Development 129, 4193-4203 (2002)
Printed in Great Britain © The Company of Biologists Limited
DEV7972

Formation of neuroblasts in the embryonic central nervous system of *Drosophila melanogaster* is controlled by *SoxNeuro*

Marita Buescher[1,*], Fook Sion Hing[2] and William Chia[1]

[1]MRC Centre for Developmental Neurobiology, King's College London, Guy's Campus, New Hunts House, London SE1 1UL, UK
[2]Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609, Republic of Singapore
*Author for correspondence (e-mail: marita.buescher@kcl.ac.uk)

Accepted 11 June 2002

SUMMARY

Sox proteins form a family of HMG-box transcription factors related to the mammalian testis determining factor SRY. Sox-mediated modulation of gene expression plays an important role in various developmental contexts. *Drosophila* SoxNeuro, a putative ortholog of the vertebrate Sox1, Sox2 and Sox3 proteins, is one of the earliest transcription factors to be expressed pan-neuroectodermally. We demonstrate that *SoxNeuro* is essential for the formation of the neural progenitor cells in central nervous system. We show that loss of function mutations of *SoxNeuro* are associated with a spatially restricted hypoplasia: neuroblast formation is severely affected in the lateral and intermediate regions of the central nervous system, whereas ventral neuroblast formation is almost normal. We present evidence that a requirement for SoxNeuro in ventral neuroblast formation is masked by a functional redundancy with Dichaete, a second Sox protein whose expression partially overlaps that of SoxNeuro. Genetic interactions of *SoxNeuro* and the dorsoventral patterning genes *ventral nerve chord defective* and *intermediate neuroblasts defective* underlie ventral and intermediate neuroblast formation. Finally, the expression of the *Achaete-Scute* gene complex suggests that *SoxNeuro* acts upstream and in parallel with the proneural genes.

Key words: *SoxNeuro*, *Dichaete*, *vnd*, *ind*, Neurogenesis, *Drosophila*

INTRODUCTION

The relatively simple embryonic central nervous system (CNS) of *Drosophila melanogaster* represents an excellent model system in which to study the mechanisms of neural progenitor formation and the generation of cellular diversity. Extensive genetic studies have led to the identification of many molecular components and a picture of the key steps in neurogenesis has emerged. Neurogenesis begins in the blastoderm embryo with the determination of the ventrolateral region as the presumptive neuroectoderm (NE) and requires the products of the *short-gastrulation* (*sog*) and *brinker* (*brk*) genes to exclude anti-neural Dpp-activity (Biehs et al., 1996; Jazwinska et al., 1999). At later stages, single cells within the NE are selected to become neuronal progenitor cells, called neuroblasts (NBs) (for a review, see Campos-Ortega, 1995). NBs delaminate from the ectoderm and undergo a stereotyped program of successive divisions to generate intermediate progenitor cells, called ganglion mother cells (GMCs). Each GMC divides once to produce a pair of post-mitotic neurons or glia.

NB selection requires the interaction of two phenotypically opposite classes of genes: the proneural genes, which promote NB formation, and the neurogenic genes, which inhibit NB formation. Prior to NB formation, three proneural genes of the *achaete/scute* gene complex (*AS-C*), *achaet*e (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*) are expressed in cell clusters at invariant positions within the NE (Campuzano et al., 1985; Martin-Bermudo et al., 1991; Skeath et al., 1992). Proneural gene expression confers neural potential to these cells and single NBs delaminate from within these clusters. Loss of the entire *AS-C* results in the loss of NBs (Jimenez and Campos-Ortega, 1990). The singling out of NBs from within proneural clusters is accomplished through lateral inhibition and requires the function of the neurogenic genes. The interaction of the receptor Notch and its ligand Delta results in an accumulation of the gene products of the *Enhancer of split* gene complex *E(spl)-C*. The *E(spl)-C* antagonizes the maintenance and upregulation of proneural gene expression and promotes the adoption of the non-neural fate (Martin-Bermudo et al., 1995). The cell that is singled out to adopt the neuronal pathway is thought to accumulate lower levels of *E(spl)-C* gene product and therefore is able to upregulate proneural gene expression in a positive auto-feedback loop. Loss of any one neurogenic gene leads to the production of excess NBs.

In total, ~30 NBs per hemisegment delaminate from the NE in five successive waves (SI-SV) and form a stereotypical array of seven anteroposterior and three dorsoventral columns (for a review, see Goodman and Doe, 1993). Each NB has a unique identity and generates an invariant cell lineage (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997). NB identity is specified by the activity of anteroposterior and dorsoventral patterning genes. Overlapping expression of these genes subdivides the NE into a grid of positional information which is established prior to the appearance of proneural clusters (for

a review, see Skeath, 1999). Anteroposterior patterning is mediated by the segment polarity genes which are expressed in transverse stripes within each segment (for a review, see Bhat, 1999). Subdivision of the NE along the DV axis is accomplished through the activity of the homeobox genes *ventral nervous system defective* (*vnd*) (Jimenez et al., 1995; McDonald et al., 1998; Mellerick and Nirenberg, 1995; Skeath et al., 1994), *intermediate neuroblasts defective* (*ind*) (Weiss et al., 1998) and *muscle segment homeobox* gene (*msh*) (Buescher and Chia, 1997; D'Alessio and Frasch, 1996; Isshiki et al., 1997). These genes are expressed in adjacent longitudinal columns and confer ventral, intermediate and lateral specificity respectively, to the NBs that arise from within these domains. Moreover, *vnd* and *ind* play a crucial role in the formation of NBs: loss of *vnd* or *ind* results in the loss of ventral or intermediate NBs, respectively.

Comparative analysis of neurogenesis in vertebrates and *Drosophila* has revealed a remarkable conservation of the mechanisms that underlie the determination of the presumptive NE, which mediate the selection of neural progenitors from within the NE and which govern certain aspects of DV patterning (for a review, see Chitnis, 1999). Many of the key molecular components originally identified in *Drosophila* were found to have orthologs in vertebrate species as diverse as *Xenopus* and zebrafish. Recent studies in *Xenopus* have been aimed at the identification of genes that link neural induction and primary neurogenesis (for a review, see Sasai, 1998). Differential screens designed to uncover genes that are upregulated by the Sog ortholog Chordin have led to the identification of genes of the Sox family (Mizuseki et al., 1998). Sox proteins are transcription factors that contain a high mobility group (HMG) domain and bind to DNA in a sequence-specific manner. Sox proteins have been shown to play a role in many developmental processes (for a review, see Pevny and Lovell-Badge, 1997; Wegner, 1999). However, the mechanisms by which vertebrate Sox proteins promote neurogenesis are as yet poorly understood. Two Drosophila Sox genes – *SoxN* and *Dichaete* are expressed in the developing CNS and thus represent valuable models to study the function of Sox proteins in neurogenesis (Cremazy et al., 2000; Nambu and Nambu, 1996; Russell et al., 1996). *Dichaete* is required for development of the ventral midline, segmentation of the abdomen and the formation of several NBs in the thorax (Ma et al., 1998; Nambu and Nambu, 1996; Russell et al., 1996; Soriano and Russell, 1998; Zhao and Skeath, 2002). We present a study of the function of *SoxN* in the development of the embryonic CNS. *SoxN* is a member of the group B family of Sox proteins and is expressed in a pan-neuroectodermal manner throughout embryonic neurogenesis. We show that mutations of *SoxN* result in a severe hypoplasia in the intermediate and lateral regions of the CNS and demonstrate that *SoxN* and *Dichaete* function is partially redundant with respect to the formation of ventral and intermediate NBs. We show that *SoxN* genetically interacts with the DV patterning genes *vnd* and *ind*. Finally, we present evidence suggesting *SoxN* acts upstream and in parallel to the proneural genes of the *AS-C*.

MATERIALS AND METHODS

Flystocks

Wild-type expression patterns were analyzed in Canton-S embryos or embryos heterozygous for $SoxN^{GA1192}$. The *SoxN* alleles GA1192, C463 and C2139 have been generated by EMS mutagenesis (Seeger et al., 1993) and were subsequently balanced over $CyO^{ftzlacZ}$ or $CyO^{pAct\text{-}GFP}$ (obtained from the Bloomington stock center) to facilitate the identification of homozygous mutant embryos. The following mutant stocks were used: $vnd^{\Delta 38}$ (Chu et al., 1998); $ind^{16.2}$ (Weiss et al., 1998); $Dichaete^{87}$ (Mukherjee et al., 2000); msh^{lttEMS} (Buescher and Chia, 1997); and $E(spl)^{R1}$ (Mari-Beffa et al., 1991). For the genetic mapping of the EMS-induced mutations the second chromosome deficiency kit from the Bloomington stock center was used.

Immunohistochemistry

Embryos were collected, fixed and immunostained as previously described (Yang et al., 1997). Primary antibodies were anti-Eve (1:2000) (Patel et al., 1989), anti-Ftz (1:500) (Doe et al., 1988), anti-Eagle (1:500) (Higashijima et al., 1996), anti-Wor (1:1000) (Yu et al., 2000), anti-Vnd (1:1000) (Chu et al., 1998), anti-Msh (1:500) (Isshiki et al., 1997), anti-L'sc (1:500) (Martin-Bermudo et al., 1991), anti-Ac (Skeath et al., 1992) (Developmental Studies Hybridoma Bank, University of Iowa), anti-Repo (1:500) (Xiong et al., 1994), anti-Ase (1:3000) (Jarman et al., 1993) and anti-β-gal (1:3000) (Promega). Histochemical detection was performed using Jackson Immunoresearch Inc HRP-conjugated secondary antibodies and visualized using the glucose-oxidase-DAB-nickel method as previously described (Yang et al., 1997).

