Sloppy paired acts as the downstream target of Wingless in the Drosophila CNS and interaction between sloppy paired and gooseberry inhibits sloppy paired during neurogenesis

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Accepted 10 November 1999; published on WWW 12 January 2000

SUMMARY

Wingless (Wg) and other Wnt proteins play a crucial role in a number of developmental decisions in a variety of organisms. In the ventral nerve cord of the Drosophila embryo, Wg is non-autonomously required for the formation and specification of a neuronal precursor cell, NB4-2. NB4-2 gives rise to a well-studied neuronal lineage, the RP2/sib lineage. While the various components of the Wg-signaling pathway are also required for generating NB4-2, the target gene(s) of this pathway in the signal-receiving cell is not known. In this paper, we show that sloppy paired 1 and sloppy paired 2 function as the downstream targets of the Wg signaling to generate the NB4-2 cell. Thus, while the loss-of-function mutations in wg and slp have the same NB4-2 formation and specification defects, these defects in wg mutants can be rescued by expressing slp genes from a heterologous promoter. That slp genes function downstream of the Wg signaling is also indicated by the result that expression of slp genes is lost from the neuroectoderm in wg mutants and that ectopic expression of wg induces ectopic expression of slp. Finally, previous results show that Gooseberry (Gsb) prevents Wg from specifying NB4-2 identity to the wg-expressing NB5-3. In this paper, we also show that gsb interacts with slp and prevents Slp from specifying NB4-2 identity. Overexpression of slp overcomes this antagonistic interaction and respecifies NB5-3 as NB4-2. This respecification, however, can be suppressed by a simultaneous overexpression of gsb at high levels. This mechanism appears to be responsible for specifying NB5-3 identity to a row 5 neuronblast and preventing Wg from specifying NB4-2 identity to that cell.

Key words: Sloppy paired, Gooseberry, Drosophila melanogaster, Neurogenesis

INTRODUCTION

The relatively simple central nervous system (CNS) of the Drosophila embryo provides an experimentally advantageous model system for investigating the mechanisms that generate and pattern the eukaryotic CNS. In the Drosophila embryo, within the developing CNS hundreds of different cell types are generated from a relatively uniform two-dimensional epithelial sheet (see Bate, 1976; Doe, 1992; Goodman and Doe, 1993; Bhat, 1999). During neurogenesis, about 30 neuroblast (NB) cells in each hemisegment delaminate from this sheet in approximately five successive waves along the mediolateral (M-L) and anterior-posterior (A-P) axes in rows and columns in a stereotyped spatio-temporal pattern. By the time it is formed, each of these neuroblasts has acquired a unique fate and the neuroblast that forms in a given position at a given time always acquires the same fate. A neuroblast then functions as a stem cell and divides by asymmetric mitosis, renewing itself with each division and producing a chain of secondary neuronal precursor cells called ganglion mother cells (GMCs).

A GMC does not self-renew; instead it divides asymmetrically to generate two distinct neurons. These postmitotic neurons then undergo cyto-differentiation. At the end of neurogenesis, each of the hemineuromeres has approximately 320 neurons and 30 glia (see Hartenstein and Campos-Ortega, 1984; Doe, 1992; Goodman and Doe, 1993; Bossing et al., 1996; Schmidt et al., 1997). Studies over the past few years indicate that the identity of a neuroblast determines the number and type of neurons generated by a neuroblast. Thus, it is critical to dissect the genetic regulatory mechanisms that specify the identity of individual neuroblasts and determine how a given neuroblast acquires its specific identity in a field of cells.

In recent years, the developing embryonic nervous system has also proved to be an excellent paradigm for understanding the functioning of the signaling pathway mediated by wingless (wg). Over several years, the role of wg in neurogenesis has been intensely studied (reviewed in Bhat, 1998a, 1999). During neurogenesis, it has been shown that wg is expressed in row 5 cells in each segment and is nonautonomously required for the formation and specification of row 4 and for the formation of
row 6 neuroblasts (Chu-LaGraff and Doe, 1993; Bhat, 1996; Bhat and Schedl, 1997). It appears that Wg is also required for the formation of at least one neuroblast in row 5, a wg expressing cell (Hartenstein et al., 1994). Among those neuroblasts that are affected in wg mutants, a row 4 neuroblast, NB4-2, has been studied in much detail. NB4-2 gives rise to the well-studied neuronal lineage, the NB4-2→GMC-1→RP2/sib lineage. The Wg-signaling pathway is shown to be required for both the formation and the specification of this neuroblast (Chu-LaGraff and Doe, 1993; Bhat, 1996, 1998b). Recent studies indicate that, as in the epidermis, the Wg signal is transduced in the receiving NB4-2 precursor cell via its receptor complex (Bhat, 1998b) to prevent Shaggy (Sgg) kinase from phosphorylating Armadillo (Arm)/β-Catenin. The hypophosphorylated Arm, the pangolin (pan) gene product (the TCF-1/LEF-1 homolog), translocates to the nucleus and activates cell-autonomous target effector genes (see reviews by Klingensmith and Nusse, 1994; Bhat, 1998a, 1999). A major stumbling block to further extending our knowledge of the functioning of this developmentally important pathway is the fact that the genes that are regulated by this signaling cascade in the signal-receiving cell remain largely unknown.

