	E	Weak to moderate hypomorph
P-1K1-8	Lethal imprecise hop of *P1*	Pai et al., 1998	E	Strong hypomorph (near protein null)
hth$^{4-4}$	Lethal excision of *P1422-4* (removes approx. 3 kb upstream of *P1422-4*)	This work	ND	Weak hypomorph
hth$^{31-2}$	Lethal excision of *P1422-4* (removes approx 4.2 kb upstream of *P1422-4*)	This work	ND	Moderate
hth$^{52-3}$	Lethal excision of *P1422-4* (size of deletion not determined)	This work	ND	Strong hypomorph
hth$^{6-2}$	Lethal excision of *P1422-4* (removes approx. 4.5 kb to both sides of *P1422-4*)	This work	ND	Strong hypomorph
hth$^{69-3}$	Lethal excision of *P1422-4* (removes approx. 2.4 kb downstream of *P1422-4*)	This work	E	Strong hypomorph
hth$^{73-3}$	Lethal excision of *P1422-4* (removes approx. 1.1 kb down stream of *P1422-4*)	This work	E	Moderate hypomorph
hth$^{64-1}$	Lethal excision of *P1422-4* (removes approx. 5.5 kb to both sides of *P1422-4*)	This work	E	Strong hypomorph (near protein null)

*E, embryonic, L2 second instar larva.
[a]These alleles were originally termed *dtl*^{H321}, *dtl*^{J186}, *dtl*$^{P1422-4}$, and *dtl*$^{P1323-7}$.

CTTGTG-3′, respectively. Lethal excision strains that did not contain *P*-element sequences (based on the PCR data) were further characterized by Southern analysis to identify deletions caused by imprecise excision events. Seven strains harboring such deletions were identified (Table 1).

The mutant homeotic alleles *iab-2^{D24}* and *Df(3R)Ubx^{109}* are described in Lindsley and Zimm (1992) and the Flybase (1997). *abd-A^{K622}* is a hypomorphic allele described in Salzberg et al. (1994). Embryos lacking both maternal and zygotic *exd* expression were generated using *yw; exd^{XP11} f FRT 18D/OVO^{D2} FRT 18D; hs-FLP38/+* mothers as described in Rauskolb et al. (1993). *exd*$^{-}$ embryos were recognized by the abnormal shape of the clypeolabrum. The *UAS-hth* strain carries a *UAS-hth* transgene on the second chromosome and is described in Pai et al. (1998).

To determine the lethal phase of the various *hth* alleles, flies of the balanced mutant strains were crossed out to wild-type flies. Eggs were collected from heterozygous *hth*/+ parents, and 100-200 eggs of each strain were monitored.

Molecular techniques

Plasmid rescue from fly genomic DNA was performed as described in Wilson et al. (1989). Northern and Southern analysis, library screening and other molecular procedures were performed using standard techniques (Sambrook et al., 1989). Two genomic libraries were screened: a λDASH II *ry^{506}/ry^{506}* library (kindly provided by Hugo Bellen) and a λCh4A library (Maniatis et al., 1978). For the isolation of embryonic cDNA clones, a 9- to 12-hour embryonic library (Zinn et al., 1988) was screened. Multiple cDNA clones were isolated, four of which were either fully or partially sequenced.

Whole-mount in situ hybridization

For the preparation of digoxigenin-labeled *hth* probe, a 0.88 kb *Xba*I-*Sal*I fragment from *hth* cDNA was used as a template. The probe was prepared as described in the DNA labeling and detection kits (nonradioactive) from Boehringer Mannheim. In situ hybridization to whole-mount embryos was performed as described by Tautz and Pfeifle (1989).

Antibody production and immunohystochemistry

Rabbit-anti-HTH serum (AS1924) was raised against a bacterially produced HTH protein. The ORF of *hth* was amplified by PCR and cloned into the *Bam*HI site of the pET15B vector (Novagen) downstream to the 6×His tag (Pai et al., 1998). The His-HTH fusion protein was produced in *E. coli* strain BL21.DE3 following the pET System Protocol (Novagen). The His-HTH protein was purified on a Ni^{2+}-NTA-Agarose column (Qiagen) following the manufacturer's protocol. The eluted protein was renatured by dialysis into 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.05% Tween-20 and 20% glycerol. The purified protein (100-150 μg) was mixed with Freund's adjuvant and injected five times, once a month, to New Zealand rabbits. The preimmune and immune sera were tested on embryos and western blots.

The AS1924 serum was used in a 1:1,000 dilution for whole-mount embryo staining, and 1:5,000 for western blot analysis. Other antibodies used for the analysis of embryonic phenotypes are listed below. mAb 22C10 (Goodman et al., 1984) was used in a 1:20 dilution. Monoclonal anti-Cut antibody (Blochlinger et al., 1990) was used in a 1:20 dilution. mAb BP102 (Seeger et al., 1993) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa and was used in a 1:50 dilution. mAb 21A6 was obtained from Dr. S. Benzer and used in a 1:10 dilution. mAb B11M (anti-EXD) (Aspland and White, 1997) was used in a 1:5 dilution. mAb Dmabd-A.1 (anti-Abd-A) (Kellerman et al., 1990) was used in a 1:1,000 dilution. Anti-Atonal serum (Jarman et al., 1994) was used in a 1:5,000 dilution. Secondary antibodies were biotinylated anti-mouse IgG, or anti-rabbit IgG from Vector Laboratories. Signal detection was carried out with Vecta-Stain Elite ABC-HRP kit (Vector Laboratories). Prior to use, all polyclonal sera were preabsorbed to 0- to 20-hour-old embryos for 2 hours at room temperature, or overnight at 4°C. Embryos were fixed and processed for whole-mount antibody staining using standard techniques (Patel, 1994). Stained embryos were cleared with 70% glycerol, mounted and examined using Nomarski optics on a Zeiss Axioskop. To prepare protein extracts for Western analysis embryos were homogenized in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl and 1% NP-40. Protein were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Signal detection was performed with HRP-conjugated goat anti-rabbit IgG (Sigma) using enhanced chemiluminescense (Amersham Corp.).

RESULTS

dtl is allelic to *posterior* and *hth* and has pleiotropic effects on embryonic development

Two embryonic lethal EMS-induced alleles of *dtl* (*dtl^{H321}* and *dtl^{J186}*) were isolated in a mutagenesis screen aimed at identifying genes required for normal pattern formation in the embryonic PNS (Salzberg et al., 1994). Two insertional alleles, *dtl$^{P1422-4}$* and *dtl$^{P1323-7}$*, were identified subsequently in a similar screen (Salzberg et al., 1997). Both *P*-element insertions were mapped to cytological position 86C1-4 (Salzberg et al., 1997). The *P*-elements inserted in *dtl$^{P1422-4}$* and *dtl$^{P1323-7}$* are of the *P{lacZ, w^{+}}* type carrying the *white* gene as a dominant eye color marker. The pigmentation in the eyes of *dtl$^{P1422-4}$* and

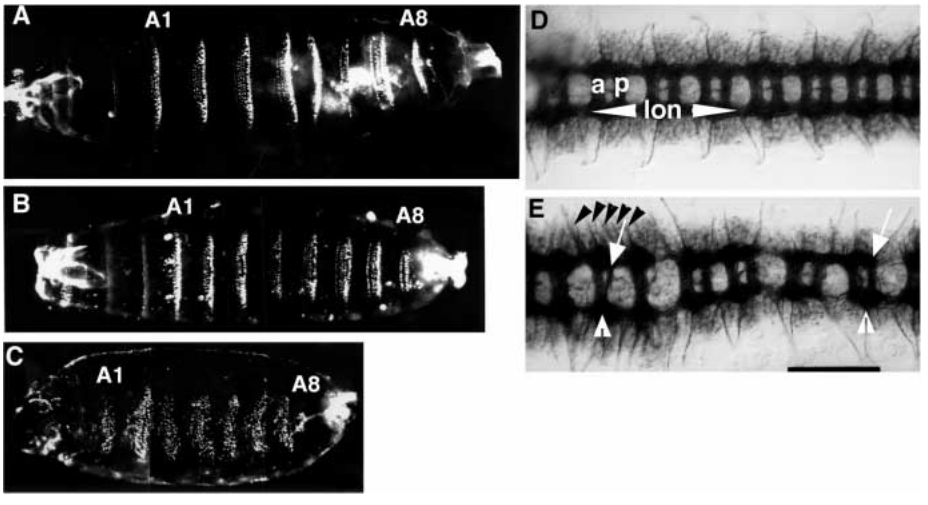

Fig. 1. Cuticle and CNS phenotypes of *hth* mutants. (A-C) Ventral view of cuticle preparations of wild-type (A), *hth^{P1422}/hth^{P1422}* (B) or *hth^{P2}/hth^{P2}* (C) embryos. Abdominal segments A1 and A8 are indicated. (D,E) Dissected VNCs of wild-type (D) or *hth^{P2}/hth^{P2}* (E) embryos immunostained with mAb BP102. In homozygous *hth^{P2}* embryos the longitudinal tracts (lon) are reduced or missing and the posterior commissures are often reduced (arrows). The spacing between the commissures is reduced, most notably in thoracic segments (white arrowheads), and abnormal outgrowth of multiple nerve roots is evident (black arrowheads). Also note the widening of the thoracic neuromers in the mutant. a and p, anterior and posterior commissures, respectively.