RNA in situ hybridization was carried out as described before (Tautz and Pfeifle, 1989). An plasmid for the generation of an *ind*-specific riboprobe was provided by T. von Ohlen; a plasmid for the generation of a *SoxN*-specific riboprobe was provided by F. Girard.

A GST fusion protein containing a region of the SoxN protein (amino acids 1-432) was produced in *E. coli* using the pGEX4T-1 vector (Pharmacia). The fusion protein was used to immunize mice. Homozygous embryos deficient for the *SoxN* gene (DfN-22) did not show anti-SoxN immunoreactivity demonstrating the specificity of the antibody.

RESULTS

Mutations in *SoxN* cause neural hypoplasia in the intermediate and lateral regions of the CNS

Three mutant alleles of the *SoxN* gene (GA1192, C463 and C2139) (see below) were generated by EMS-mutagenesis in a large screen that was aimed at the identification of novel genes which play a role in axon guidance (Seeger et al., 1993). Mutations in *SoxN* are associated with multiple defects in axon morphology, as evidenced by thinner, interrupted connectives and incompletely formed commissures (data not shown). Moreover, mutant embryos show severe defects in head formation and gut constrictions (Fig. 1I,J). In this study, we have focused on the role of *SoxN* in neurogenesis. All experiments were performed using GA1192, a null mutation of *SoxN*.

Analysis of mutant embryos with antibodies that recognize marker gene expression in subsets of neurons revealed a drastic loss of neurons. During late stages of embryonic development, the protein Even-skipped (Eve) is expressed in ~20 neurons per hemisegment (Fig. 1A): the aCC/pCC and the CQ neurons, which derive from the ventral part of the NE; the RP2 neuron, which derives from the intermediate region; and the El neuron cluster, which arises in a more lateral region (Patel et al., 1989). *SoxN* mutant embryos show a near complete loss of Eve-positive RP2 neurons (98% loss) and EL neuron clusters (100%

loss), whereas the aCC/pCC neurons are only slightly affected (3% loss) and the CQ neurons remain unaffected (0% loss) (*n*=200 hemisegments; Fig. 1B). As all Eve-positive neurons derive from GMCs, which themselves express Eve, we analyzed early mutant embryos for the presence of Eve-positive GMCs. We observed a loss of Eve-expressing parental GMCs occurring with frequencies comparable with that of the loss of their respective neuronal progeny (Fig. 1C,D). To determine if the observed loss of neurons is specific only for Eve-expressing cells, we stained *SoxN* mutant embryos with an antibody against Fushi Tarazu (Ftz) (Doe et al., 1988), a protein that is transiently expressed in large number of GMCs and neurons. Anti-Ftz staining revealed a severe loss of Ftz-positive GMCs/neurons. Strikingly, the loss occurs predominantly in the intermediate and lateral regions of the CNS while the ventralmost region forms almost normally (Fig. 1E,F).

The failure to form specific GMCs/neurons could be explained by loss or mis-specification of the respective parental NBs. To assess NB formation in *SoxN* mutant embryos, we used an antibody against Worniu (Wor), a protein which is expressed in all NBs (Ashraf et al., 1999; Cai et al., 2001; Yu et al., 2000). In wild-type embryos, ~30 NBs delaminate from the NE during embryonic stages 8-11 in five waves (SI-SV). SI NBs form three discrete columns: the ventral column which is made up of three NBs and the MP2 precursor; the intermediate column with two NBs; and the lateral column, which comprises four NBs. At later stages (SII-SV) additional NBs fill the space between these columns.

Anti-Wor staining of stage 9 *SoxN* mutant embryos indicated that SI NB formation in the lateral and intermediate columns is severely impaired (Fig. 2A-C). In the lateral column instead of the wild-type set of four NBs per hemisegment only one/two NBs are formed. Different lateral NBs are differentially affected. For example, NB3-5 fails to form in 82% of the hemisegments, whereas NB2-5 fails to form in only 22% of the hemisegments (for all NB between 50-100 hemisgements scored). We made similar observations with respect to NB formation in the intermediate column, which in wild type is composed of NB-5-3 and NB3-2. Both NBs frequently fail to form in *SoxN* mutant embryos (NB5-3, 14% loss; NB3-2, 67% loss). By contrast, the four NBs of the ventral column form almost normally. Analysis of older mutant embryos with anti-Wor revealed that *SoxN* is also required for the formation of late arising NBs. Stage 11 embryos exhibit drastically reduced numbers of NBs; NBs that do form, appear predominantly in the ventral region. (Fig. 2D,E). These results were confirmed using antibodies against three additional NB marker genes – *hunchback* (Cabrera and Alonso, 1991), *snail* (Alberga et al., 1991) and *klumpfuss* (Yang et al., 1997) (data not shown). Staining of stage 11 *SoxN* mutant embryos with anti-Engrailed antibody revealed no difference to the wild-type Engrailed expression pattern, suggesting that the loss of NBs is not due to segmentation defects (Fig. 2I).

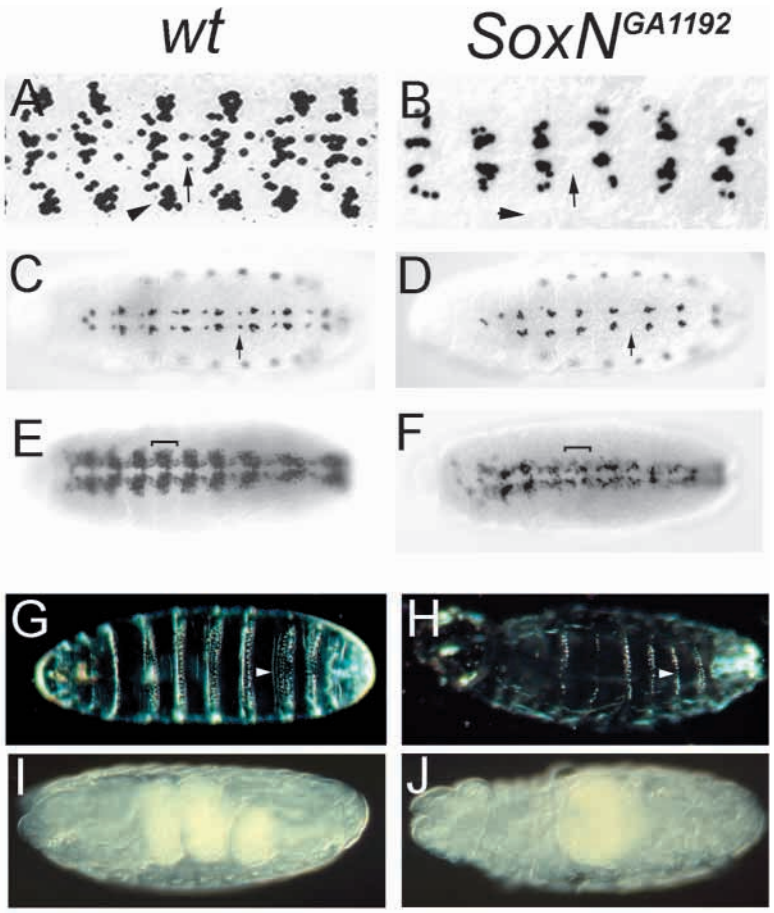

Fig. 1. Mutations in *SoxN* result in the loss of neurons/GMCs and cause multiple morphological defects. (A-D) Immunostaining with anti-Eve antibody. Dorsal view of (A) wild type and (B) *SoxN* dissected stage 16 embryos. Arrows indicate the RP2 neuron (A) or the RP2 neuron position (B); arrowheads mark the EL neuron cluster (A) or the position of the EL neuron cluster (B). Ventral view of (C) wild-type and (D) *SoxN* whole-mount embryos. Arrows indicate GMC4-2a (C) or the GMC4-2a position (D). (E) Wild-type and (F) *SoxN* stage 11 embryos stained with anti-Ftz antibody. Brackets encompass one hemisegment each. Note the drastic loss of neurons/GMCs in intermediate and lateral regions of the CNS in F. (G,H) Ventral view of the cuticle of first instar larva, (G) wild-type and (H) *SoxN*. Arrowheads indicate a denticle belt. Note the reduction of the denticle belts along the AP axis in H. (I,J) Lateral view of stage17 whole-mount embryos. (I) Wild type and (J) *SoxN*. Note the defects in head and gut formation in J. Anterior is towards the left.