Our previous results indicate that Gsb prevents the autocrine Wg pathway from specifying NB4-2 identity to NB5-3, a row 5 NB (Bhat, 1996; Bhat and Schedl, 1997; see also Duman-Scheel et al., 1997). Thus, in gsb mutants, Wg transforms NB5-3 into NB4-2. Moreover, in engrailed, invected (en, inv) mutants, Gsb expression is lost from NB5-3 and its precursor cells leading to a transformation of this NB into NB4-2 by Wg (Bhat and Schedl, 1997). By contrast, in ptc mutants, expression of gsb is expanded into row 4 and this prevents Wg from specifying NB4-2 identity to this row 4 cell (Bhat, 1996; Bhat and Schedl, 1997; Duman-Scheel et al., 1997). Furthermore, when gsb is ectopically expressed from a heat shock promoter, this prevents the specification of NB4-2 identity (Zhang et al., 1993; Li and Noll, 1994). It was, however, not clear how Gsb can prevent the Wg pathway that specifies NB4-2 identity during neurogenesis.

In genetic screens for mutations that affect the specification of the NB4-2→GMC-1→RP2/sib lineage, we identified sloppy paired 1 (slp1) and sloppy pared 2 (slp2), two sister genes of the forhead group of transcription factor genes (Grossniklaus et al., 1992; Cadigan et al., 1994a,b), as required for the specification of this lineage. In this paper we show that the slp genes are the targets of the Wg pathway in the Wg-receiving cells in CNS and function downstream of wg during NB4-2 formation and specification (as opposed to upstream of wg in the ectoderm). Thus, the expression of slp genes from the

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**Fig. 1.** Loss of slp genes affects the formation and specification of NB4-2 lineage. The vertical lines indicate the midline, arrowheads indicate missing NB4-2 or RP2/sib lineage. Anterior end is up. In (A-C) (only half segments shown), embryos are stained with anti-LacZ to detect Hkb-LacZ expression. In (C-F), embryos are stained with anti-Eve and in (G,H), embryos are double stained with anti-Eve and 22C10. (A) Approx. 5.5-hour-old buckebein enhancer trap embryo (hkb5953). In approx. 5- to 5.5-hour-old wild-type embryos, NB1-1, NB2-2, NB2-5 are also Hkb-LacZ positive, in addition to NB4-2. (B) Approx. 5.5-hour-old slp; hkb5953 embryo. Note that ‘NB4-2’ is negative for Hkb expression. (C) Approx. 5.5-hour-old slp; hkb5953 embryo. NB4-2 fails to form in this hemisegment (arrowhead). Line drawings adjacent to A-C describe the NB map; the numbers inside these neuroblasts indicate their identities. M, midline. (D,E) Anti-Eve stained wild-type embryos. Eve is expressed in GMC-1, RP2 and a newly formed sib (D); however, the sib loses Eve expression by about 11 hours of development while the RP2 retains it (E). (F,G) slp mutant embryos; the Eve-positive GMC-1→RP2/sib cells are missing. (H) Wild-type embryo. Note that 22C10 stains several other neurons in the RP cluster; however, the particular fixation condition is well suited for anti-Eve and 22C10 staining of the RP2 and aCC neurons. (Moreover, the 22C10 staining of the other RP neurons, RP1, RP3 and RP4, is the strongest in a stage 17 embryo (about 16 hours old); in earlier stages, the staining of these RP neurons is weak and difficult to photograph and they are also located in a different focal plane than RP2.) (I) slp mutant embryos missing the RP2 motoneuron.
Interaction between Wg, Slp and Gsb in the CNS

heterologous and inducible heat shock 70 promoter rescues CNS defects in wg mutants. This is consistent with the fact that loss of wg activity leads to a loss of slp expression from the wg-receiving row 4 cells and a ubiquitous induction of wg also induces a ubiquitous expression of slp. We have also found that

Gsb prevents Wingless (Wg) from specifying the identity of NB4-2 by blocking Slp activity. Our genetic experiments show that gsb interacts with slp and inhibits slp from activating target genes that specify NB4-2 identity. Overexpression of slp overcomes this antagonistic interaction with gsb and respecifies NB5-3 as NB4-2. This respecification, however, can be suppressed by a simultaneous overexpression of gsb at high levels. Our results show that this mechanism is responsible for specifying NB5-3 identity to a row 5 neuroblast and also preventing Wg from specifying NB4-2 identity to that cell.

MATERIALS AND METHODS

Mutants, genetics

The wg alleles used are the two null alleles, wgCX4 and wgIG22 and a temperature-sensitive allele wgIL114. For slp mutation, a small internal deletion that removes most of slp1 and slp2 genes (Cadigan et al., 1994b) was used. The transgenic lines with hs-slpl and hs-slp2 genes have been previously described (Cadigan et al., 1994b). To determine the formation and specification of NB4-2 identity, the hkb enhancer-trap line, hkb5953 (see Doe, 1992), was introduced into slp mutant

Fig. 2. Loss of slp activity affects the expression of wg. The vertical lines in (B,D,F,H) indicate the midline and the anterior end of the CNS is up in these panels. Arrows in (E-H) indicate even-numbered parasegments. Note the decaying or missing wg stripes. Sagittal views of entire embryos are shown in wild type (A,C) and slp mutant (E,G) while wild type (B,D) and slp mutant (F,H) show ventral views. (A-D) Expression of wg stripes in wild type is shown. (E-H) Decaying or missing wg stripes in even-numbered parasegments in slp mutant embryos are shown.