dtl$^{P1323\text{-}7}$ flies is restricted to the posterior region of the eye, suggesting the presence of a genomic silencer in the vicinity of the insertions. This unique eye pigmentation pattern was identified previously in a screen for silencers in the *Drosophila* genome (*posterior* insertions *P1* and *P2*; Sun et al., 1995). Complementation tests between lethal *posterior* insertions and lethal *dtl* alleles revealed that they belong to the same complementation group. In addition, complementation tests between lethal *dtl* and *hth* alleles (Jürgens et al., 1984; *hth*^{5E}, obtained from R. Mann) revealed that these mutations are also allelic. Therefore, we refer to all the mutations in this complementation group as *hth* alleles. Additional mutations in the *hth* locus were generated by imprecise excisions of the *P{lacZ, w*$^{+}$*}1422-4* insertion. The various *hth* alleles form an allelic series ranging from mild to very severe (Table 1).

Mutations in *hth* have pleiotropic effects on embryonic development. Cuticle preparations of loss-of-function alleles of *hth* reveal defects in segmentation and head involution (Fig. 1A-C). The head skeleton was reduced or absent. Thoracic segments appeared deranged and the thoracic denticle belts were eliminated. Abdominal denticle belts appeared more dispersed and less differentiated than normal and exhibited a weak *engrailed*-like phenotype. In addition, the width of the denticle belts in anterior abdominal segments appeared reduced and similar to that of the seventh or eighth abdominal segment. The central nervous system (CNS) of *hth* mutant embryos was examined using mAb BP102 (Seeger et al., 1993). Two distinct phenotypes were evident in the ventral nerve cord (VNC) of mutant embryos: substantial widening of the VNC in thoracic segments and abnormal scaffold of CNS

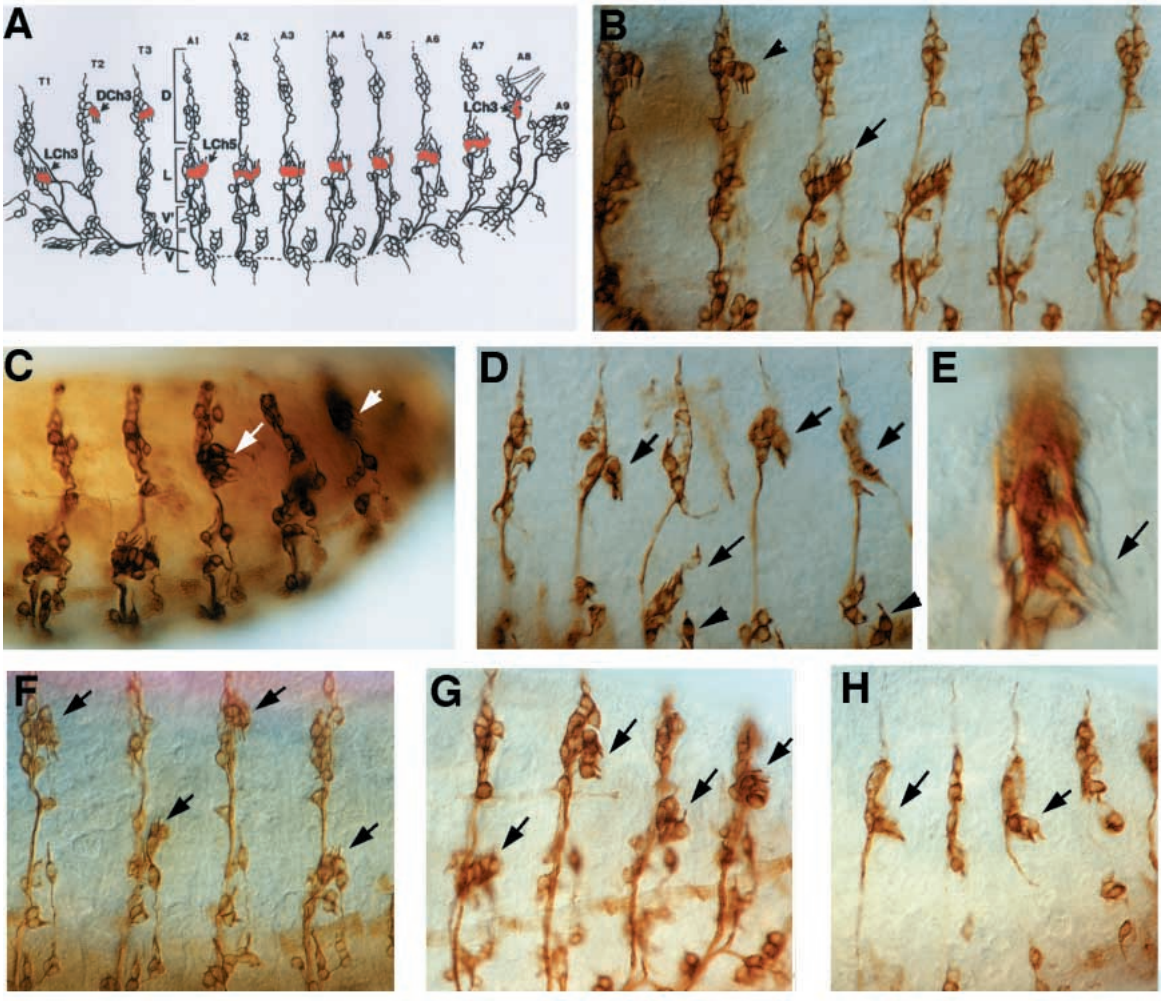

Fig. 2. The 'dorsal chordotonals' phenotype associated with *hth*, *abd-A* and *exd* mutations. (A) A lateral view of the embryonic PNS pattern (adapted from Ghysen et al., 1986). The Ch neurons originating in the posterior compartment of abdominal or thoracic segments are depicted in red. D, dorsal PNS cluster; L, lateral PNS cluster; V′ and V, ventral PNS clusters. (B-H) Lateral view of PNS neurons as visualized with mAb 22C10. Here and in all other micrographs anterior is to the left and posterior to the right. (B) Segments T2-A4 of a wild-type embryo. The LCh5 neurons of segment A1 are indicated by an arrow. The DCh3 neurons of segment T3 are indicated by an arrowhead. (C) Abdominal segments of a homozygous *hth*^{P1422} embryo. The aberrantly positioned LCh5 neurons are indicated by arrows. (D) Abdominal segments of a homozygous *hth*$^{52\text{-}3}$ embryo. In addition to the dorsal position of the LCh5 neurons, their number is reduced (arrows). Ventral Ch neurons are indicated by arrowheads. (E) A closeup view of one cluster of 'dorsalized' LCh5 neurons. The arrow points to the normal-looking scolopales (viewed with Nomarski optics). (F) Abdominal segments of a homozygous *abd-A*^{K622} embryo. The aberrantly positioned neurons are indicated by arrows. (G) Abdominal segments of an embryo lacking maternal contribution of *exd*. The PNS phenotype is similar to that of weak *hth* alleles (compare C and G). (H) A similar view of an embryo lacking both maternal and zygotic expression of *exd*. The PNS phenotype is similar to that of strong *hth* alleles (compare D and H).

axons throughout the VNC (Fig. 1D,E). As shown in Fig. 1E, the longitudinal pathways were absent or reduced in all thoracic and abdominal segments. The anterior commissure was present, but the posterior commissure was often reduced (arrow in Fig. 1E). The spacing between the anterior and posterior commissures was also reduced, most notably in thoracic segments (white arrowheads in Fig. 1E). Another phenotype observed in the CNS of *hth* mutant embryos is the outgrowth of multiple nerve roots on each side of the CNS as compared to two nerve roots in wild-type embryos (black arrowheads in Fig. 1E).

The *hth* gene is required for proper localization of chordotonal organs

In wild-type embryos the LCh5 neurons are located invariably in the lateral PNS cluster of abdominal segments A1-A7 (Fig. 2A,B). In contrast, these neurons were situated in a more dorsal position in 25% or 36% of the abdominal segment in the PNS of embryos homozygous for hth^{H321} (n=91) or hth^{J186} (n=56) respectively. This phenotype was also observed in more than 30% of abdominal segments in homozygous hth^{P1422} or hth^{P1323} embryos (Fig. 2C). The affected Ch neurons remained associated with the dorsal PNS cluster, or occasionally, were positioned between the dorsal and lateral PNS clusters. The orientation of the affected neurons was also abnormal. Whenever the affected LCh5 neurons remained associated with the dorsal PNS cluster their dendrites pointed ventrally or posteriorly instead of dorsally (Fig. 2C). The 'dorsal chordotonals' phenotype could be detected in all the abdominal segments in varying frequencies. In weak alleles, such as hth^{P1422} or hth^{4-4}, it was observed more frequently in the posterior abdominal segments (A5-A7). Stronger alleles affected all the abdominal segments in similar frequencies. Weak *hth* alleles did not affect any PNS neurons other than the LCh5 neurons.