To characterize the *SoxN* phenotype with respect to the formation of late arising NBs, we stained mutant embryos with antibodies that label subsets of NBs. Anti-Vnd labels all ventral NBs (Chu et al., 1998), anti-Eagle labels four late forming NBs in the lateral region (Higashijima et al., 1996) and anti-Huckebein-*lacZ* (5953) (Doe, 1992) labels early- and late-forming NBs in the ventral, intermediate and lateral regions. Anti-Eagle and Huckebein-*lacZ* staining are shown in Fig. 2F-I, anti-Vnd staining is not shown. In addition, we used anti-Odd-skipped (Coulter et al., 1990) and anti-Repo (Xiong et al., 1994) to score the MP2

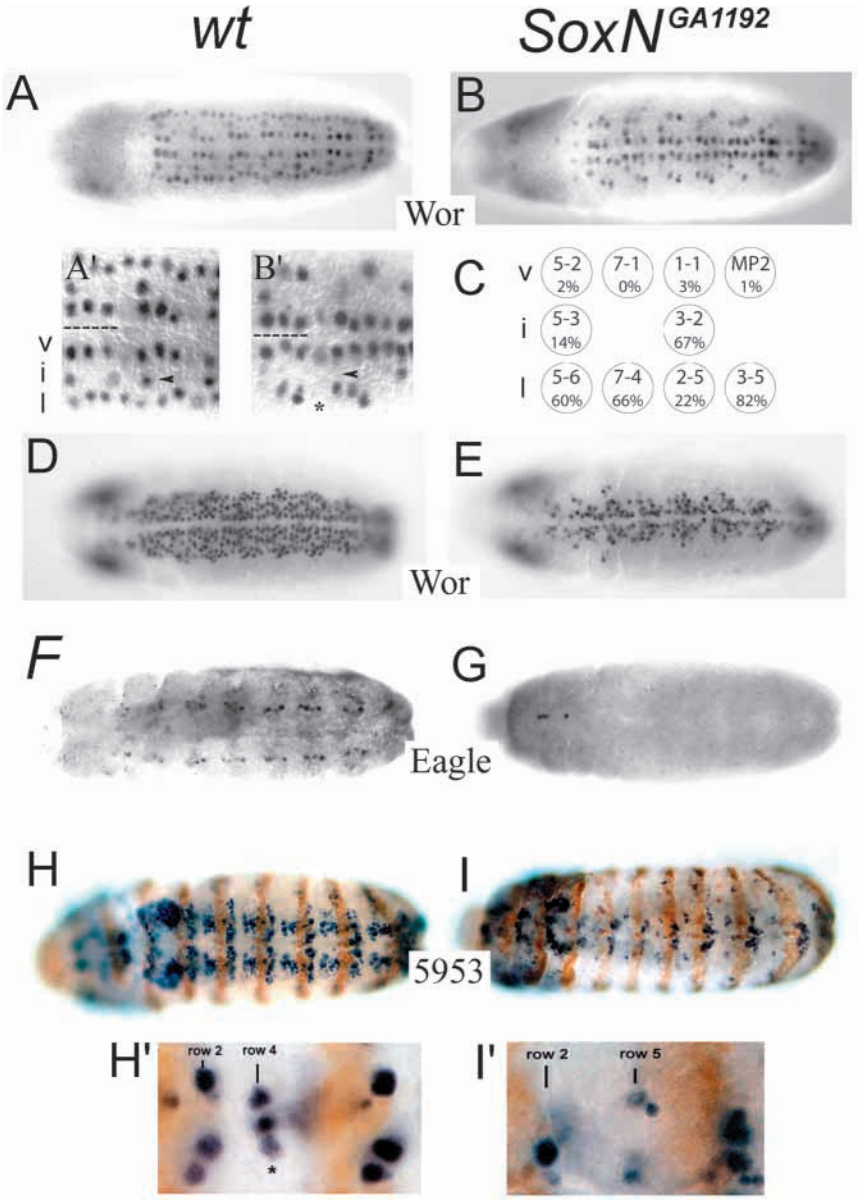

Fig. 2. *SoxN* mutation leads to the loss of lateral and intermediate neuroblasts. (A-E) Immunostaining with anti-Wor antibody. (A) Wild-type and (B) *SoxN* stage 9 embryos; (A′,B′) higher magnification of two consecutive segments of A,B. The broken line indicates the ventral midline; (v) ventral, (i) intermediate and (l) lateral column of NBs. The arrowhead marks NB5-3 (A′) or the position of NB5-3 (B′); the asterisk indicates the position of NB3-5. (C) Quantification of the SI NB phenotype: the percentages of samples showing loss of each NB are given. Note that the loss of NBs predominantly occurs in the intermediate and lateral column. (D) Wild-type and (E) *SoxN* stage 11 embryos. Note the drastic loss of NBs in E. (F) Wild-type and (G) *SoxN* stage 11 embryos stained with anti-Eagle antibody. Note the complete absence of Eagle-positive NBs in G. (H) Wild-type and (I) *SoxN* stage 11 embryos stained with anti-β-gal to detect hkb-*lacZ* (5953) expression (black) and anti-Engrailed (brown) to facilitate the identification of positions along the AP axis. (I) Note the strong reduction of hkb-*lacZ* expression in the intermediate and lateral regions of the neuroectoderm. (H′,I′) Higher magnification of one hemisegment of H,I. Note the loss of NB2-4 in row 2 and the complete absence of hkb-*lacZ* expression in row 4. Ventral views with anterior towards the left.

precursor and the lateral glioblast, respectively (data not shown).

The results are shown in Table 1 and can be summarized as follows: the loss of *SoxN* causes a severe hypoplasia. However, specific spatial and temporal aspects are observed.

(1) *SoxN* is required for the formation of NBs that derive from the lateral and intermediate regions of the NE, but does not appear to play a major role in ventral NB formation. For example, compare the formation of the ventral NB2-1 (9% loss) with that of the lateral NB2-4 (98% loss), both of which are formed at the same time (SIV) and at the same position along the AP axis but at different positions along the DV axis (see anti-Huckebein-*lacZ* staining Fig. 2H-I′).

(2) Late arising NBs are more severely affected than early arising NBs. Compare the moderate frequencies of the loss of SI NBs with the near complete loss of SIV/SV NBs. Accordingly, the most extreme phenotype is observed for late arising, lateral NBs (note the complete loss of anti-Eagle expressing NBs, Fig. 2F,G).

(3) NBs that arise at the same time and in the same column are differentially affected by the loss of *SoxN* (compare the loss of intermediate SI NBs NB3-2(67%) and NB 5-3(14%).

In addition to the CNS, the NE gives rise to the ventral epidermis. To study possible defects of the ventral epidermis, we analyzed the cuticle of unhatched *SoxN* larvae. In wild-type first instar larvae, denticle belts are formed on the ventral side of the eight abdominal segments (Fig. 1G). Each denticle belt is made up of five rows of setae. In *SoxN* mutant larvae, we observed a severe loss of anterior setae, which results in a reduction of the AP expansion of the denticle belts (Fig. 1H). These results indicate that *SoxN* mutations lead to defects in both tissues that derive from the NE: the CNS and the ventral epidermis.

GA1192, C463 and C2139 are loss of function alleles of the *SoxN* gene

From the same EMS stock collection, we recovered three lines GA1192, C463 and C2139, which fail to complement each other and exhibit similar morphological defects. However, the morphological defects observed in C2139 mutant embryos are less severe than those of GA1192 and C463. All three alleles display similar CNS phenotypes either in homozygosity or in heterozygosity with each other. Using deficiencies, we mapped lethality and all phenotypic defects to the cytological position

Table 1. *SoxN* affects the formation of early and late arising neuroblasts

	Loss of neuroblasts*		
Delamination wave	Ventral†	Intermediate†	Lateral†
S1 (stage 8)	NB1-1 (3%)	NB3-2 (67%)	NB2-5 (22%)
	MP2 (1%)	NB5-3 (14%)	NB5-6 (82%)
	NB5-2 (2%)		NB3-5 (60%)
	NB7-1 (0%)		NB7-4 (66%)
S2 (stage 9)	NB2-2 (8%)	NB4-2 (98%)	
		NB6-2 (65%)	
		NB7-2 (86%)	
S3 (stage 10)	NB3-1 (10%)		NB6-4 (97%)
	NB4-1 (7%)		aGB (0%)
	NB6-1 (12%)		
S4 (early stage 11)	NB2-1 (9%)		NB2-4 (98%)
			NB3-3 (100%)
			NB4-4 (99%)
			NB5-4 (24%)
S5 (late stage 11)	NB5-1 (6%)		NB4-3 (100%)
			NB5-5 (95%)
			NB7-3 (100%)

*The loss of individual neuroblasts in % is indicated in parenthesis; for each neuroblast 50-100 hemisegments were scored.

The following markers were used to identify individual neuroblasts:

anti-Wor (Ashraf et al., 1999) – to identify all S1 and S2 neuroblasts

anti-Vnd (Chu et al., 1998) – to identify all ventral neuroblasts, NB6-2 and NB7-2;

anti-Eagle (Higashijima et al., 1996) – to identify NB2-4, NB3-3, NB6-4 and NB7-3;

anti-Hkb-*lacZ* (5953, Doe 1992) – to identify NB1-1, NB2-1, NB2-2, NB4-2, NB4-3, NB4-4, NB5-4, NB5-5 and NB7-3;

anti-Odd-skipped (E. Ward and D. Coulter, unpublished) – to identify MP2; and

anti-Repo (Halter et al., 1995) – to identify aGB.

†The classification of neuroblasts as ventral, intermediate and lateral is based on the expression pattern of *vnd* (Chu et al., 1998), *ind* (Weiss et al., 1998) and *msh* (Isshiki et al., 1997) in the neuroectoderm at stages 8-9.

29F. The phenotype of a homozygous deficiency that removes 29F (DfN-22, breakpoints: 29C;30C) is identical to that of GA1192 and C463, while the weaker CNS phenotype of C2139 is enhanced in heterozygosity with DfN-22. These data strongly suggest that GA1192 and C463 represent amorphic alleles, while C2139 appears to be a hypomorphic allele.