Fig. 3. Loss of wg activity affects the expression of slp. The vertical lines in (B,C,E-H) indicate the midline, anterior end is up and numbers along the midline mark rows of neuroblasts. (A,D) Sagittal views of an entire embryo; the remaining panels show ventral views of the CNS. (A,C) Expression of slp1 in wild type. (D,F) Expression of slp1 in wg null embryos. (B,E) The neuroectodermal expression of slp1 in wild type and wg mutant, respectively. Note that the expression of slp1 in the anterior rows of neuroectodermal cells is lost in wg mutants. In C (wild type) and F (wg mutant), the expression of slp1 in neuroblasts is shown. Note that the expression of slp1 in row 4 neuroblasts in not significantly affected in wg mutants except in NB4-2 (F, arrow). (G,H) Ubiquitous expression of wg induces ubiquitous expression of slp1 during neurogenesis. Ventral views of anti-Slp1 stained embryos. (G) hs-wg embryo where the wg gene is ubiquitously induced. (H) hs-slpl transgenic embryo where the slp1 transgene is ubiquitously induced. The endogenous slp1 stripe is masked by the ubiquitous expression of slp1.
background using standard genetics. To rescue the NB4-2 lineage defect, hs-slp1 and hs-slp2 were introduced into wg\(^{CX4}\) and wg\(^{BH22}\) alleles. The various mutant combinations were constructed using standard genetics. Staging of embryos was done according to Wieschaus and Nusslein-Volhard (1986).

**Immunohistochemistry**

Embryos from various lines were collected and stained with antibodies as described previously (Bhat, 1996; Bhat and Schedl, 1997). Modifications to the general fixation conditions can be obtained by request. The antibodies used were against LacZ (to visualize Hkb\(^{5953}\) (1:200 dilution), Eve (1:2000), 22C10 (1:4), Slp1 (1:300), Gsb-distal (1:5), Wg (1:300); Svp (1:10); Runt (1:1). The wg\(^{CX4}\);hs-slp1, wg\(^{CX4}\);hs-slp2 or wg\(^{BH22}\);hs-slp1 or wg\(^{BH22}\);hs-slp2 embryos were identified by double staining with anti-Wg (1:200) and anti-Eve. A separate batch of wg;hs-slp1 embryos were also stained with anti-Eve and anti-Slp1 to correlate the rescue with the presence of high levels of Slp1 protein. To analyze rescue at the NB4-2 level, wgs;hkb\(^{5953}\);hs-slp1 embryos were heat shocked (see below) and double-stained with anti-LacZ and anti-Wg. In order to determine whether the hemisegments in slp mutants were missing a differentiated RP2 neuron (with its axonal projection), slp embryos were double stained with anti-Eve and mAb 22C10. The nuclear Eve staining was developed by the DAB reaction whereas the membrane 22C10 staining by the alkaline phosphatase reaction. hs-slp;hkb\(^{5953}\) embryos were double stained with anti-LacZ and anti-Gsb-distal and analyzed using confocal microscopy. While the intensity of staining of either Hkb-LacZ or Gsb-distal was weaker in double-staining experiments than in single-staining experiments due to less than ideal fixation conditions, the results were similar to the single staining results.

**Experiments with hs-slp transgenes**

wg;hs-slp1, wg;hs-slp2, wg\(^{CX4}\);hkb\(^{5953}\);hs-slp1, hs-slp1, hs-slp2 or hkb\(^{5953}\);hs-slp1 embryos were collected, dechorionated, immersed in halocarbon oil, and different stages were hand picked. Embryos aged between stage 7 to early stage 8 were transferred to tubes containing halocarbon oil and shifted to a 37°C water bath for 25 minutes. They were then shifted back to room temperature and allowed to develop until stage 13 for Eve staining or stage 9-11 for Hkb, Gsb-d, Runt, Svp and Wg staining. The hs-slp1 embryos were also stained with Slp1 antibody to confirm that the induction of Slp1 antibody to confirm that the induction of Svp and Wg staining. The expression pattern, physical sizes and position within the half-segment.

Initially, we examined embryos lacking the slp genes using an antibody against Even-skipped (Eve). In wild type, as shown in Fig. 1D,E, Eve is expressed in the NB4-2 lineage initially in the G2 phase of the GMC-1 cell cycle. Eve continues to be expressed in newly formed RP2 and sib. The smaller sib cell, however, soon loses Eve expression and only the larger RP2 maintains Eve throughout further development (Fig. 1E). Thus, the GMC-1, RP2 and sib can be reliably identified by their dynamics of Eve (and also several other marker genes) expression as well as the difference in their physical sizes. Examination of slp loss-of-function mutant embryos with Eve antibody indicates that these embryos lack the Eve-positive GMC-1 or its progeny, RP2 and RP2-sib (Fig. 1F,G). The absence of Eve-positive GMC-1→RP2/sib cells could mean that the GMC-1 has not formed from NB4-2. Alternatively, the identity of GMC-1 is not specified and thus, this ‘GMC-1’ has not generated a bona fide RP2 and a sib cell. Therefore, we examined the late stage CNS of slp mutants by double staining embryos with anti-Eve and a neuronal marker mAb 22C10. MAb 22C10 is raised against a neuronal marker mAb 22C10. MAb 22C10 is raised against an antibody against Even-skipped (Eve). In wild type, hkb is expressed in NB4-2 (Fig. 1A), thus it serves as an excellent marker for this neuroblast (see Chu-LaGraff and Doe, 1993; Bhat, 1996; Bhat and Schedl, 1997). Hkb is also expressed in several other neuroblasts (see Fig. 1A), thus, combined with the position of each of these neuroblasts, one can reliably identify NB4-2 within a hemisegment. When the NB4-2 in slp mutant embryos was examined for hkb expression using an enhancer-trap line in the hkb gene,
hkb<sup>5953</sup> (Doe, 1992), this neuroblast was found to be negative for Hkb (Fig. 1B). This indicates that <i>slp</i> genes are required for the specification of NB4-2 identity. We observed this identity specification defect in approximately 47% of the hemisegments (<i>n</i>=54). In the remaining 53% of the hemisegments, however, NB4-2 was not formed (Fig. 1C). This result indicates that <i>slp</i> genes are required not only for the specification of NB4-2 identity but also for the formation of NB4-2.