Strong hypomorphic mutations in *hth*, such as hth^{K1-8} or hth^{64-1}, affected not only the position and orientation of the LCh5 neurons, but also caused a reduction in their number. Only three dorsal Ch neurons were observed in nearly 100% of abdominal segments of homozygous hth^{K1-8} or hth^{64-1} embryos (Fig. 2D). Most of the affected neurons remained associated with the dorsal PNS cluster and their dendrites pointed ventrally. In spite of their abnormal location and orientation, the affected Ch neurons appeared fully differentiated, as judged by their overall morphology and the presence of normal-looking scolopales at the tip of their dendrites (Fig. 2E). Based on anti-Atonal staining we concluded that the precursors of the LCh5 neurons are born in a normal dorso-lateral position in *hth* mutant embryos (data not shown). Due to the lack of appropriate markers we could not detect the 'dorsal chordotonals' phenotype in mutant embryos earlier than stage 12. In addition to the 'dorsal chordotonals' phenotype, we observed a reduction in the total number of PNS neurons and defects in the axonal pathways in the PNS of strong hypomorphic *hth* mutants (Fig. 2D). One of the most severe alleles (hth^{64-1}) caused on average a loss of 3-4 dorsal PNS neurons, 3-4 lateral PNS neurons and 10-12 ventral PNS neurons. The remaining neurons are not organized in a highly stereotyped pattern and it was difficult to asses their identity. However, based on anti-Cut staining we concluded that in the dorsal cluster one dorsal ES neuron and 2-3 Cut-negative MD neurons are lost (data not shown). The ventral Ch neurons were only rarely lost in hth^{64-1} mutant embryos (arrowhead in Fig. 2D).

hth mutations do not alter *abd-A* expression

The number, position and orientation of the LCh5 neurons affected by *hth* mutations resembled the number position and orientation of the thoracic DCh3 neurons, or to lesser extent, the position and orientation of the A8-LCh3 neurons (see Discussion). A similar phenotype was observed in embryos homozygous for mutations in the homeotic selector gene *abd-A* (Heuer and Kaufman, 1992; Salzberg et al., 1994). In the absence of *abd-A* activity, the LCh5 neurons were transformed into DCh3 neurons (Heuer and Kaufman, 1992), and as such they remained associated with the dorsal PNS cluster and their dendrites pointed ventrally (Fig. 2F). Only rarely were the transformed neurons observed in a lateral or dorso-lateral position in embryos homozygous for a null allele of *abd-A* ($iab\text{-}2^{D24}$, 4-5%, n=105). In embryos homozygous for both the $iab\text{-}2^{D24}$and the hth^{P1422} mutations, the LCh5 neurons exhibited a DCh3-like morphology and number. However, quite surprisingly these neurons were positioned lateraly in 17-19% of the abdominal segments (n=173 or 149 in two independent double mutant strains). This observation suggests that, at least in some aspects, mutations in *hth* and *abd-A* have opposite effects on Ch neuron development.

Since the PNS phenotype associated with loss of *hth* function suggested a homeotic transformation of LCh5 neurons towards the identity of DCh3 or A8-LCh3 neurons, which do not depend on *abd-A* for their development, we examined the expression pattern of the Abd-A protein in *hth* mutant embryos. Abd-A is normally expressed in the ectoderm of abdominal segments from PS7 to the anterior region of PS13 (Karch et al., 1990). In addition, Abd-A is expressed in the LCh5 neurons of segments A1-A7 (Fig. 3A) and in the VNC in segments A2-A7 (Karch et al., 1990). The spatial distribution of the Abd-A protein is not altered in the ectoderm or CNS of embryos homozygous for the hth^{K1-8} allele as compared to wild-type embryos, although a slight reduction in the level of the protein was observed (Fig. 3). Since the LCh5 neurons are positioned aberrantly in hth^{K1-8} mutants, we double-stained these embryos

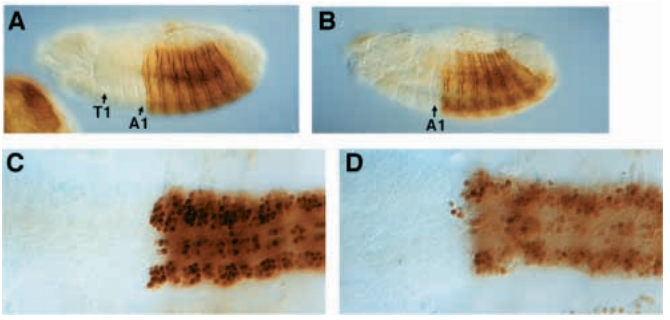

Fig. 3. Mutations in *hth* do not alter Abd-A expression. (A,B) Lateral view of wild-type (A) or homozygous hth^{K1-8} (B) stage-13 embryos immunostained with anti-Abd-A antibody. The mutant embryos can be identified by the abnormal morphology of head and thorax. (C,D) Dissected VNCs of wild-type (C) or homozygous hth^{K1-8} (D) stage-16 embryos immunostained with anti-Abd-A antibody. The VNC of mutant embryos can be identified by the abnormal width of the thoracic neuromers.

with an antibody against Abd-A and with mAb 21A6, which labels the scolopales of Ch neurons. This double-labeling experiment allowed us to identify unambiguously the misplaced LCh5 neurons and establish whether or not they express Abd-A. We found that the abdominal Ch neurons express the *abd-A* gene in the absence of *hth* activity (data not shown). We therefore concluded that *hth* may be required for the activity of Abd-A rather than its expression.

The PNS phenotype of *exd* mutant embryos

Mutations in the *exd* gene were shown to cause homeotic transformations in the embryonic cuticle without altering homeotic gene expression (Peifer and Wieschaus, 1990). The EXD protein affects the morphological consequences of homeotic gene activity by modulating the DNA binding specificity of homeotic proteins (Rauskolb et al., 1993; van Dijk and Murre, 1994; Chan et al., 1994). The involvement of *exd* in homeotic determination and pattern formation in the PNS was not addressed however, and the PNS pattern of embryos lacking normal *exd* activity has not been described previously.

To determine whether *exd* is required for normal pattern formation in the embryonic PNS we examined embryos lacking either zygotic, or maternal, or both zygotic and maternal expression of *exd* using mAb 22C10 as a marker. Embryos lacking zygotic expression of *exd* were obtained from attached-X mothers (C(1)DX, y f /Y) and exd^{XP11}/Dp(1:Y)Y#1 males (Rauskolb et al., 1993). All of the embryos from this cross contained high levels of maternally derived EXD, but one quarter of them lacked zygotic *exd* expression. Embryos lacking a maternal contribution of *exd* were derived from exd^{XP11}/exd^{XP11} germline clones (Rauskolb et al., 1993). Half of these embryos received a wild-type copy of *exd* from their fathers. The other half did not receive a copy of *exd* from their fathers and thus lacked both maternal and zygotic *exd* expression.

Embryos lacking only zygotic expression of *exd* appeared normal when stained with mAb 22C10 and did not exhibit any obvious defects in their PNS. In contrast, embryos with normal zygotic expression, but no maternal contribution of *exd*, exhibited a ‘dorsal chordotonal’ phenotype in approximately 5% of the examined abdominal segments (*n*=77; Fig. 2G).