Analysis of the genomic sequence of the 29F region prompted us to choose the *SoxN* locus as a likely candidate gene (Cremazy et al., 2000). SoxN belongs to a family of sequence-specific DNA binding proteins whose common feature is the HMG box. The HMG box of SoxN shares more than 90% amino acid identity with the human group B Sox1, Sox2 and Sox3 proteins, and with *Xenopus* and chicken Sox2. Sox1, Sox2 and Sox3 have been implicated in vertebrate neural development (Collignon et al., 1996; Nishiguchi et al., 1998; Rex et al., 1994; Streit et al., 1997; Uwanogho et al., 1995). Moreover, Drosophila *SoxN*-RNA expression can be detected early in the embryo (stage4), is later found in a pan-neuroectodermal pattern and expression persists until NB formation is completed (Cremazy et al., 2000) (Fig. 4). Thus, the *SoxN* gene expression pattern coincides with the developmental defects that are observed in GA1192, C463 and C2139 mutant embryos. Immunostaining with a polyclonal anti-SoxN antibody (see below) revealed that homozygous GA1192 and C463 embryos are non-immunoreactive, consistent with them being phenotypic null alleles. By contrast, C2139 mutant embryos show a near wild-type expression pattern of SoxN, although the overall expression level is reduced, supporting the notion that C2139 represents a hypomorphic allele (data not shown).

Sequencing of genomic DNA from homozygous C463 embryos revealed an internal deletion of 311 bp (from position 1373-1684; AJ252124), which introduces a frame-shift. The deduced 234 amino acid mutant polypeptide shares the first 215 amino acids with wild-type SoxN protein followed by 19 amino acids of novel peptide sequence. This mutation removes the C-terminal part of the HMG box and all SoxN sequences C-terminal of it (Fig. 3). This polypeptide is most probably non-functional.

SoxN protein expression pattern

To determine the SoxN protein expression pattern, we raised a polyclonal antibody to SoxN protein. Immunostaining with this antibody showed that RNA and protein expression patterns in the NE are virtually identical (Fig. 4A-H) [for a detailed description of the *SoxN* RNA expression pattern see Cremazy et al. (Cremazy et al., 2000)]. We did not observe maintenance of SoxN expression in delaminating NBs; rather SoxN protein levels in NBs are low and transient; they may represent a 'carry-over' of neuroectodermally expressed protein (Fig. 4I). However, a small number of neural progenitor cells in the intermediate region continue to express SoxN and give rise to SoxN-positive progeny (Fig. 4H). It is noteworthy that anti-SoxN staining in stage 9-11 NE appears patchy, suggesting that protein expression – although ubiquitous – is not uniform (Fig. 4J).

SoxN and Dichaete both contribute to the formation of ventral and intermediate neuroblasts

In addition to SoxN a second HMG box protein, Dichaete is expressed prior to and during NB formation (Nambu and Nambu, 1996). Within the NE, Dichaete is expressed from stage 7 to stage 12 in two longitudinal stripes that encompass the ventral and intermediate but not the lateral region (Cremazy et al., 2000). *Dichaete* mutant embryos display severe defects in CNS development (Nambu and Nambu, 1996). Recently, it has been shown that *Dichaete* plays a role in the formation of several late arising ventral and intermediate NBs (Zhao and Skeath, 2002). However, as observed in *SoxN* mutants, *Dichaete* mutant embryos do not show significant defects in ventral SI NB formation. This raises the question of whether *Dichaete* and *SoxN* function redundantly with respect to early ventral NB formation. We generated a double mutant stock *Dichaete*^{87}*;SoxN*^{GA1192} and stained stage 9 embryos with anti-Wor (Fig. 5). As homozygous *Dichaete* mutants show severe segmentation defects in the abdomen, we restricted our analysis to the thoracic segments and found that in double

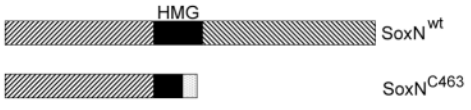

Fig. 3. The C463 allele contains an internal deletion. The C463 allele was found to have an internal deletion of 311 bp (position 1373-1684), which introduces a frame-shift. The deduced C463 234 amino acid polypeptide shares the first 215 amino acids with wild-type *SoxN* protein followed by 19 amino acids of novel sequence.

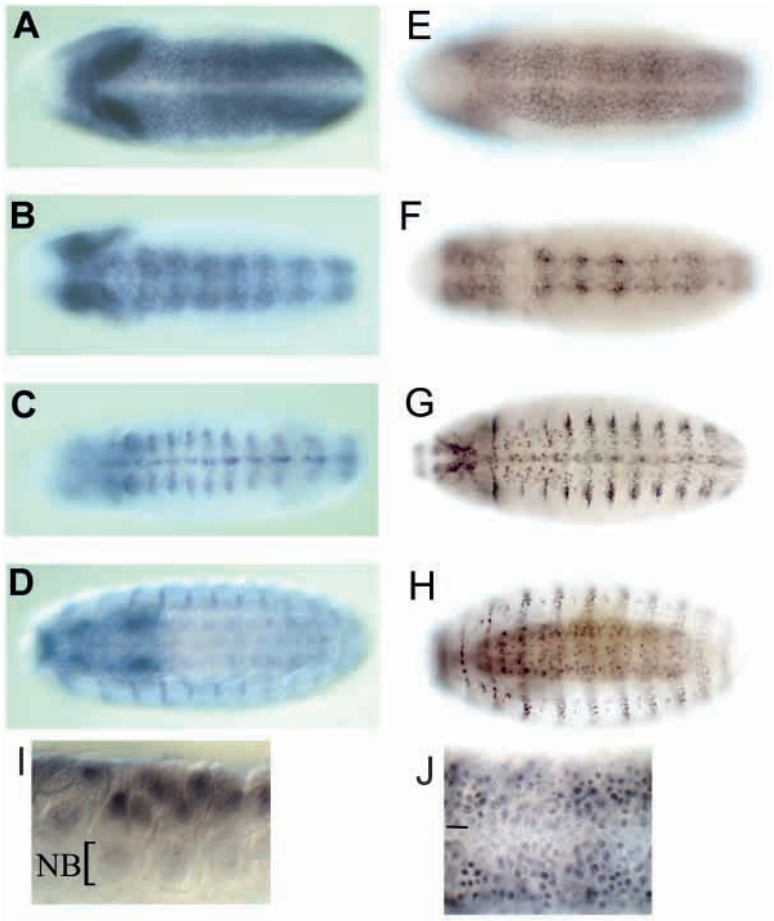

Fig. 4. *SoxN* RNA and protein expression pattern. (A-D) RNA in situ with a *SoxN*-specific probe. (E-J) Immunostaining with anti-*SoxN* antibody. (A,E) Stage 8: *SoxN* is expressed in the entire neuroectoderm with exception of the ventral midline. (B,F) Late stage 10: the staining has become metameric. (C,G) Stage 12: *SoxN* is expressed in ectodermal stripes that extend laterally. Strong expression is seen in the ventral midline. (D,H) Ectodermal stripes extend the entire circumference of the embryos. *SoxN* is expressed in a subset of CNS and PNS cells. (I) At stage10, SoxN levels are high in neuroectodermal cells and low in delaminated NBs. (H) Stage 10: *SoxN* protein distribution in the neuroectoderm is ubiquitous but not uniform. Ventral views with anterior towards the left are shown except I, which is a lateral view.

mutant embryos, ventral SI NB formation is severely impaired: e.g. in *SoxN* and *Dichaete* single mutant embryos, the formation of NB1-1 is hardly affected (3% and 2% loss, respectively), while in double mutant embryos NB1-1 fails to form in 48% of the hemisegments. Thus, *SoxN* and *Dichaete* function is at least partially redundant with respect to early ventral NB formation.

SoxN and Dichaete expression also overlaps in the intermediate region of the NE and therefore both proteins may contribute to early intermediate NB formation. We analyzed the formation of the intermediate S1 NB5-3, which is moderately affected in *SoxN* single mutants (14% loss) and hardly affected in *Dichaete* single mutants (1%). In $SoxN^{GA1192}/Dichaete^{87}$ double mutant embryos, we observed an enhanced loss of NB5-3 (25%) (Fig. 5) and thus conclude that *SoxN* and *Dichaete* both contribute to the formation of the intermediate NB5-3.

SoxN genetically interacts with *vnd* and *ind*

Prior to and during NB formation, three homeobox genes, *vnd*, *ind* and *msh*, are expressed in adjacent longitudinal columns and subdivide the NE along the DV axis. *vnd* and *ind* play a crucial role in NB formation: loss of *vnd* or *ind* results in the loss of ventral or intermediate NBs, respectively. To determine if *SoxN* plays a role in the initiation or maintenance of Vnd, Ind or Msh expression, we stained stage 8 *SoxN* mutant embryos with anti-Vnd (Chu et al., 1998) and anti-Msh antibodies (Isshiki et al., 1997), or an *ind*-specific RNA probe (Weiss et al., 1998). The staining patterns of these genes were found to be identical to that of wild-type embryos, indicating that *SoxN* is dispensable for their expression (data not shown). Conversely, staining of *vnd*, *ind* or *msh* mutant embryos with an anti-SoxN antibody revealed no role for *vnd*, *ind* or *msh* in the maintenance of SoxN expression prior to and during NB formation (data not shown).