**The loss of NB4-2 lineage in sloppy paired mutants is not due to the effect of sloppy paired on wingless**

In the epidermis, mutation in <i>slp</i> genes result in a fusion of abdominal segments A1-A2, A3-A4, A5-A6 and A7-A8 (characteristic of pair-rule mutants) and replacement of naked cuticle by denticle belts, a wg-type of segment polarity phenotype (Grossniklaus et al., 1992; Cadigan et al., 1994a,b). Previously, it has been shown that during the patterning of the epidermis, <i>slp</i> genes function upstream of <i>wg</i> to maintain <i>wg</i> expression (Cadigan et al., 1994a,b). Since <i>wg</i> is also required for the formation and specification of NB4-2 identity, it was possible that the effect of loss of <i>slp</i> genes on NB4-2 is mediated via its effect on <i>wg</i> expression. Therefore, to determine the precise temporal requirement of <i>slp</i> for maintaining <i>wg</i> expression during neurogenesis, the expression of <i>wg</i> in <i>slp</i> mutant embryos was first examined. As shown in Fig. 2E,F. in <i>slp</i> mutants the <i>wg</i> expression begins to fade from the neuroectoderm initially in even-numbered parasegments (marked by arrows) approximately 3.75 hours of development (stage 7, early germ band extension). This fading is particularly prominent in abdominal segments (Fig. 2E, see arrows). By approximately 4.5 hours of development (early stage 9), <i>wg</i> expression in these parasegments is completely lost (Fig. 2G,H). By contrast, in odd numbered parasegments, <i>wg</i> expression is nearly as high as in wild type during early stage 9 (approximately 4.5 hours of development; compare Fig. 2C, wild type with Fig. 2G, mutant), and is only lost by approximately 6-6.5 hours of development (stage 10; data not shown). These results are consistent with the previous findings (Cadigan et al., 1994a,b) and show that <i>slp</i> genes function upstream of <i>wg</i> and positively regulate <i>wg</i> expression.

Previous studies using a temperature-sensitive allele of <i>wg</i> revealed that Wg activity is required for the specification of NB4-2 identity at approximately 4 hours of development (between early to mid-stage 8, at 22°C; see Chu-LaGraff and Doe, 1993; Bhat, 1996). However, in <i>slp</i> mutants the expression of <i>wg</i> is still high in the odd-numbered parasegments around the time of NB4-2 specification and the expression of <i>wg</i> is lost in these parasegments only by approximately 6.5 hours of development (stage 10; see Fig. 2G), nearly 2.5 hours after the specification of NB4-2 identity. Thus the loss of Wg expression from odd-numbered parasegments is well past the temporal requirement of <i>wg</i> for NB4-2 specification. Therefore, at least in the odd-numbered parasegments, the specification of NB4-2 identity in <i>slp</i> mutants must occur earlier than the decay of <i>wg</i> expression. Therefore, we conclude that the loss of NB4-2 identity in <i>slp</i> mutants is unlikely due to the loss of <i>wg</i> expression, at the least in the odd-numbered parasegments, and possibly in the even-numbered parasegments as well.

**wingless is required for the expression of sloppy paired in the neuroectodermal cells from which row 4 neuroblasts are delaminated**

The above results raise the possibility that in NB4-2 lineage, <i>slp</i> genes function either downstream of <i>wg</i> or in parallel to <i>wg</i> (in a pathway that does not involve <i>wg</i>), but not upstream of <i>wg</i>. If <i>slp</i> genes function downstream of <i>wg</i>, they are expected to be present in NB4-2 and its precursor cells (i.e. in Wg-receiving row 4 cells). Therefore, we examined whether <i>slp</i> genes are expressed in row 4 cells using an antibody against Slp1 (Cadigan et al., 1994a,b). In the wild-type embryo, previous results indicated that <i>slp</i> genes are expressed in Wg-expressing row of cells as well as in the row of cells immediately anterior to the Wg-expressing cells (Cadigan et al., 1994a,b). This would suggest that <i>slp</i> genes are expressed in Wg-receiving row 4 cells. Anti-Slp1 staining indicates that this is indeed the case. In the wild type, <i>slp1</i> is expressed in four rows of neuroectodermal cells (Fig. 3B). The posterior two rows of cells correspond to the Wg expressing domain from which row 5 neuroblasts are formed, whereas the anterior two rows correspond to cells from which row 4 neuroblasts are delaminated. At the neuroblast level, both row 5 and row 4 neuroblasts express <i>slp1</i> (Fig. 3C). The identity of rows of cells that express <i>slp1</i> was confirmed by double staining wild-type embryos with anti-Slp1 and anti-En antibodies (<i>en</i> is expressed in row 6 and row 7 neuroblasts and the precursor neuroectoderm from which these neuroblasts are derived; data not shown).