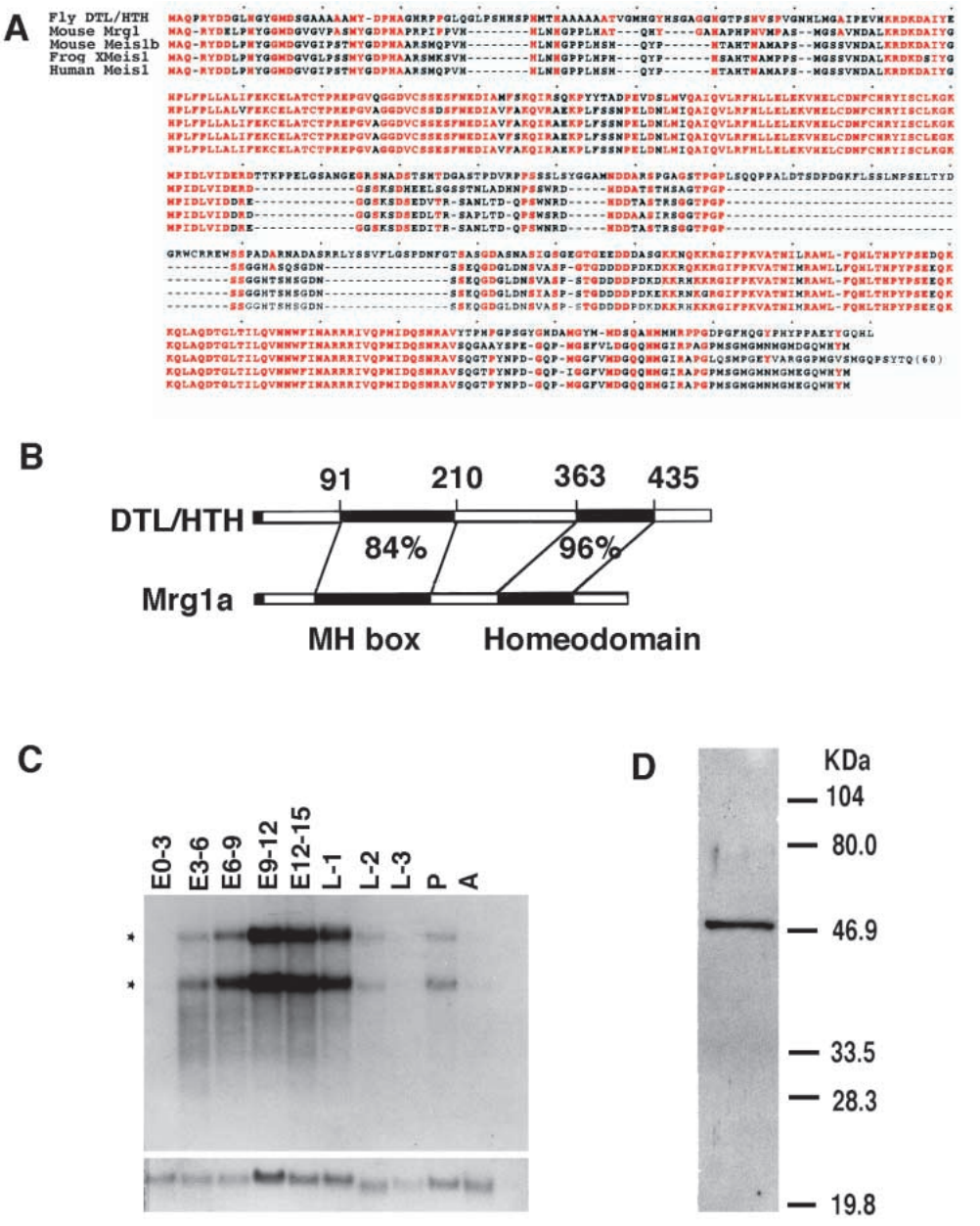

Fig. 4. The *dtl/hth* gene encodes a homeodomain protein of the Meis1 family. (A) Sequence similarity between the predicted DTL/HTH protein and vertebrate proteins of the Meis1 family. Residues that are conserved between the fly and vertebrate proteins are depicted in red. Accession numbers: Fly, AF032865; Mouse, U68383; Frog, U68386; Human, U85707. (B) A comparison between the general structure of the fly DTL/HTH and the mouse Mrg1a proteins. The homeodomain and the MH box are indicated by black boxes. The percentage of identical amino acids within the conserved regions is shown. (C) Developmental northern blot probed with *hth* cDNA (cDNA #7, Pai et al., 1997). 2.5 μg of poly(A)$^+$ RNA was loaded in each lane. The sizes of the two major transcripts are 4.0-4.5 and 6.0-6.5 kb (asterisks). The *guf* gene was used as a standard to evaluate the amount of RNA loaded. E, embryos (the numbers denote age of embryos in hours); L, larva; P, pupa; A, adult. (D) Western blot analysis of embryonic protein extract probed with anti-HTH serum (AS1924, 1:5,000).

Neurons other than LCh5 were not affected. This phenotype appears identical to the PNS phenotype of weak hypomorphic alleles of *hth*, although the expressivity was lower. Embryos lacking both maternal and zygotic expression of *exd* exhibited a PNS phenotype very similar to that caused by strong loss-of-function *hth* alleles (Fig. 2H). In these embryos, the LCh5 neurons were positioned in the dorsal PNS cluster in more than 65% of the abdominal segments and their number was reduced. A reduction in the number of other PNS neurons and axonal pathway defects were also observed (Fig. 2H).

hth is the Drosophila homologue of *meis1*

To clone *hth*, genomic fragments flanking the *P{lacZ, w+}1422-4* insertion site were plasmid-rescued and used as probes to screen genomic and embryonic cDNA libraries. Genomic phage clones that span more than 25 kb were isolated and a partial restriction map of the *hth* locus was constructed (data not shown). Southern analysis and comparisons of cDNA and genomic sequences revealed that the *hth* transcription unit spans more than 25 kb of genomic DNA and contains at least four exons. Multiple embryonic cDNA clones were isolated and sequenced. The embryonic cDNA clones contained a single large open reading frame (ORF) of 1461 nucleotides, identical to the ORF reported by Pai et al. (1998). No alternatively spliced variants were identified. Rescue experiments demonstrated that the identified ORF corresponds to the *hth* gene and can rescue the lethality associated with the hth^{P1422} mutation (Pai et al., 1998).

The predicted translation product is a protein of 487 amino acids of M_r 52.8×10^3. The predicted HTH protein displays extensive sequence similarity with a family of PBX-related homeobox-containing proteins that includes the murine proto-oncogene Meis-1 (Moskow et al., 1995), the murine Meis1-related proteins (Nakamura et al., 1996a; Steelman et al., 1997), xMeis1 from *Xenopus laevis* and the human MEIS1 protein (Smith et al., 1997) (Fig. 4A). The highest similarity between the HTH and Meis-1 proteins is found in the homeodomain, with up to 96% identity over a 69-amino-acid stretch (Fig. 4B). The similarity between these proteins extends beyond the homeodomain and spans almost their entire length. Most notably, a stretch of 120 amino acids in the amino-terminal region of the proteins exhibits 84% of sequence identity (Fig. 4B). This domain, which does not share sequence similarity with any other protein in the available data bases, is referred to as MH-box (Meis-homology box, or Meis-HTH box).

Developmental northern blot analysis with *hth* cDNA probes revealed two major transcripts that are 4.0-4.5 kb and 6.0-6.5 kb long (Fig. 4C). The gene is transcribed only zygotically. Transcripts of *hth* are relatively abundant in mRNA samples of 3- to 15-hour-old embryos, first instar larvae and pupae. These transcripts are less abundant in mRNA samples of second instar larvae and are present in very low levels in 0- to 3-hour-old embryos, third instar larvae and adults (Fig. 4C).

A polyclonal antiserum was raised against a bacterially expressed HTH protein. The anti-HTH serum recognized a single protein with an approximate molecular mass of 50 kDa in extracts prepared from Drosophila embryos (Fig. 4D).

The embryonic expression pattern of *hth*

To determine the spatial pattern of *hth* expression we performed *in situ* hybridization to whole embryos using *hth* cDNA as a probe. *hth* expression begins at cellular blastoderm. It is expressed in the region that gives rise to the segmented portion of the embryo and is excluded from the anterior and posterior tips (Fig. 5A). During gastrulation, *hth* expression is detected throughout the ectoderm, but is still excluded from the procephalon (Fig. 5B). Starting at stage 11, high levels of *hth* mRNA are detected in the thoracic region whereas *hth* expression in the abdominal segments start to decline (Fig. 5C,D). Two of the head segments, the mandible and maxilla, express moderate levels of *hth*, whereas expression is absent from the labium. At the same time *hth* expression becomes evident in the developing visceral mesoderm and a very high level of expression appears in the clypeolabrum. At stages 13-14, the levels of *hth* transcripts remain high in the head and thorax and decline further in the abdomen. *hth* expression appears also in the rudiments of the Malpighian tubules and the developing central nervous system (Fig. 5E). During stages 15-17 the ectodermal expression of *hth* declines whereas expression in the CNS becomes more prominent (Fig. 5F). The expression of *hth* in the CNS is graded; it is high in the anterior portion of the VNC and weak in its posterior portion.