These results demonstrate that the expression of *SoxN* and the DV patterning genes is regulated independently. However, the *vnd* and *ind* mutant and the *SoxN* mutant phenotypes exhibit strikingly similar phenotypes with respect to ventral and intermediate NB formation. Moreover, SoxN and Vnd/Ind are co-expressed during NB formation. This prompted us to study whether *SoxN* genetically interacts with *vnd* and/or *ind* in the NE. We chose the *SoxN* allele C2139, which appears to be a hypomorph and tested whether removal of one copy of *vnd* or *ind* dominantly enhances the phenotype of *SoxN*. We generated the stocks $vnd^{\Delta 38}/+;SoxN^{C2139}/SoxN^{C2139}$ and $ind^{16.2}/+;\ SoxN^{C2139}/SoxN^{C2139}$, and scored the formation of NBs using anti-Wor for the ventral SI NBs and the intermediate NB5-3 (Fig. 6). In addition, we used anti-Eve to score the RP2 neuron, the progeny of the intermediate SIII NB4-2 (data not shown). Anti-Wor staining of stage 9 $vnd^{\Delta 38}/+;SoxN^{C2139}/SoxN^{C2139}$ embryos revealed an enhanced loss of ventral SI neuroblasts, ranging from 12% to 18% (Fig. 6C). In $ind^{16.2}/+;\ SoxN^{C2139}/SoxN^{C2139}$ mutant embryos we observed an increased loss of NB5-3 ($SoxN^{C2139}$ homozygous embryos: 12% loss, $ind^{16.2}/+;\ SoxN^{C2139}/SoxN^{C2139}$ embryos: 46% loss; Fig. 6D) and an increased loss of the RP2 neuron ($SoxN^{C2139}/SoxN^{C2139}$ 75% loss versus 99% loss for $ind^{16.2}/+;\ SoxN^{C2139}/SoxN^{C2139}$; data not shown). Thus, *SoxN* interacts genetically with *vnd* in ventral and with *ind* in intermediate NB formation.

The lateral column of NBs derives from a stripe of *msh*-expressing NE. *msh* has been shown to play an important role in the specification of lateral NBs, but does not appear to play a role in NB formation (Buescher and Chia, 1997; Isshiki et al., 1997). To analyze whether the loss of *SoxN* uncovers a function of *msh* in NB formation, we generated $SoxN^{GA1192};msh^{lttEMS}$ double homozygous mutant embryos and scored the formation of lateral S1 NBs with anti-Wor antibody. We did not observe an enhancement of the

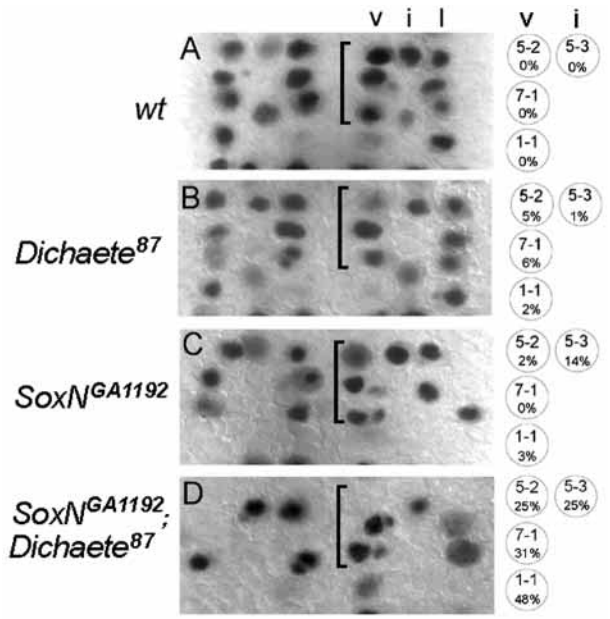

Fig. 5. *SoxN* and *Dichaete* both contribute to the formation of ventral S1 neuroblasts. (A-D) Whole-mount stage 9 embryos stained with anti-Wor antibody. Each panel shows one segment. Anterior is upwards. The bracket encompasses the ventral NBs. v, ventral; i, intermediate; l, lateral. (A) Wild type; (B) *Dichaete*^{87}; (C) *SoxN*^{GA1192}; and (D) *Dichaete*^{87}*; SoxN*^{GA1192}

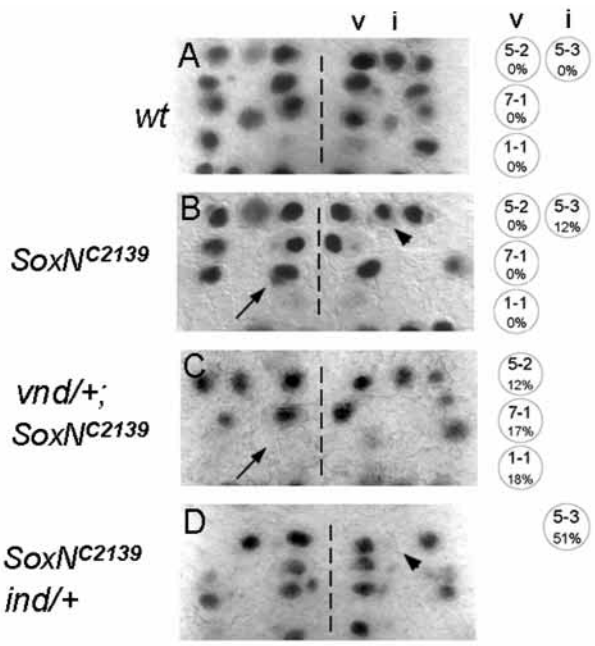

Fig. 6. *SoxN* genetically interacts with *vnd* and *ind*. (A-D) Whole-mount stage 9 embryos stained with anti-Wor antibody. Each panel shows one segment. Anterior is upwards; the broken line indicates the midline. The arrowhead indicates the position of NB5-3 in B,D; the arrow indicates the position of NB1-1 in B,C. v, ventral; i, intermediate. (A) Wild-type; (B) *SoxN*^{C2139}; (C) *vnd /+*; *SoxN*^{C2139}; and (D) *SoxN*^{C2139}*;ind/+*. The percentages of samples showing loss of each NB are given; ~50 hemisegments were counted for each NB.

SoxN^{GA1192} homozygous phenotype (data not shown) and therefore conclude that even in the absence of *SoxN*, *msh* has no role in NB formation.

SoxN is required for the singling out of neuroblasts

We have shown that loss of *SoxN* results in a severe loss of NBs. Our expression studies show that SoxN protein is present in the NE before and during the entire process of neurogenesis. Hence, the expression pattern provides no clue as to which step(s) depend on *SoxN* function. To approach this question, we studied two key steps in neurogenesis: (1) the establishment cell clusters with neural potential and (2) the 'singling out' of NBs.

The proneural genes of the AS-C have been shown to be essential for the promotion of NB formation and deletion of the entire gene complex results in the loss of ~75% of all NBs (Campos-Ortega, 1993). Many NBs that normally derive from clusters of neuroectodermal cells, which express either *ac*, *sc*, *l'sc* or a combination of these genes, fail to form in *SoxN* mutant embryos. This raises the question of whether proneural genes are still expressed in a *SoxN* mutant background in clusters of ectodermal cells, and, if so, do they still confer neural potential to these cells? In wild-type embryos, prior to NB segregation (stage 8), Ac protein is found in cell clusters in rows 3 and 7 in the ventral and lateral column of the NE, while L'sc is found in stripes of two to three cell widths that transverse the entire NE (Martin-Bermudo et al., 1991). Staining of stage 8 *SoxN* mutant embryos with anti-L'sc antibody revealed no appreciable difference from wild-type L'sc expression (Fig. 7A,B). Staining with anti-Ac antibody showed that Ac expression is initiated in both ventral and lateral clusters, but expression levels appear reduced and show significant variation in lateral cell clusters (Fig. 7C,D).

In wild-type embryos, the process of lateral inhibition results in the singling out of one cell per proneural cluster which will enter the neural pathway. This process is accompanied by an upregulation of proneural gene expression, delamination of the NB from the neuroectodermal layer and the initiation of expression of a set of neuronal precursor genes. In stage 9 *SoxN* mutant embryos, we frequently observed a failure in the upregulation of Ac expression in lateral proneural clusters (Fig. 7E,F). In those instances in which Ac was still upregulated, expression was less robust than in wild type and varied significantly among different hemisegments. Variation of Ac expression levels was also apparent in ventrally delaminating cells. The failure to upregulate Ac expression was accompanied by a failure in cell delamination. Moreover, the expression of neuronal precursor genes was severely affected: in wild type, one of the earliest precursor genes to be expressed is *asense* (*ase*); *ase* is expressed in all delaminating NBs (Jarman et al., 1993). In SoxN mutant embryos, *ase* expression was strongly reduced (Fig. 7G,H). These results suggest that in *SoxN* mutant embryos the establishment of proneural clusters is impaired but not abolished. The subsequent process of singling out NBs is severely defective.