We reasoned that if the <i>slp</i> genes are downstream of <i>wg</i> in the CNS, the expression of <i>slp</i> in <i>wg</i> mutants must be affected. Indeed, as shown in Fig. 3D-F, the expression of <i>slp</i> was affected in <i>wg</i> null embryos. When stage 8 <i>wg</i> mutant embryos were stained with anti-Slp1, the expression of <i>slp1</i> was absent in those rows of neuroectodermal cells from which NB4-2 and other row 4 neuroblasts are delaminated (Fig. 3D-F). Interestingly, the expression of <i>slp1</i> in row 4 neuroblasts in <i>wg</i> mutants follows a different pattern. We found that although the expression of <i>slp1</i> in row 4 neuroectodermal cells is absent, in row 4 neuroblasts, it is more or less unaffected. One exception, however, is NB4-2. That is, whenever NB4-2 was present in <i>wg</i> mutant embryos (see below), it had no <i>slp1</i> expression (Fig. 3F).

A previous study by Chu-LaGraff and Doe (1993) indicated that <i>wg</i> is required for the formation of NB4-2 and other row 4 neuroblasts. For example, it was reported that in a temperature-sensitive allele of <i>wg</i> (<i>wg<sup>Il114</sup></i>), none of the hemisegments examined had NB4-1 or NB4-3 and only 4% of the hemisegments had NB4-4. NB4-2, on the other hand, was present in about 20% of the hemisegments. By anti-Slp1 staining, we observed that 84% of the hemisegments (the number of hemisegments examined, <i>n</i>=89) had NB4-1, of which 90% had moderately high levels of <i>slp1</i> expression (see Fig. 3F) whereas about 10% had very little of <i>slp1</i> expression. On the other hand, all the hemisegments examined had NB4-3 (see Fig. 3F). Moreover, NB4-4 was present in none of the hemisegments examined (<i>n</i>=89), although they were Slp1-negative (Fig. 3F). In summary, these results indicate that <i>wg</i> is required for the expression of <i>slp1</i> in those neuroectodermal cells from which row 4 neuroblasts are formed. While <i>wg</i> is also required for
the expression of \textit{slp1} in NB4-2, it is not required for the expression of \textit{slp1} in other row 4 neuroblasts.

The effect of loss of function for \textit{wg} on \textit{slp1} expression in row 5 appears to be only modest in stage-8 embryos. Thus, both the neuroectodermal cells as well as the row 5 neuroblasts that are derived from these cells had \textit{slp1} expression at this stage, although the level might be slightly reduced (Fig. 3D-F). However, at later stages of development, \textit{wg} is found to be required for the expression of \textit{slp1} in these cells. Thus, by stage 10, row 5 cells in most parasegments have lost most of their \textit{slp1} expression (data not shown). These immunohistochemistry results were also confirmed by western blotting analysis with anti-Slp1. The western analysis of embryo extracts from \textit{wg} mutant embryos revealed that the level of expression of Slp1 was considerably reduced in these embryos compared to wild type (data not shown). These results

**Fig. 4.** Induction of \textit{slp} from a heterologous promoter rescues the loss of NB4-2\rightarrow\text{GMC-1}\rightarrow\text{RP2\text/sib} lineage in \textit{wg} mutants. (A,B) CNS from embryos stained with anti-LacZ to visualize Hkb staining. (A) Wild-type embryo; (B) \textit{wg};\textit{hs-slp1;hkb}^{595} embryo where the \textit{slp1} transgene was induced (see text). Only half segments are shown and numbers along the midline indicate rows of neuroblasts in these two panels. (C-H) Embryos are double stained with anti-Eve and anti-Wg. Midline is marked by vertical lines; arrows, GMC-1/RP2/sib cells; arrowheads, lack of these cells. Anterior end is up. (C,D) Wild-type embryos. Note that in some hemisegments in A, the GMC-1 has divided to generate an RP2 and a sib. (E,F) \textit{wg} null embryos. All the hemisegments are missing the RP2/sib lineage. (G,H) \textit{wg};\textit{hs-slp} embryos. Only some of the GMC-1s in G have divided to generate RP2 and sib cells.

**Fig. 5.** Overexpression of \textit{slp1} respecifies additional neuroblasts as NB4-2. The midline is marked by vertical lines, anterior end is up. Half-segments are shown in all panels. (a-d) Anti-Eve stained, (e-h) anti-LacZ stained (to detect Hkb-LacZ) and (i,j) anti-Gsb stained embryos. (a) Approx. 8-hour-old wild-type embryo, showing an RP2 (larger, intensely stained cell) and a sib (smaller, weakly stained cell). (b) Approx. 13-hour-old wild-type embryo. Note that the sib cell has lost Eve expression. (c) Approx. 7.5-hour-old \textit{hs-slp1} embryo. One of the two GMC-1s has divided to generate an RP2 and a sib cell while the other has not yet divided. (d) Approx. 13-hour-old \textit{hs-slp1} embryo. Note that there are three RP2 neurons in this hemisegment. (e) Approx. 5.5-hour-old wild-type embryo stained for Hkb-LacZ. (f-h) Approx. 5.5- to 6-hour-old \textit{hs-slp1;hkb}^{595} embryos. Note that NB5-3 (f,g), NB3-5 (g,h) and NB5-6 (h) have Hkb-LacZ expression, indicating that these neuroblasts have adopted a NB4-2 identity. (i) Expression of Gsb in wild-type embryo. Row 6 neuroblasts also express Gsb; however, they have not yet formed at this stage. (j) Expression of Gsb is not affected in \textit{hs-slp1} embryos in row 5 neuroblasts.
therefore indicate that \( wg \) is required to maintain a high level of \( slp \) expression and also reveal an opposite relationship between \( wg \) and \( slp \) during neurogenesis from the one that exists during the patterning of the epidermis (see Cadigan et al., 1994a,b).