To test whether any of the enhancer detectors inserted in the *hth* locus is a good reporter of *hth* expression we stained embryos of all the insertional strains with an anti-β-

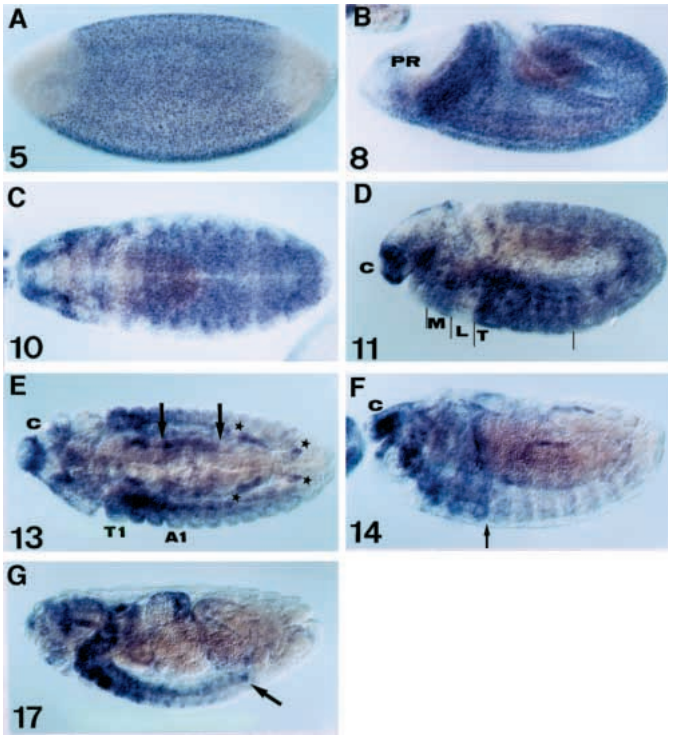

Fig. 5. The expression pattern of *hth.* In situ hybridization to whole embryos with digoxigenin-labeled *hth* cDNA probe. (A) Cellular blastoderm. (B) Gastrulation; PR, procephalon. (C) Ventral view of a stage-10 embryo. (D) A stage-11 embryo; C, clypeolabrum; M, mandible; L, labium; T, thorax. (E) Dorsal view of a stage-13 embryo. The rudiments of the Malpighian tubules are indicated by asterisks, and the visceral mesoderm with arrows. (F) A stage-14 embryo. The border between the domain of strong *hth* expression (in thoracic segments) and weak *hth* expression (in abdominal segments) is indicated by an arrow. (G) A stage-17 embryo; the arrow indicates the posterior end of the VNC.

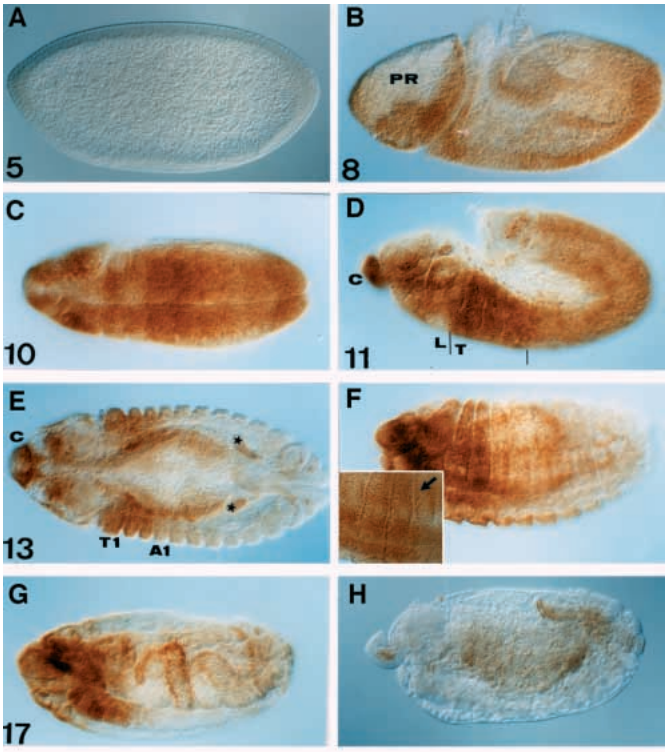

Fig. 6. HTH protein distribution. (A-G) Wild-type embryos immunostained with polyclonal anti-HTH serum (AS1924). (A) Cellular blastoderm. (B) Gastrulation; PR, procephalon.(C) Ventral view of a stage-10 embryo. Note that at this stage the HTH protein is already detected in nuclei. (D) Late stage-11 embryo; C, clypeolabrum; L, labium; T, thorax. (E) Dorsal view of a stage-13 embryo. The rudiments of the Malpighian tubules are indicated by asterisks. (F) A stage-14 embryo. The insert shows a closeup view of the ectodermal staining in segments T1-A. Note that the staining is nuclear and that the HTH protein appears on both sides of the T3/A1 segmental groove. (G) A stage-17 embryo. (H) A homozygous hth^{K1-8} embryo immunostained with the anti-HTH serum. HTH immunoreactivity is not detected in homozygous mutant embryos, which can be identified by the abnormal morphology of their clypeolabrum and CNS.

galactosidase mAb. We found that the *P{lacZ, w+}1422-4* insertion is an excellent reporter of *hth* expression (data not shown).

To determine the distribution of the HTH protein, we stained whole embryos with anti-HTH serum. The distribution of the HTH protein was very similar to the distribution of *hth* mRNA (compare Figs 5 and 6), although the protein was not detected prior to the beginning of gastrulation (stage 6). During gastrulation (stages 6-8), the HTH protein was localized to the cytoplasm (Fig. 6B). At stage 9 (germband extension) HTH started to accumulate in nuclei in a spatially regulated fashion (Fig. 6C-G). Nuclear localization was detected in the ectoderm, and in specific cells within the thoracic portion of the VNC (see also Fig. 8E) and within the visceral mesoderm (data not shown).

To determine whether strong loss-of-function alleles of *hth* abolish *hth* transcription or protein production, we examined the distribution of *hth* mRNA and protein in mutant embryos. As shown in Fig. 6H, hth^{K1-8}/hth^{K1-8} embryos lack detectable levels of the HTH protein. These embryos lacked also detectable levels of *hth* transcripts (data not shown).

hth expression is regulated by genes of the *bithorax* complex

Starting at germband extension, and throughout the rest of embryonic development, *hth* expression is modulated in a segment-specific fashion. Most notable is the repression of *hth* expression in the ectoderm and VNC of abdominal segments during late stages of embryonic development (Fig. 7A). The genes of the homeotic complexes are the major regulators of segmental identity (reviewed by Lewis, 1978). We therefore examined *hth* expression in embryos deficient for the *abd-A* gene (iab^{D24}), or the *abd-A* and *Ubx* genes together ($Df(3R)Ubx^{109}$). In the absence of *abd-A* activity *hth* expression was derepressed in the abdominal ectoderm in cells along the segment boundaries (Fig. 7B-D). In the absence of both *abd-A* and *Ubx*, the derepression was more prominent; *hth* was expressed in ectodermal cells throughout the segment (Fig. 7E). In addition, a uniform level of the HTH protein was observed in all the thoracic and abdominal neuromers of $Df(3R)Ubx^{109}$ homozygous embryos (Fig. 8M).

HTH is required and sufficient for nuclear localization of EXD

The spatial distribution of the HTH protein in wild-type embryos, from stage 10 until the end of embryonic development, is very similar to the expression pattern of the EXD protein (Rauskolb et al., 1993; Flegel et al., 1993; Aspland and White, 1997). Unlike *exd*, *hth* is not expressed maternally. The subcellular localization of HTH also resembles the subcellular localization of EXD (Mann and Abu-Shaar, 1996; Aspland and White, 1997).

The similar PNS phenotypes associated with mutations in the *hth* and *exd* genes, and the colocalization of the HTH and EXD proteins, prompted us to test whether one of these proteins regulates the other protein's spatial distribution. To determine whether EXD is required for proper distribution of HTH we stained *exd*-deficient embryos with anti-HTH serum. We found that embryos lacking both maternal and zygotic expression of EXD exhibited very low levels of HTH-specific immunoreactivity as compared to their siblings (with zygotic

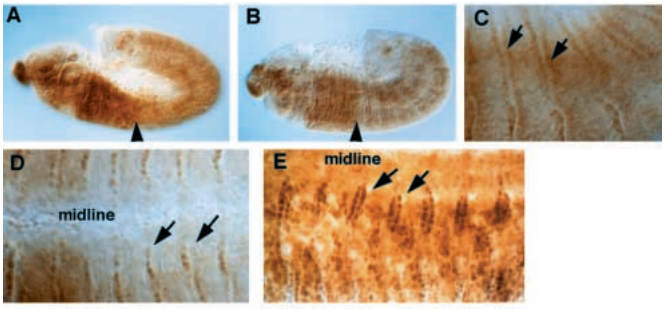

Fig. 7. HTH expression is regulated by Abd-A and Ubx. Embryos immunostained with polyclonal anti-HTH serum (AS1924). (A) Late stage-11 wild-type embryo. The border between the domain of strong thoracic HTH expression and weak abdominal HTH expression is indicated by an arrowhead. (B-D). Stage-12 embryos homozygous for a null mutation in *abd-A* ($iab\text{-}2^{D24}$). Note that HTH is expressed along the segment boundaries in all abdominal segments (arrows). C and D are lateral and ventral closeup views respectively. (E) Ventral view of an embryo homozygous for the $Df(3R)Ubx^{109}$ deletion. HTH expression along abdominal segment boundaries is indicated by arrows.