SoxN does not act to antagonize Notch signaling

The singling out of neuronal progenitor cells from cell clusters with neuronal potential requires the action of the neurogenic genes. Productive Notch signaling results in the accumulation

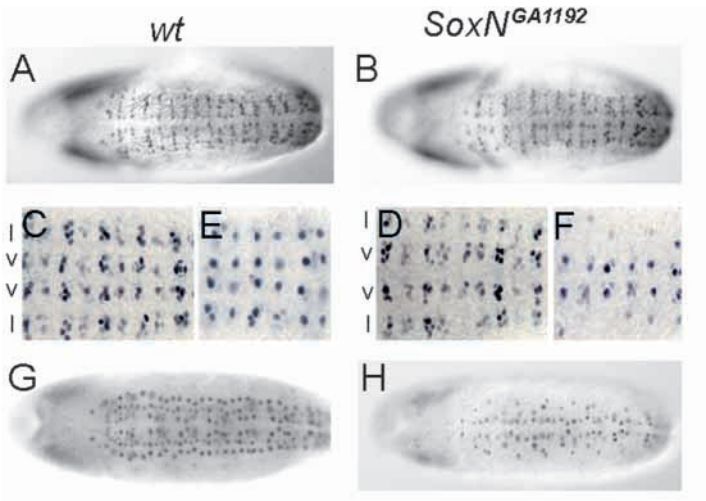

Fig. 7. *SoxN* is required for the upregulation of Ac expression and the initiation of Ase expression. (A) Wild-type and (B) *SoxN* whole-mount early stage 8 embryos stained with anti-L'sc antibody. (C) Wild-type and (D) *SoxN* dissected late stage 8 embryos stained with anti-Ac antibody. Note the slight reduction of Ac expression in lateral clusters. (E) Wild-type and (F) *SoxN* dissected late stage 9 embryos stained with anti-Ac antibody. Note the strong reduction of Ac expression in the lateral column. (G) Wild-type and (H) *SoxN* whole-mount stage 9 embryos stained with anti-Ase antibody. Anterior is towards the left.

of E(spl) gene products, which negatively regulate the expression of the proneural genes and initiates the non-neuronal differentiation pathway. Cells that enter the neuronal pathway are thought to accumulate only low levels of E(spl)gene products, a prerequisite for the upregulation of proneural gene expression (Martin-Bermudo et al., 1995). In *SoxN* mutant embryos, we observed a failure to upregulate proneural gene expression. Thus, it is conceivable that SoxN normally acts during lateral inhibition to antagonize the accumulation of E(spl) gene products.

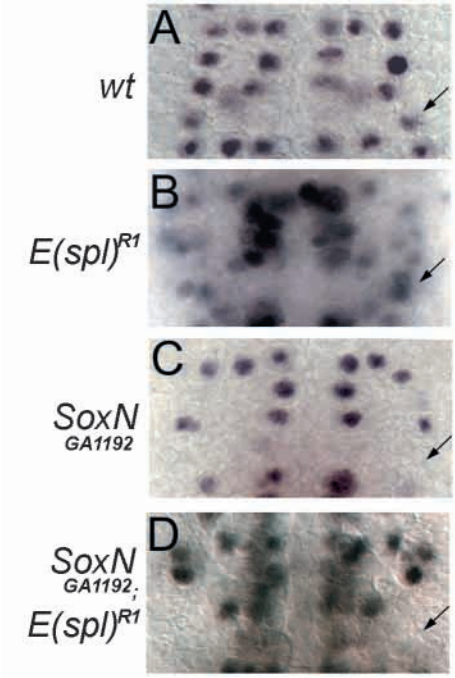

Fig. 8. *SoxN* does not act to antagonize productive Notch signaling. (A-D) Immunostaining of stage 9 embryos with anti-Wor antibody. Anterior is upwards. Each panel shows the SI NB pattern of one segment. Anterior is upwards. The arrow indicates one NB in the 3-5 position in wild type (A), multiple NBs in the 3-5 position in the *E(spl)* mutant (B), and the lack of a NB in the 3-5 position in *SoxN* (C) and *SoxN/E(spl)* double mutant (D).

To test this idea, we investigated whether *SoxN* function is still required in a genetic background where Notch signaling is non-productive: in a deletion mutant in which the entire *E(spl)* gene complex is removed. If *SoxN* indeed functions to antagonize Notch signaling, concomitant loss of *E(spl)* should restore NB formation in the lateral and intermediate regions. $E(spl)^{R1}$ mutant embryos display a severe neurogenic phenotype (Mari-Beffa et al., 1991). Fig. 8B shows the SI NB pattern: the typical arrangement of S1 NBs in three columns is maintained; however, instead of the wild-type set of ten NBs, additional NBs are found in each position, indicating that more than one cell per proneural cluster has entered the neural pathway. $E(spl)^{R1}$;$SoxN^{GA1192}$ double mutant embryos display a combination of the neurogenic $E(spl)^{R1}$ phenotype and the anti-neural *SoxN* phenotype (Fig. 8D): the neurogenic phenotype is apparent in the ventral column of SI NBs, which normally does not require *SoxN* function. In the lateral column, the anti-neural phenotype of *SoxN* remains unchanged: e.g. in *SoxN* single mutant embryos, NB3-5 fails to from in 82% of the hemisegments and in the double mutant NBs still fail to form with comparable frequency in the 3-5 position (Fig. 8C,D, arrows). Similar results were observed for other lateral and intermediate NBs. Therefore, in a *SoxN* mutant background the concomitant loss of E(spl) function does not restore NB formation in the lateral column. Based on these results, we conclude that *SoxN* does not function to antagonize Notch signaling during lateral inhibition. Rather, *SoxN* appears to act in a parallel pathway with the proneural and neurogenic genes and in the absence of *SoxN*, proneural gene expression is less efficient at conferring neural potential to ectodermal cells.

DISCUSSION

SoxN is required for neuroblast formation and acts in parallel to the genes of the AS-C

Sox genes are expressed in spatially and temporally regulated patterns during embryogenesis and several Sox genes have been shown to play key roles in development (for reviews, see Pevny and Lovell-Badge, 1997; Wegner, 1999). In this study, we have demonstrated that *SoxN* is essential for the proper development of the embryonic CNS. In *SoxN* mutant embryos

~70% of all NBs fail to form, similar to the loss of NBs following chromosomal deletions that remove all proneural genes of the AS-C (for a review, see Campos-Ortega, 1995). Interestingly, the manner in which NBs are lost in *SoxN* and *AS-C* mutants appears mechanistically different. In *AS-C* mutants only a small proportion of NBs fails to be singled out and fails to delaminate from the NE (~25% of early NBs) (Jimenez and Campos-Ortega, 1990). The majority of NBs still segregates and later may be subject to cell death. By contrast, in *SoxN* mutant embryos, neuroectodermal cells fail to be singled out as NBs and delamination does not take place. Thus, it appears that loss of *SoxN* affects NBs formation at an earlier step than the loss of proneural genes. We demonstrate that proneural gene expression is regulated largely independently of *SoxN*, as loss of *SoxN* does not affect the neuroectodermal expression of L'sc and does not abolish that of Ac. We suggest that *SoxN* acts upstream and in parallel to the proneural genes. Comparison of the NB phenotypes of *AS-C* mutant and *SoxN* mutant embryos revealed that overlapping but not identical subsets of NBs were affected (data not shown). This result suggests that SoxN function – as we understand it at this time – does not explain why some NBs do not require the proneural genes of the AS-C. The binary decision of neuroectodermal cells to adopt the neural or the epidermal fate requires Notch signaling. Our analysis of the *E(spl);SoxN* double mutant phenotype demonstrates that *SoxN* does not promote neural fate by antagonizing Notch signaling.

It would be interesting to determine if neuroectodermal cells in *SoxN* mutants are still able to adopt the epidermal fate. However, owing to the lack of appropriate markers, which would indicate early epidermal differentiation, we examined the formation of the ventral denticle belts at the first instar larval stage. Denticle belt formation is severely impaired in *SoxN* mutant embryos indicating that epidermal development is disturbed. Hence, in the absence of *SoxN*, the ability of neuroectodermal cells to undergo neural or epidermal development may both be compromised.

SoxN and *Dichaete* both contribute to ventral SI neuroblast formation

The *SoxN* mutant phenotype shows a strong spatial aspect with respect to the DV axis: loss of *SoxN* severely affects the formation of NBs that derive from the lateral and intermediate regions of the NE but has little effect on ventral NB formation. This DV effect of *SoxN* mutations is not mirrored in a corresponding DV *SoxN* expression pattern. Thus, the mutant phenotype rather reflects a differential requirement for *SoxN* in different regions. Our analysis of ventral NB formation in *SoxN;Dichaete* double mutant embryos provides at least a partial explanation for these regional differences as the concomitant loss of *SoxN* and *Dichaete* results in a strong loss of ventral NBs. This suggests that *SoxN* and *Dichaete* may functionally substitute for each other. A functional redundancy of *SoxN* and *Dichaete* is not unexpected as the proteins have structural similarities and overlapping expression patterns. Like *SoxN*, *Dichaete* has been classified as a group B Sox protein and the HMG domains of both proteins show 87% amino acid identity. As the ability of sequence-specific DNA binding resides within the HMG domain, it is likely that *SoxN* and *Dichaete* bind to the same DNA motif present in an identical set of target genes. This is supported by studies that have shown that various vertebrate Sox proteins can bind to the same DNA sequence. Neuroectodermal *Dichaete* and *SoxN* expression overlaps in the ventral and intermediate region and therefore a functional redundancy would be expected to occur in ventral and intermediate NB formation. However, the severe phenotype of *SoxN* single mutants in intermediate NB formation suggests that *Dichaete* cannot always substitute for *SoxN* function. Additional evidence that *SoxN* and *Dichaete* function is not equivalent stems from the observation that loss of *Dichaete* or *SoxN* has different effects on Ac expression in the intermediate region of the NE: in *Dichaete*, but not in *SoxN* mutant embryos, Ac expression is partially derepressed in the intermediate column (Zhao and Skeath, 2002) (this paper).