That the \( Wg \) signaling activates \( slp \) genes in the CNS is also illustrated by another result. As shown in Fig. 4G, ectopic induction of \( wg \) in a ubiquitous manner from a \( hs-wg \) transgene (Noordermeer et al., 1992) results in a ubiquitous expression of \( slp1 \). The endogenous \( slp1 \) stripes in such embryos appear to be masked by the ectopic expression of \( slp \), which is indicated by the fact that a similar masking of the endogenous \( slp \) stripe is observed in embryos where \( slp1 \) is ectopically induced using \( hsp70 \) promoter, see Fig. 3H). Moreover, by western blotting analysis, the extract from \( hs-wg \) transgenic embryos that were subjected to heat shock had a very high level of \( Slp1 \) compared to the extract prepared from noninduced \( hs-wg \) embryos (data not shown). These results show that \( wg \) is a positive regulator of \( slp \) during neurogenesis, and that \( wg \) not only functions upstream of \( slp \) but can ectopically activate \( slp \) expression in the neuroectoderm.

Expression of \textit{sloppy paired} from heterologous promoter rescues the NB4-2 defects in \textit{wingless} mutants

Given the above results, it seemed possible that \( slp \) genes are the targets of the \( Wg \)-signaling pathway in the \( Wg \)-receiving NB4-2 and that it is the \( slp \) genes that mediate the formation and the specification of NB4-2 identity. This possibility was directly tested by determining whether or not the expression of either the \( slp1 \) or the \( slp2 \) genes from a heterologous promoter can rescue the NB4-2 defects in \( wg \) mutant embryos. Initially, the \( slp1 \) transgenic gene under the control of the heterologous, inducible \( hsp70 \) gene promoter was introduced into a \( wg \) null mutant. The \( slp1 \) gene was then induced in the neuroectoderm of \( hs-slp1;wg \) embryos during the time when \( wg \) is shown to be required for NB4-2 specification (see Chu-LaGraff and Doe, 1993; Bhat, 1996). As shown in Fig. 4G, H, Eve staining of the \( wg;hs-slp1 \) embryos indicates that such an induction of \( slp1 \) rescues the RP2/sib lineage in as many as 85% of the hemisegments (\( n=240 \)). We further examined this NB4-2→RP2/sib lineage rescue at the NB4-2 level by using the
NB4-2 specific marker, hkb<sup>5953</sup>. Staining of embryos of the genotype wg; hs-slp1; hkb<sup>5953</sup>, where the hs-slp1 was induced prior to the formation of NB4-2, with anti-LacZ indicates that an Hkb-LacZ positive neuroblast in the position of NB4-2 was indeed observed in these embryos (Fig. 4B). Such Hkb-positive NB4-2 in wg; hs-slp1 embryos was observed in approximately 43% (n=56) of the hemisegments (see section below to account for the discrepancy observed in the percentage of hemisegments showing the rescue between Hkb and Eve staining experiments). Since the rescue of NB4-2 was observed in approximately 43% of the hemisegments, hs-slp1 must rescue both the NB4-2 formation and specification defects in wg mutant embryos.

Since previous results indicate that the sister gene of slp1, slp2, is also expressed in the same domain as slp1 (Grossniklaus et al., 1992; Cadigan et al., 1994a,b), we sought to determine whether the slp2 gene can also rescue the loss of NB4-2 in wg mutants. A similar induction of slp2 in wg null mutant embryos (wg; hs-slp2) is also found to rescue the RP2 lineage (data not shown). The rescue was, however, not as efficient as slp1 and thus approximately 50% of the hemisegments (n=90) were found to be rescued in hs-slp2/wg mutant embryos. It must be pointed out that we examined the rescue effect of slp genes using two different alleles of wg, wg<sup>CX4</sup> and wg<sup>G22</sup>; both are considered either true null or phenotypic null alleles of wg. Also, more than one transgenic line was tested for the rescue and the rescue effects of different slp transgenic lines were found to be similar.

**Ectopic expression of slp genes in the neuroectoderm generates additional GMC→RP2/sib lineages in the CNS**

In order to determine if the slp genes have an instructive role in the specification of NB4-2 identity, we ectopically expressed wild-type slp1 or slp2 genes in embryos derived from hs-slp1 and hs-slp2 transgenic lines using the heat-inducible heat shock 70 (hsp70) promoter. A brief heat induction of slp genes at different stages of development (25 minutes) indicates that ectopic expression of these genes at high levels within the neuroectoderm between 2.5 and 4.0 hours of development (at 22°C) results in generating additional GMC-1→RP2/sib lineages. For instance, with anti-Eve staining, a brief induction of slp1 gene (see Materials and Methods) during this time resulted in the duplication of the RP2/sib lineage in 18% of the hemisegments (n=77). In 17% of the hemisegments, triplication of the lineage was observed (Fig. 5c,d). A similar result was also observed in hs-slp2 embryos, although the frequency of transformation was about half that of hs-slp1 (data not shown).