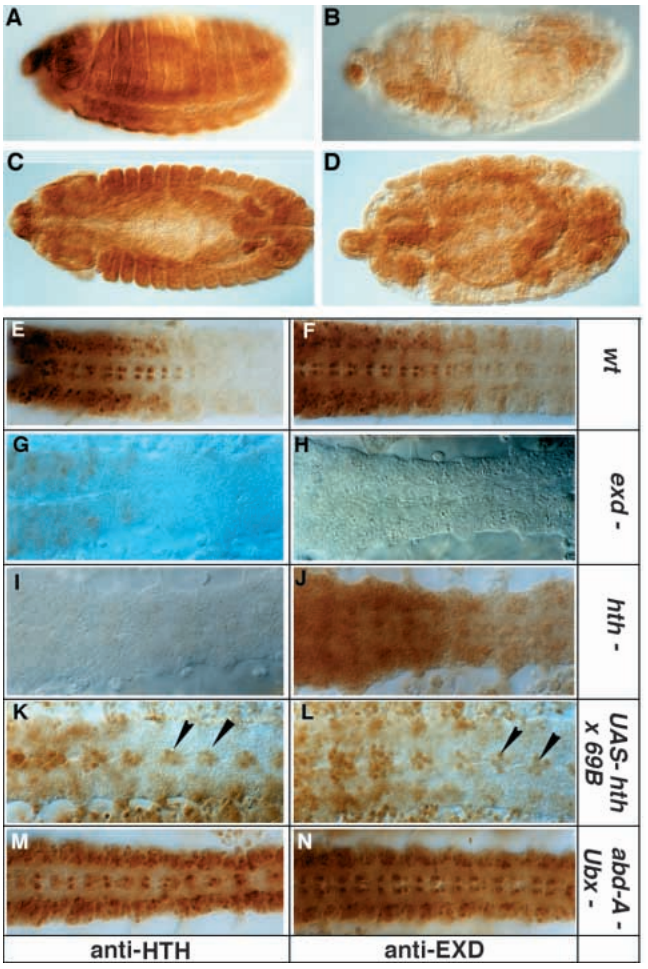

Fig. 8. Regulatory interactions between HTH and EXD. (A,B) Embryos derived from exd^{XP11}/exd^{XP11} germline clones immunostained with polyclonal anti-HTH serum. The embryo in A received a wild-type copy of *exd* from its father. The embryo in B did not receive zygotic contribution of *exd* from its father and exhibits very reduced levels of HTH immunoreactivity. (C,D) Embryos derived from $hth^{K1-8}/TM6$ parents immunostained with anti-EXD antibody. The embryo in C is heterozygous and the embryo in D is homozygous for the hth^{K1-8} mutation. Note the lack of nuclear accumulation of EXD in D. (E-N) Dissected VNCs of stage-16 embryos immunostained with anti-HTH (E,G,I,K,M) or anti-EXD antibodies (F,H,J,L,N). (E,F) Wild-type embryos. (G,H) VNC of embryos deficient for maternal and zygotic *exd*. (I,J) VNC of embryos homozygous for the hth^{K1-8} mutation. (K,L) VNC of embryos with ectopic expression of *hth*. The arrowheads point to neuronal cells that normally do not express *hth*. Note that the same cells exhibit nuclear localization of EXD. (M,N) VNC of embryos deficient for the *abd-A* and *Ubx* genes ($Df(3R)Ubx^{109}$). EXD and HTH are expressed in all the neuromers and exhibit very similar subcellular distribution.

expression of *exd*) or wild-type embryos (Fig. 8A,B and E,G). These results demonstrate that EXD is required for maintaining normal levels of the HTH protein in the embryo. We do not know at present whether EXD is required for *hth* transcription, protein production or stabilization of the HTH protein.

To determine whether HTH is required for proper distribution of EXD we stained *hth* mutant embryos with anti-EXD antibody. The overall level of the EXD protein was only slightly reduced in homozygous hth^{K1-8}/hth^{K1-8} embryos as compared to $hth^{K1-8}/+$ embryos from the same preparation (Fig. 8C,D). However, a dramatic change was observed in the subcellular localization of EXD. In homozygous hth^{K1-8}/hth^{K1-8} embryos the EXD protein was localized mainly to the cytoplasm and did not accumulate in nuclei (Fig. 8C,D and F,I,J). This data indicates that HTH is required, either directly or indirectly, for the nuclear localization of EXD.

To establish whether *hth* expression is sufficient for driving nuclear localization of EXD, we examined the distribution of the EXD protein in embryos expressing *hth* ectopically. To drive ectopic *hth* expression we crossed flies carrying a *UAS-hth* transgene (Pai et al., 1998) to flies of the 69B-GAL4 strain, which drives GAL4 expression in multiple embryonic tissues (Brand and Perrimon, 1993). We found that in most embryonic tissues, ectopic expression of *hth* resulted in ectopic nuclear localization of EXD (Fig. 8K,L). In embryos lacking *abd-A* and *Ubx* activity, both HTH and EXD were expressed in all the abdominal neuromers and exhibited very similar patterns of nuclear localization (Fig. 8M,N). These data indicate that HTH is sufficient to drive nuclear localization of EXD. Further experiments are required to establish whether the ectopic HTH protein induces both expression and nuclear localization of EXD, or leads only to a transport of cytoplasmic EXD to the nucleus.

DISCUSSION

The effects of *hth* on LCh5 identity

Loss-of-function mutations in the *hth* gene lead to a dorsal instead of lateral localization of the abdominal LCh5 neurons. In addition, the number of the affected neurons is reduced from five to three and their orientation is altered. Similar, but not identical effects on the number, position and orientation of these neurons are caused by mutations in the *abd-A* gene that transform the LCh5 neurons into DCh3 neurons. One possibility is therefore that mutations in *hth* cause some kind of homeotic transformation of the LCh5 neurons toward the identity of DCh3 neurons. Another possibility is that mutations in *hth* transform the LCh5 neurons into A8-LCh3 neurons, which exhibit an intermediate DCh3/LCh5 position and orientation (Fig. 2A). Whether the 'dorsal chordotonals' phenotype of *hth* reflects a transformation of the LCh5 neurons into a DCh3 or A8-LCh3 identity remains an open question. The position of the affected neurons within the segment suggests a transformation towards a DCh3 identity. However, the observation that posterior segments are more susceptible to this phenotype, and the cuticular phenotype caused by *hth* mutations, supports a posterior transformation toward an A8-LCh3 identity. In addition, the observation that *abd-A/hth* double mutants exhibit an intermediate phenotype may support a posterior transformation by *hth* mutations as opposed to anterior transformation by *abd-A* mutations. In any case, both the thoracic DCh3 neurons and the A8-LCh3 neurons differ from the LCh5 neurons in that they do not depend on *abd-A* for their development. The thoracic DCh3 neurons require the *Ubx* gene for their development (Heuer and Kaufman, 1992). It is not known which homeotic genes are required for the development of the A8-LCh3 neurons (*abd-B* mutations have no effect on the pattern of the PNS (Heuer and Kaufman, 1992).

The cuticular phenotype of *hth* mutant embryos is similar to the cuticular phenotype of embryos that lack maternal and zygotic expression of *exd* (Peifer and Wieschaus, 1990). This cuticular phenotype is not consistent with the chordotonals phenotype of *hth* (or *exd*) in terms of which segments are transformed phenotypically to which. However, although sensory organs and epidermal structures are derived from the same ectodermal tissue, the PNS phenotypes of homeotic mutations do not always conform with simple homeotic transformations. For example, whereas mutations in the *Antennapedia* gene transform the cuticle of thoracic segments T2 and T3 to a T1 identity (Wakimoto and Kaufman, 1981), no parallel phenotype is observed in the PNS (Heuer and Kaufman, 1992). Moreover, null mutations in the *abd-A* gene transform the cuticle of segments A2-A7 toward the identity of A1 (Sanchez-Herrero et al., 1985), but the LCh5 neurons are transformed into DCh3 neurons of T3 identity (Karch et al., 1990; Heuer and Kaufman, 1992; Salzberg et al., 1994). It is therefore possible that *exd* and *hth* lead to posterior homeotic transformations in the cuticle of abdominal segments (A1 to A3, A2 to A5 etc, see Peifer and Wieschaus, 1990), but lead to a different type of transformation of the LCh5 neurons (i.e. A1-A7 to A8).