SoxN genetically interacts with *vnd* and *ind* in ventral and intermediate neuroblast formation

The loss of one copy of *vnd* or *ind* in a *SoxN* homozygous mutant background dominantly enhances the *SoxN* phenotype, suggesting that *SoxN* genetically interacts with *vnd* and *ind*. As the expression of Vnd and Ind does not require *SoxN* function, we conclude that *SoxN* does not act upstream of *vnd* and *ind*, but rather in parallel. In *ind* mutant embryos, Ac expression in the NE is derepressed in the intermediate region. Nevertheless, NBs fail to form within this region (Weiss et al., 1998). *vnd* is required for Ac expression in the ventral NE. However, there seems to be no causal relationship between the loss of Ac expression and the subsequent loss of NBs as ectopic expression of Ac does not rescue NB formation (Chu et al., 1998). Thus, it appears that expression of the genes of the AS-C can confer neural potential to the NE only when *SoxN*, *vnd* and *ind* expression is intact.

Molecular studies of vertebrate Sox proteins and, more recently, *Drosophila* Dichaete, have provided evidence that modulation of target gene expression requires heterodimerization of Sox with other transcription factors. For example, Dichaete interacts with the Single-minded and Drifter proteins both genetically and physically during midline development (Ma et al., 2000). This raises the question of whether SoxN forms functional heterodimers with Vnd and Ind. The co-expression of these factors in the NE and their parallel functions in NB formation do support a model in which SoxN physically associates with Vnd and Ind. Recently, Zhao and Skeath have shown that *Dichaete* genetically interacts with *vnd* and *ind* to promote NB formation and have postulated that a physical interaction of Dichaete/Vnd and Dichaete/Ind may occur (Zhao and Skeath, 2002). Further experiments are required to delineate the molecular relationships between these proteins.

Sox gene function in the development of neural tissue may be conserved across species

Comparative studies of the key steps in neural development have revealed a remarkable conservation across a wide range of species. Common features include early neural determination, which depends on the antagonistic action of positive (Sog, Chordin) and negative (Dpp, BMP) acting factors; the singling out of neural progenitor cells and aspects of DV patterning. The results we present in this paper suggest that conservation extends to the function of Sox proteins in neural development. Based on sequence homology, the closest vertebrate relatives of SoxN and Dichaete are Sox1, Sox2 and Sox3. These proteins

are closely related in structure throughout their entire length and are expressed in overlapping patterns in developing neural tissues (Collignon et al., 1996). These features, taken together with the observation that mice carrying a homozygous *Sox1* mutation display rather mild defects in neural development, have led to the hypothesis that the functions of *Sox1*, *Sox2* and *Sox3* function is at least partially redundant (Nishiguchi et al., 1998). Our analysis of *SoxN;Dichaete* double mutant embryos confirms this hypothesis in *Drosophila*, as *SoxN* and *Dichaete* function is indeed redundant with respect to the formation of a subset of NBs.

Interestingly, the regulation of *SoxN* and Sox2 expression appears to be conserved in *Drosophila* and *Xenopus*: both are negatively regulated by Dpp (BMP4) and positively regulated by the Dpp antagonist Sog (Chordin) (for a review, see Sasai, 2001). Experiments using dominant-negative forms of Sox2 in animal cap ectoderm have shown that Sox2 is required for the maintenance rather than the initial induction of neural tissue (Kishi et al., 2000). This is in agreement with our observations that loss of *SoxN* does not alter the early expression of Brk, Sog and Dpp (M. B., unpublished) and thus does not seem to promote neurogenesis through the determination of the ventrolateral region in the blastoderm embryo. Despite indications for a role for vertebrate Sox genes in neural differentiation, its mode of action remains unclear as neither target genes nor CNS interaction partners have been identified. Our observations that *SoxN* genetically interacts with *vnd* and *ind* suggest the vertebrate homologs of Vnd [Nkx2.2 family (Pabst et al., 1998)] and Ind [Gsh1/2 (Hsieh-Li et al., 1995; Valerius et al., 1995)] as potential CNS partners for Sox1, Sox2 and Sox3. Like Vnd and Ind, Nkx2.2 and Gsh1 are expressed in developing neural tissue and govern aspects of regional specification. Further studies will demonstrate whether Sox gene function represents a neuralizing pathway that is conserved across species.

We thank the following people for providing flystocks, antibodies and other reagents: M. Frasch, J. Skeath, C. Doe, J. Nambu, G. Technau, T. Isshiki, T. von Ohlen, F. Girard, Y.N. Jan, S. Romani and D. Mellerik. Our special thanks to R. Tuxworth for help with the image collection. We thank Sami Bahri, X. Morin, V. Rodrigues and R. Tuxworth for critical comments on the manuscript. We thank the Bloomington stock center for providing stocks and the Developmental Studies Hybridoma Bank for providing cell lines. A special thanks to Guy Tear for providing the EMS mutation collection and comments on the manuscript. M. B. and W. C. are supported by the Wellcome Trust.

REFERENCES

Alberga, A., Boulay, J. L., Kempe, E., Dennefeld, C. and Haenlin, M. (1991). The snail gene required for mesoderm formation in Drosophila is expressed dynamically in derivatives of all three germ layers. *Development* **111**, 983-992.

Ashraf, S. I., Hu, X., Roote, J. and Ip, Y. T. (1999). The mesoderm determinant snail collaborates with related zinc-finger proteins to control Drosophila neurogenesis. *EMBO J.* **18**, 6426-6438.

Bhat, K. M. (1999). Segment polarity genes in neuroblast formation and identity specification during Drosophila neurogenesis. *Bioessays* **21**, 472-485.

Biehs, B., Francois, V. and Bier, E. (1996). The Drosophila short gastrulation gene prevents Dpp from autoactivating and suppressing neurogenesis in the neuroectoderm. *Genes Dev.* **10**, 2922-2934.

Bossing, T., Udolph, G., Doe, C. Q. and Technau, G. M. (1996). The embryonic central nervous system lineages of Drosophila melanogaster. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Dev. Biol.* **179**, 41-64.

Buescher, M. and Chia, W. (1997). Mutations in lottchen cause cell fate transformations in both neuroblast and glioblast lineages in the Drosophila embryonic central nervous system. *Development* **124**, 673-681.

Cabrera, C. V. and Alonso, M. C. (1991). Transcriptional activation by heterodimers of the achaete-scute and daughterless gene products of Drosophila. *EMBO J.* **10**, 2965-2973.

Cai, Y., Chia, W. and Yang, X. (2001). A family of snail-related zinc finger proteins regulates two distinct and parallel mechanisms that mediate Drosophila neuroblast asymmetric divisions. *EMBO J.* **20**, 1704-1714.

Campos-Ortega, J. A. (1993). Early neurogenesis in *Drosophila melanogaster*. In *The Development of* Drosophila melanogaster. Vol. II (ed. B. A. Martinez-Arias), pp. 1131-1206. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

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

Campuzano, S., Carramolino, L., Cabrera, C. V., Ruiz-Gomez, M., Villares, R., Boronat, A. and Modolell, J. (1985). Molecular genetics of the achaete-scute gene complex of D. melanogaster. *Cell* **40**, 327-338.

Chitnis, A. B. (1999). Control of neurogenesis–lessons from frogs, fish and flies. *Curr. Opin. Neurobiol.* **9**, 18-25.

Chu, H., Parras, C., White, K. and Jimenez, F. (1998). Formation and specification of ventral neuroblasts is controlled by vnd in Drosophila neurogenesis. *Genes Dev.* **12**, 3613-3624.

Collignon, J., Sockanathan, S., Hacker, A., Cohen-Tannoudji, M., Norris, D., Rastan, S., Stevanovic, M., Goodfellow, P. N. and Lovell-Badge, R. (1996). A comparison of the properties of Sox-3 with Sry and two related genes, Sox-1 and Sox-2. *Development* **122**, 509-520.

Coulter, D. E., Swaykus, E. A., Beran-Koehn, M. A., Goldberg, D., Wieschaus, E. and Schedl, P. (1990). Molecular analysis of odd-skipped, a zinc finger encoding segmentation gene with a novel pair-rule expression pattern. *EMBO J.* **9**, 3795-3804.

Cremazy, F., Berta, P. and Girard, F. (2000). Sox neuro, a new Drosophila Sox gene expressed in the developing central nervous system. *Mech. Dev.* **93**, 215-219.

D'Alessio, M. and Frasch, M. (1996). msh may play a conserved role in dorsoventral patterning of the neuroectoderm and mesoderm. *Mech. Dev.* **58**, 217-231.

Doe, C. Q. (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the Drosophila central nervous system. *Development* **116**, 855-863.

Doe, C. Q., Hiromi, Y., Gehring, W. J. and Goodman, C. S. (1988). Expression and function of the segmentation gene fushi tarazu during Drosophila neurogenesis. *Science* **239**, 170-175.

Goodman, C. S. and Doe, C. Q. (1993). Early neurogenesis in Drosophila melanogaster. In *The Development of* Drosophila melanogaster, Vol. II (ed. B. A. Martinez-Arias), pp. 1131-1206. Cold Spring Harbour: Cold Spring Harbour Laboratory Press.