The presence of additional RP2/sib lineages in hs-slp embryos could be due to other GMCs assuming a GMC-1 identity and generating the RP2/sib lineage or, alternatively, they might be arising from other neuroblasts adopting a NB4-2 identity. Previous results indicate that NB4-2 identity is determined in the neuroectoderm itself prior to its delamination (Chu-LaGraff and Doe, 1993; Bhat, 1996; reviewed in Bhat, 1999). Neuroblasts begin to form during stage 8 (at about 5 hours of development; NB4-2 is formed at about 5.5 hours of development as an S2 NB); Since the timing of the effect of ectopic slp in producing additional RP2/sib lineages is prior to the formation of any neuroblasts (i.e. 2.5-4.0 hours of development), it seems highly likely that the effect is at the NB level and not at the GMC level. Thus, other NBs must be adopting NB4-2 identity in hs-slp embryos (see below).

**NB5-3 adopts NB4-2 identity in embryos expressing slp genes at high levels**

The presence of additional RP2/sib lineages in hs-slp1 (or hs-slp2) embryos suggests that inappropriate expression of slp1 results in other neuroblasts adopting a NB4-2 fate. To determine if this is true, the NB4-2 marker, hkb<sup>5953</sup>, was introduced into the hs-slp1 background and the effect of induction of hs-slp1 (see Materials and Methods) was determined by examining the Hkb-LacZ expression pattern. As shown in Fig. 5f-h, consistent with the possibility that the additional Eve-positive GMC-1→RP2/sib lineages were generated by NBs that have adopted NB4-2 identity, we observed several other NBs that are normally negative for Hkb now expressing Hkb-LacZ. These neuroblasts include NB5-3, NB3-5 and NB5-6 (Fig. 5h). Interestingly, we find that the row 5 neuroblast, NB5-3, is the most frequently transformed neuroblast in these embryos (approximately 35% of the hemisegments, n=44). This NB is formed during the first wave of neuroblast delamination during early stage 8 (at about 5 hours of development at 22°C). The frequency of transformation of this NB was highest when the transgene was induced between 3.0 and 3.5 hours of development. This indicates that the NB5-3 identity is specified nearly 1-1.5 hours prior to its delamination from the neuroectoderm and that ectopic induction of hs-slp1 during this window of time specifies NB5-3 as NB4-2. A similar result was also observed with hs-slp2 (data not shown), although this frequency was approximately 50% lower than hs-slp1.

**The NB5-3 that changes its identity to NB4-2 in embryos ectopically expressing high levels of slp retains expression of gsb**

The specification of NB4-2 identity to NB5-3 by overexpression of slp1 at high levels was remarkable on two counts. First, in wild type, NB5-3 and its precursor cells normally express slp and this does not lead to an NB5-3→NB4-2 transformation. This suggests that the specification of NB5-3 identity is sensitive to the levels of Slp. Second, NB5-3 and its precursor cells express gsb, and our previous results show that whenever Gsb is present in a cell, it prevents Wg from specifying NB4-2 identity to that cell (Bhat, 1996; Bhat and Schedl, 1997; see also Duman-Scheel et al., 1997). Therefore, the specification of NB4-2 identity to NB5-3 in hs-slp1 embryos suggests two possibilities. (1) Overexpression of slp in NB5-3 and its precursor cells, leads to a loss of gsb expression in these cells and this in turn allows the autocrine Wg to specify NB4-2 identity to NB5-3. (2) Slp and Gsb antagonistically interact with one another in NB5-3 precursor cells and an excess of slp overcomes this inhibitory interaction with Gsb and specifies NB4-2 identity to NB5-3.

Initially, we determined if the expression of gsb is lost in NB5-3 in hs-slp1 embryos by examining the hs-slp1 embryos with anti-Gsb. As shown in Fig. 5j, the level of Gsb in NB5-3 was found to be unaffected in these embryos. Moreover, expression of another NB5-3 specific marker, runt (see Doe, 1992), was also unaffected in these embryos (data not shown). These results would, therefore, argue that a high level of Slp
protein overcomes the inhibitory effects of Gsb (and other NB5-3 specific genes) and re-specifies NB5-3 as NB4-2.

**Slp and Gsb proteins antagonistically interact with each other in a dose-dependent manner**

To further establish the possibility that Gsb and Slp antagonistically interact with each other, we overexpressed *slp1* and *gsb* genes simultaneously using hs-sslpl and hs-gsb transgenic lines (Fig. 6). Previous studies have shown that ectopic induction of *gsb* results in the loss of RP2 lineage (Zhang et al., 1993; Li and Noll, 1994; Bhat, 1996; Bhat and Schedl, 1997). If Slp and Gsb proteins antagonistically interact with each other, embryos carrying one copy of the *hs-sslpl* and two copies of the *hs-gsb* are expected to have the RP2/sib lineage missing as in *hs-gsb* embryos. As shown in Fig. 6C,D, when there are two copies of the *hs-gsb* and only one copy of the *hs-sslpl*, the RP2/sib lineage was found to be missing from 97% of the hemisegments (n=196). By contrast, when the copy numbers of each of the transgenes are the same (either one or two), both the phenotypes (loss and gain of RP2s) were observed in a given embryo in a partially penetrant manner (Fig. 6E,F). These results indicate that the penetrance of the loss of NB4-2 lineage (*hs-gsb* phenotype) or the re-specification of NB5-3 as NB4-2 (the gain of RP2/sib lineage, the *hs-sslpl* phenotype) is dependent on the levels of the two proteins. Thus, the respecification of NB5-3 as NB4-2 by a high level of *sslpl* can be effectively countered by simultaneously increasing the levels of *gsb*. These results lend additional support to the conclusion that Slp and Gsb proteins antagonistically interact with one another during NB4-2 specification.