The precursors of the LCh5 neurons originate from five individual ectodermal cells in a two-phase process that requires the activity of the *rhomboid* gene and the EGF-receptor signaling pathway (Okabe and Okano, 1997). The first three precursors are determined by the activity of *atonal* and the neurogenic genes. In the second phase, the three precursors express the *rhomboid* gene, which induces the recruitment of two additional precursors through the activity of the EGF-receptor pathway. Thus, an alternative possibility we need to consider is that *hth* affects the EGF-dependent Ch neural induction. The loss of ES and MD neurons in the anterior compartment of abdominal segments, which is caused by strong hypomorphic alleles of *hth*, cannot be correlated with a simple homeotic transformation. Further analyses are required to establish whether *hth* is required for the formation of these neurons, for their survival, or for the determination of their segmental identity.

Requirement for EXD in PNS development

Zygotic expression of *exd* is not required for normal pattern formation in the PNS, indicating that *exd* is required for this process prior to stage 10 when zygotic expression can be detected for the first time (Rauskolb et al., 1993). The observation that maternal expression of *exd* is necessary for normal PNS development further supports this notion, because maternally derived *exd* mRNA does not persist in the embryo beyond stage 8 (Rauskolb et al., 1993). Whereas zygotic expression of *exd* is not absolutely required for normal PNS development, it can partially compensate for the loss of maternally expressed *exd*. These data suggest that the developmental time window during which *exd* is required for proper pattern formation in the PNS is between stages 8-10. During the same time window, both EXD and HTH start to accumulate in nuclei. Starting at stage 11, the level of EXD and HTH expression in the abdominal segments start to decrease. Thus, EXD and HTH are required for LCh5 development prior to, and/or concurrently with, the formation of the first LCh5 precursors. We did not detect *hth* expression in the LCh5 precursors themselves after their delamination; however, the ectodermal cells from which the precursors are derived do express it.

Why do the LCh5 neurons remain dorsal in the absence of *hth* activity?

Although the process of Ch organ migration and rotation is not understood, we can divide the system conceptually into two components: the neuronal cells and their environment (or the receiving and signaling components of the pathway, respectively). We can think of two scenarios that are not mutual exclusive. One is that *hth* affects the homeotic identity of the LCh5 neurons themselves. The other possibility is that *hth* affects the environment in which these neurons form and migrate. In midgut development *abd-A* and *Ubx*, which are expressed in neighboring parasegments of the visceral mesoderm, were shown to regulate *dpp* and *wingless* expression, which affect the underlying endoderm (Manak et al., 1995). It is possible that the influence of HTH and EXD on Abd-A activity in the ectoderm affects signaling molecules such as Wingless and DPP, which in turn affect the localization of the Ch neurons. For example the *dpp* gene was recently shown to control tracheal cell migration along the dorso-ventral axis of the embryo (Vincent et al., 1997). Support for this idea comes from phenotypic analysis of *hth* mutations in adult flies. Loss of *hth* activity in eye imaginal discs results in ectopic eye formation in the ventral head tissue, whereas ectopic expression of *hth* suppressed normal eye development (Pai et al., 1998). These phenotypes are consistent with a role for HTH in activating Wingless and/or repressing DPP signaling (Ma and Moses, 1995; Treisman and Rubin, 1995; Chanut and Heberlein, 1997; Pignoni and Zipurski, 1997). In addition, *wingless* expression was reduced in the ectoderm of stage 11 hth^- embryos (data not shown).

Does *hth* function solely through *exd*?

The relationship between *hth* and *exd* is probably not that simple, as both of them are required for each other's normal distribution in the embryo. HTH is required for nuclear localization of EXD, but we do not know whether EXD is required for nuclear localization of HTH. When HTH was ectopically expressed in the embryo it generally assumed a nuclear localization. However, in such experiments a concomitant ectopic nuclear localization of EXD was observed. The subcellular distribution of ectopically expressed HTH in an exd^- background has not been established yet and the level of endogenous HTH is not maintained in such a genetic background. *hth* transcription starts at cellular blastoderm, long before EXD becomes nuclear. Therefore it is not likely that *exd* is required for *hth* transcription. Moreover, HTH production is not completely abolished in the absence of *exd*. One possible scenario is that a direct interaction between EXD and HTH stabilizes the latter.

Is the nuclear localization of EXD the only role of HTH? Further experiments are required to answer this question. However, the data gathered so far suggests that if not the only role, this is probably the major role of *hth*. The loss-of-function phenotypes of *hth* and *exd* are extremely similar both in the embryo and in adults (Pai et al., 1998). We did not observe any phenotype in *hth* mutants that was not detected in *exd* mutants. In addition, the *dpp* enhancers known to be directly regulated

by Ubx and Abd-A (Capovilla et al., 1994; M. Capovilla and J. Botas, unpublished) responded similarly in vivo to the loss of either *exd* or *hth* function (Chan et al., 1994; M. Capovilla, personal communication). As a homeodomain-containing protein, HTH is expected to bind DNA and affect transcription of effector genes. The mammalian homologue of *hth*, *meis1*, was shown to be coactivated with the homeotic genes *Hoxa7* and *Hoxa9* in murine myeloid leukemia formation (Nakamura et al., 1996b). However it is not known whether *meis1* cooperates with homeotic genes under normal physiological conditions. Whether HTH functions (like EXD) as a transcription regulator on its own and whether it interacts directly with homeotic genes, or binds to homeotic genes in a complex with EXD, are some of the most interesting questions remaining to be elucidated.

We are truly grateful to Hugo Bellen in whose laboratory this project was initiated, and we thank Sharyl Martini for technical assistance in the initial stages of this work. We thank S. Benzer, M. Capovilla, S. Cohen, D. Duncan, I. Duncan, A. Jarman, R. Mann and R. A. H. White for antibodies and fly strains. We thank M. Capovilla and J. Botas for sharing unpublished data, and M. Capovilla for a helpful discussion. This work was supported by grants to A. S. from the 'Israel Cancer Association' and 'The Israel Science Foundation' founded by the Israel Academy of Sciences and Humanities – Charles H. Revson Foundation, and by grants to Y. H. S. from the National Science Council (NSC-86-2313-B-001-003, NSC-87-2311-B-001-106), Taiwan, Republic of China. C.-Y. P. is supported by a National Science Council Postdoctoral Fellowship.

REFERENCES

Aspland, S. E. and White, R. A. H. (1997). Nucleoplasmic localisation of *extradenticle* protein is spatially regulated throughout development in *Drosophila*. *Development* **124**, 741-747.

Blochlinger, K. R., Bodmer, R., Jack, J. W., Jan, L. Y. and Jan, Y. N. (1988). Primary structure and expression of a product from *cut*, a locus involved in specifying sensory organ identity in Drosophila. *Nature* **333**, 629-635.

Blochlinger, K., Bodmer, R., Jan, L. Y. and Jan, Y. N. (1990). Patterns of expression of Cut, a protein required for external sensory organ development in wild-type and *cut* mutant Drosophila embryos. *Genes Dev.* **4**, 1322-1331.

Blochlinger, K., Jan, L. Y. and Jan, Y. N. (1991). Transformation of sensory organ identity by ectopic expression of Cut in Drosophila. *Genes Dev* **5**, 1124-1135.

Bodmer, R., Barbel, S., Shepherd, S., Jack, J. W., Jan, L. Y. and Jan, Y. N. (1987). Transformation of sensory organs by mutations of the *cut* locus of *D. melanogaster*. *Cell* **51**, 293-307.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Campos-Ortega, J. A. (1995). Genetic mechanisms of early neurogenesis in *Drosophila melanogaster*. *Mol. Neurobiol.* **10**, 75-89.

Campos-Ortega, J. A. and Hartenstein, V. (1985). *The Embryonic Development of* Drosophila melanogaster. Springer-Verlag: Berlin, Heidelberg, New York, Tokyo.

Capovilla, M., Brandt, M., and Botas, J. (1994). Direct regulation of *decapentaplegic* by *Ultrabithorax* and its role in Drosophila midgut morphogenesis. *Cell* **76**, 461-475.

Chan, S.-K., Jaffe, L., Capovilla, M., Botas, J. and Mann, R. (1994). The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with Extradenticle, another homeoprotein. *Cell* **78**, 603-615.

Chanut, F., and Heberlein, U. (1997). Role of *decapentaplegic* in initiation and progression of the morphogenetic furrow in the developing Drosophila retina. *Development* **124,** 559-567.

Doe, C. Q. (1996). Asymmetric cell division and neurogenesis. *Curr. Opin. Genet. Dev.* **6**, 562-566.

Flegel, W. A., Singson, A. W., Margolis, J. S., Bang, A. G., Posakony, J. W. and Murre, C. (1993). *Dpbx*, a new homeobox gene closely related to the human proto-oncogene *pbx1* Molecular structure and developmental expression. *Mech. Dev.* **41**, 155-161.

Flybase (1997). The Drosophila Genome Database. http://cbbridges.harvard.edu.