Higashijima, S., Shishido, E., Matsuzaki, M. and Saigo, K. (1996). eagle, a member of the steroid receptor gene superfamily, is expressed in a subset of neuroblasts and regulates the fate of their putative progeny in the Drosophila CNS. *Development* **122**, 527-536.

Hsieh-Li, H. M., Witte, D. P., Szucsik, J. C., Weinstein, M., Li, H. and Potter, S. S. (1995). Gsh-2, a murine homeobox gene expressed in the developing brain. *Mech. Dev.* **50**, 177-186.

Isshiki, T., Takeichi, M. and Nose, A. (1997). The role of the msh homeobox gene during Drosophila neurogenesis: implication for the dorsoventral specification of the neuroectoderm. *Development* **124**, 3099-3109.

Jarman, A. P., Brand, M., Jan, L. Y. and Jan, Y. N. (1993). The regulation and function of the helix-loop-helix gene, asense, in Drosophila neural precursors. *Development* **119**, 19-29.

Jazwinska, A., Rushlow, C. and Roth, S. (1999). The role of brinker in mediating the graded response to Dpp in early Drosophila embryos. *Development* **126**, 3323-3334.

Jimenez, F. and Campos-Ortega, J. A. (1990). Defective neuroblast commitment in mutants of the achaete-scute complex and adjacent genes of D. melanogaster. *Neuron* **5**, 81-89.

Jimenez, F., Martin-Morris, L. E., Velasco, L., Chu, H., Sierra, J., Rosen, D. R. and White, K. (1995). vnd, a gene required for early neurogenesis of Drosophila, encodes a homeodomain protein. *EMBO J.* **14**, 3487-3495.

Kishi, M., Mizuseki, K., Sasai, N., Yamazaki, H., Shiota, K., Nakanishi, S.

and Sasai, Y. (2000). Requirement of Sox2-mediated signaling for differentiation of early Xenopus neuroectoderm. *Development* **127**, 791-800.

Ma, Y., Niemitz, E. L., Nambu, P. A., Shan, X., Sackerson, C., Fujioka, M., Goto, T. and Nambu, J. R. (1998). Gene regulatory functions of Drosophila fish-hook, a high mobility group domain Sox protein. *Mech. Dev.* **73**, 169-182.

Ma, Y., Certel, K., Gao, Y., Niemitz, E., Mosher, J., Mukherjee, A., Mutsuddi, M., Huseinovic, N., Crews, S. T., Johnson, W. A. et al. (2000). Functional interactions between Drosophila bHLH/PAS, Sox, and POU transcription factors regulate CNS midline expression of the slit gene. *J. Neurosci.* **20**, 4596-4605.

Mari-Beffa, M., de Celis, J. F. and Garcia-Bellido, A. (1991). Genetic and developmental analyses of chaetae pattern formation in Drosophila tergites. *Roux Arch. dev. Biol.* **200(3)**, 132-142.

Martin-Bermudo, M. D., Martinez, C., Rodriguez, A. and Jimenez, F. (1991). Distribution and function of the lethal of scute gene product during early neurogenesis in Drosophila. *Development* **113**, 445-454.

Martin-Bermudo, M. D., Carmena, A. and Jimenez, F. (1995). Neurogenic genes control gene expression at the transcriptional level in early neurogenesis and in mesectoderm specification. *Development* **121**, 219-224.

McDonald, J. A., Holbrook, S., Isshiki, T., Weiss, J., Doe, C. Q. and Mellerick, D. M. (1998). Dorsoventral patterning in the Drosophila central nervous system: the vnd homeobox gene specifies ventral column identity. *Genes Dev.* **12**, 3603-3612.

Mellerick, D. M. and Nirenberg, M. (1995). Dorsal-ventral patterning genes restrict NK-2 homeobox gene expression to the ventral half of the central nervous system of Drosophila embryos. *Dev. Biol.* **171**, 306-316.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S. and Sasai, Y. (1998). Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* **125**, 579-587.

Mukherjee, A., Shan, X., Mutsuddi, M., Ma, Y. and Nambu, J. R. (2000). The Drosophila sox gene, fish-hook, is required for postembryonic development. *Dev. Biol.* **217**, 91-106.

Nambu, P. A. and Nambu, J. R. (1996). The Drosophila fish-hook gene encodes a HMG domain protein essential for segmentation and CNS development. *Development* **122**, 3467-3475.

Nishiguchi, S., Wood, H., Kondoh, H., Lovell-Badge, R. and Episkopou, V. (1998). Sox1 directly regulates the gamma-crystallin genes and is essential for lens development in mice. *Genes Dev.* **12**, 776-781.

Pabst, O., Herbrand, H. and Arnold, H. H. (1998). Nkx2-9 is a novel homeobox transcription factor which demarcates ventral domains in the developing mouse CNS. *Mech. Dev.* **73**, 85-93.

Patel, N. H., Schafer, B., Goodman, C. S. and Holmgren, R. (1989). The role of segment polarity genes during Drosophila neurogenesis. *Genes Dev.* **3**, 890-904.

Pevny, L. H. and Lovell-Badge, R. (1997). Sox genes find their feet. *Curr. Opin. Genet. Dev.* **7**, 338-344.

Rex, M., Uwanogho, D., Cartwright, E., Pearl, G., Sharpe, P. T. and Scotting, P. J. (1994). Sox gene expression during neuronal development. *Biochem. Soc. Trans.* **22**, 252S.

Russell, S. R., Sanchez-Soriano, N., Wright, C. R. and Ashburner, M. (1996). The Dichaete gene of Drosophila melanogaster encodes a SOX-domain protein required for embryonic segmentation. *Development* **122**, 3669-3676.

Sasai, Y. (1998). Identifying the missing links: genes that connect neural induction and primary neurogenesis in vertebrate embryos. *Neuron* **21**, 455-458.

Sasai, Y. (2001). Roles of Sox factors in neural determination: conserved signaling in evolution? *Int. J. Dev. Biol.* **45**, 321-326.

Schmid, A., Chiba, A. and Doe, C. Q. (1999). Clonal analysis of Drosophila embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development* **126**, 4653-4689.

Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J. and Technau, G. M. (1997). The embryonic central nervous system lineages of Drosophila melanogaster. II. Neuroblast lineages derived from the dorsal part of the neuroectoderm. *Dev. Biol.* **189**, 186-204.

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.

Skeath, J. B. (1999). At the nexus between pattern formation and cell-type specification: the generation of individual neuroblast fates in the Drosophila embryonic central nervous system. *BioEssays* **21**, 922-931.

Skeath, J. B., Panganiban, G., Selegue, J. and Carroll, S. B. (1992). Gene regulation in two dimensions: the proneural achaete and scute genes are controlled by combinations of axis-patterning genes through a common intergenic control region. *Genes Dev.* **6**, 2606-2619.

Skeath, J. B., Panganiban, G. F. and Carroll, S. B. (1994). The ventral nervous system defective gene controls proneural gene expression at two distinct steps during neuroblast formation in Drosophila. *Development* **120**, 1517-1524.

Soriano, N. S. and Russell, S. (1998). The Drosophila SOX-domain protein Dichaete is required for the development of the central nervous system midline. *Development* **125**, 3989-3996.

Streit, A., Sockanathan, S., Perez, L., Rex, M., Scotting, P. J., Sharpe, P. T., Lovell-Badge, R. and Stern, C. D. (1997). Preventing the loss of competence for neural induction: HGF/SF, L5 and Sox-2. *Development* **124**, 1191-1202.

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.

Uwanogho, D., Rex, M., Cartwright, E. J., Pearl, G., Healy, C., Scotting, P. J. and Sharpe, P. T. (1995). Embryonic expression of the chicken Sox2, Sox3 and Sox11 genes suggests an interactive role in neuronal development. *Mech. Dev.* **49**, 23-36.

Valerius, M. T., Li, H., Stock, J. L., Weinstein, M., Kaur, S., Singh, G. and Potter, S. S. (1995). Gsh-1: a novel murine homeobox gene expressed in the central nervous system. *Dev. Dyn.* **203**, 337-351.

Wegner, M. (1999). From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res.* **27**, 1409-1420.

Weiss, J. B., von Ohlen, T., Mellerick, D. M., Dressler, G., Doe, C. Q. and Scott, M. P. (1998). Dorsoventral patterning in the Drosophila central nervous system: the intermediate neuroblasts defective homeobox gene specifies intermediate column identity. *Genes Dev.* **12**, 3591-3602.

Xiong, W. C., Okano, H., Patel, N. H., Blendy, J. A. and Montell, C. (1994). repo encodes a glial-specific homeo domain protein required in the Drosophila nervous system. *Genes Dev.* **8**, 981-994.

Yang, X., Bahri, S., Klein, T. and Chia, W. (1997). Klumpfuss, a putative Drosophila zinc finger transcription factor, acts to differentiate between the identities of two secondary precursor cells within one neuroblast lineage. *Genes Dev.* **11**, 1396-1408.

Yu, F., Morin, X., Cai, Y., Yang, X. and Chia, W. (2000). Analysis of partner of inscuteable, a novel player of Drosophila asymmetric divisions, reveals two distinct steps in inscuteable apical localization. *Cell* **100**, 399-409.

Zhao, G. and Skeath, J. B. (2002). The Sox-domain containing gene Dichaete/fish-hook acts in concert with vnd and ind to regulate cell fate in the Drosophila neuroectoderm. *Development* **129**, 1165-1174.