Ghysen, A., Dambly-Chaudiere, C., Aceves, E., Jan, L. Y. and Jan, Y. N. (1986). Sensory neurons and peripheral pathways in Drosophila embryos. *Roux's Arch. Dev. Biol.* **195**, 281-289.

Goodman, C. S., Bastiani, M. J., Doe, C. Q., Du Lac, S., Helfand, S. L., Kuwada, K. Y. and Thomas, J. B. (1984). Cell recognition during neuronal development. *Science* **225**, 1271-1279.

Hartenstein, V. (1987). The influence of segmental compartmentalisation on the development of the larval peripheral nervous system in *Drosophila melanogaster*. *Dev. Biol.* **196**, 101-112.

Hassan, B. and Vaessin, H. (1996). Regulatory interactions during early neurogenesis in *Drosophila*. *Dev. Genet.* **18**, 18-27.

Heuer, J. and Kaufman, T. (1992). Homeotic genes have specific functional roles in the establishment of the *Drosophila* embryonic peripheral nervous system. *Development* **115**, 35-47.

Jan, L. Y. and Jan, Y. N. (1993). The peripheral nervous system. In *The Development of* Drosophila melanogaster (ed. M. Bate and A. Martinez-Arias), pp. 1207-1244. Cold Spring Harbor Laboratory Press.

Jarman, A. P., Grell, E. E., Ackerman, L., Jan, L. Y. and Jan, Y. N. (1994). *atonal* is the proneural gene for *Drosophila* photoreceptors. *Nature* **369**, 398-403.

Jürgens, G., Wieschaus, E., Nusslein-Volhard, C. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*: II. Zygotic loci on the third chromosome. *Roux's Arch. Dev. Biol.* **193**, 283-295.

Kania, A., Salzberg, A., Baht, M., D'Evelyn, D., He, Y., Kiss, I. and Bellen, H. J. (1995): *P*-element mutations affecting embryonic peripheral nervous system development in *Drosophila melanogaster*. *Genetics* **139**, 1663-1678.

Karch, F., Bender, W. and Weiffenbach, B. (1990). *Abdominal-A* expression in *Drosophila* embryos. *Genes Dev.* **4**, 1573-1578.

Kellerman, K. A., Mattson, D. M. and Duncan, I. (1990). Mutations affecting the stability of the fushi tarazu protein of Drosopila. *Genes Dev.* **4**, 1936-1950.

Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* **276**, 565-570.

Lindsley, D. L. and Zimm G. G. (1992). *The Genome of* Drosophila melanogaster. Academic Press: San Diego, CA.

Ma, C. and Moses, K. (1995). Wingless and patched are negative regulators of the morphogenetic furrow and can affect tissue polarity in the developing Drosophila compound eye. *Development* **121**, 2279-2289.

Manak, J. R., Mathies, L. D. and Scott, M. P. (1995). Regulation of a *decapentaplegic* midgut enhancer by homeotic proteins. *Development* **120**, 3605-3619.

Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K. and Efstratiadis, A. (1978). The isolation of structural genes from libraries of eucaryotic DNA. *Cell* **15**, 687-701.

Mann, R. S. and Abu-Shaar, M. (1996). Nuclear import of the homeodomain protein Extradenticle in response to Wg and Dpp signalling. *Nature* **383**, 630-633.

Moskow, J. J., Bullrich, F., Hubener, K., Daar, I. O. and Buchberg, A. M. (1995). *Meis1*, a *PBX1*-related homeobox gene involved in myeloid leukemia in BXH-2 mice. *Mol. Cell. Biol.* **15**, 5434-5443.

Nakamura, T., Jenkins, N. A. and Copeland, N. G. (1996a). Identification of a new family of Pbx-related homeobox genes. *Oncogene* **13**, 2235-2242.

Nakamura T., Largaespada, D. A., Shaughnessy, J. D., Jenkins, N. and Copeland, N. G. (1996b). Cooperative activation of *Hoxa* and *Pbx1*-related genes in murine myeloid leukaemias. *Nature Genetics* **12,** 149-153.

Okabe, M. and Okano, H. (1997). Two-step induction of chordotonal organ precursors in Drosophila embryogenesis. *Development* **124**, 1045-1053.

Pai, C.-Y., Kou, T.-S., Jaw, T. J., Kurant, E., Chen, C.-T., Bessarab, D. A., Salzberg, A. and Sun, Y. H. (1998). The Homothorax homeoprotein activates the nuclear localization of another homeoprotein, Extradenticle, and supresses eye development in Drosophila. *Genes Dev*. (in press).

Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. In Drosophila melanogaster: *Practical Uses in Cell and Molecular Biology*. (ed. L. S. B. Goldstein and E. A. Fyrberg), pp. 445-487. Academic Press Inc.: San Diego.

Peifer, M. and Wieschaus, E. (1990). Mutations in the *Drosophila* gene *extradenticle* affect the way specific homeodomain proteins regulate segmental identity. *Genes Dev.* **4**, 1209-1223.

Pignoni, F. and Zipurski, S. L. (1997). Induction of Drosophila eye development by Decapentaplegic. *Development* **124,** 271-278.
Posakony, J. W. (1994). Nature versus nurture: asymmetric cell divisions in Drosophila bristle development. *Cell* **76**, 415-418.
Rauskolb, C., Peifer, M. and Wieschaus, E. (1993). *extradenticle*, a regulator of homeotic gene activity, is a homolog of the homeobox-containing human protooncogene PBX1. *Cell* **74**, 1101-1112.
Salzberg, A., D'Evelyn, D., Schulze, K. L., Lee, J.-K., Strumpf, D., Tsai, L. and Bellen, H. J. (1994). Mutations affecting the pattern of the PNS in *Drosophila* reveal novel aspects of neuronal development. *Neuron* **13**, 268-287.
Salzberg, A., Prokopenko, S. N., He, Y.-C., Tsai, P., Deák, P., Pál, M., Maróy, P., Glover, D. M. and Bellen, H. J. (1997). *P* Element insertion alleles of essential genes on the third chromosome of *Drosophila melanogaster*: mutations affecting embryonic PNS development. *Genetics* **147**, 1723-1741.
Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Second Edition. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
Sanchez-Herrero, E., Vernos, I., Marco, R. and Morata, G. (1985). Genetic organization of *Drosophia bithorax* complex. *Nature* **313**, 108-113.
Seeger, M., Tear, G., Ferres-Marco, D. and Goodman, C. S. (1993). Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. *Neuron* **10**, 409-426.
Smith, J. E. J., Bollekens, J. A., Inghirami, G. and Takeshita, K. (1997). Cloning and mapping of the MEIS1 gene, the human homolog of a murine leukomogenic gene. *Genomics* **43**, 99-103.
Steelman, S., Moskow, J. J., Muzynski, K., North, C., Druck, T., Montgomery, J. C., Huebner, K., Daar, I. O. and Buchberg, A. M. (1997). Identification of a conserved family of *Meis1*-related homeobox genes. *Genome Res.* **7**, 142-156.
Sun, Y. H., Tsai, C.-J., Green, M. M., Chao, L.-L., Yu, C.-T., Jaw, T. J., Yeh, J.-Y. and Bolshakov, V. N. (1995). *white* as a reporter gene to detect transcriptional silencers specifying position-specific expression during *Drosophila melanogaster* eye development. *Genetics* **141**, 1075-1086.
Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98,** 81-85.
Treisman, J. E. and Rubin, G. M. (1995). *wingless* inhibits morphogenetic furrow movement in the Drosophila eye disk. *Development* **121,** 3519-3527.
van Dijk, M. A. and Murre, C. (1994). *extradenticle* raises the DNA binding specificity of homeotic selector gene products. *Cell* **78**, 617-624.
Vincent, S., Ruberte, E., Grieder, N. C., Chen, C.-K., Haerry, T., Schuh, R. and Affolter, M. (1997). DPP controls tracheal cell migration along the dorsoventral body axis of the *Drosophila* embryo. *Development* **124**, 2741-2750.
Wakimoto, B. T. and Kaufman, T. C. (1981). Analysis of larval segmentation in lethal genotypes associated with the Antennapedia complex in *Drosophila melanogaster*. *Dev. Biol.* **81**, 51-64.
Wilson, C., Pearson Kurth, R., Bellen, H. J., O'Kane, C. J., Grossniklaus, U. and Gehring, W. J. (1989). P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in Drosophila. *Genes Dev.* **3**, 1301-1313.
Zinn, K., McAllister, L. and Goodman, C. S. (1988). Sequence analysis and neuronal expression of *fasciclin I* in grasshopper and Drosophila. *Cell* **53**, 577-587.

Note added in proof

The regulation of EXD nuclear localization by HTH has also been reported.

Rieckhof, G. E., Casares, F., Don Ryoo, H., Abu-Shaar, M. and Mann, R. S. (1997). Nuclear translocation of extradenticle requires *homothorax*, whic h encodes an extradenticle-related homeodomain protein. *Cell* **91**, 171-183.