We further tested this idea genetically by examining if the NB5-3→ NB4-2 transformation in *gsb* mutants is mediated by Slp. The RP2 lineage present in wild type (Fig. 7A,B) is missing in *slp* mutants (Fig. 7C,D) and is duplicated in *gsb* and *en*, *inv* mutants (Fig. 7E,F and I,J, respectively). Consistent with the possibility that *slp* genes are responsible for the NB5-3→ NB4-2 transformation in *gsb* mutants, the RP2 lineage was found to be missing in *slp*, *gsb* mutant embryos (Fig. 7G,H). Furthermore, our previous results showed that embryos lacking *en* and *inv* genes lose Gsb expression from NB5-3 and its precursor cells and as a result, the NB5-3 is transformed into NB4-2 in *en*, *inv* mutants (Bhat and Schedl, 1997). As indicated by the examination of *en*, *inv*, *sslpl* mutant embryos, the transformation of NB5-3 to NB4-2 in *en*, *inv* mutants due to loss of expression of Gsb in NB5-3 precursor cells is found to be *sslpl*-dependent (Fig. 7K,L).

**DISCUSSION**

A previous study showed that the two *slp* genes, *sslpl* and *slp2*, which encode proteins containing the DNA binding forkhead domain, are required for segmentation (Grossniklaus et al., 1992; Cadigan et al., 1994a,b). The current study addresses the requirements of *slp* genes during neurogenesis. In this study, quite the opposite picture of the relationship between *wg* and *slp* emerges during neurogenesis from that of epidermal patterning. In the CNS, *wg* positively regulates *slp* expression, thus *slp* functions downstream of *wg*. Moreover, while the previous studies have well deduced the genetic hierarchy between *gsb*, *wg*, *ptc*, *hh* and *en*/*inv* (reviewed in Bhat, 1998a, 1999), this current study establishes the inter-relationship between these genes and *slp* in the specification of NB5-3 and NB4-2 identities (Fig. 8).

**sloppy paired genes function downstream of wingless during neurogenesis**

While the evidence to support the conclusion that *slp* genes regulate expression of *wg* in the epidermis is quite strong (see Fig. 2; also Cadigan et al., 1994a,b), the evidence that the *wg*-signaling controls the expression of *slp* in the CNS is also equally strong. First, the *slp* genes are expressed not only in the *wg*-expressing row 5 cells but also in the Wg-negative, but Wg-receiving row 4 cells (Fig. 3). Second, the expression of *slp* was affected in *wg* mutant embryos. That is, staining of *wg* mutant embryos show that the expression of *slp* is lost from the Wg-receiving row 4 neuroectodermal cells (Fig. 3). This result is also supported by the western analysis of embryo extract from *wg* mutants in which the level of Slp protein was found to be greatly reduced (data not shown). Third, consistent with the above result, the ectopic expression of *wg* induces ectopic expression of *slp* in the neuroectoderm. Fourth, in *slp* mutants, just as in *wg* mutants, the formation and identity specification of a well-studied neuronal precursor cell, NB4-2, is affected; this defect in *wg* mutants can be rescued by the expression of *slp* genes from a heterologous promoter (Fig. 4). Moreover, a similar relationship also appears to exist between *slp* and *wg* during mesoderm specification. For instance, in both *wg* and *slp* mutants, the specification of heart cells (derived from mesoderm) is affected and this defect in *wg* mutants can be rescued by expressing *slp* genes from a heterologous promoter (our unpublished results). These results therefore indicate that *wg* is a positive regulator of *slp* expression not only during neurogenesis but also in other processes such as mesoderm specification.

An intriguing aspect of regulation of *slp* genes by *wg* is the finding that this regulation is restricted primarily to the neuroectoderm but not extended to the neuroblasts that are derived from these neuroectodermal cells, with one exception, the NB4-2. Thus, while row 4 neuroectodermal cells in *wg* mutant are missing *sslpl* expression, row 4 neuroblasts other than NB4-2 have *sslpl* expression. The induction/maintenance of *sslpl* expression in these neuroblasts must, therefore, necessarily be under the control of some other pathway. Alternatively, the Wg-signaling pathway is redundant in these neuroblasts. These results are consistent with our previous finding that the induction of an ectopic *gsb*-stripe by gain-of-function *wg* occurs only in the neuroectodermal cells but not in the underneath neuroblasts (Bhat and Schedl, 1997). In summary, these results reveal a hitherto unsuspected relationship between *wg* and *slp* in the CNS that is the opposite of their relationship in the epidermis (summarized in Fig. 8).

**The activity of sloppy paired genes regulate the formation and the specification of NB4-2 identity in the CNS**

Using a temperature-sensitive allele of *wg*, it was shown that the requirement of Wg in the CNS for NB4-2 formation and specification is between late stage 7 and early stage 8 and precedes its requirement for epidermal patterning (Chu-LaGraff and Doe, 1993; Bhat, 1996). Moreover, it is the neuroectodermal expression of *wg* that regulates NB4-2 formation and identity specification. Thus, while the timing of
decay of \(wg\) expression in \(slp\) mutants in even-numbered parasegments coincides with the requirements of \(wg\) for NB4-2 formation and specification, it is not so in the odd-numbered parasegments. Thus, the odd-numbered parasegments in \(slp\) mutants have \(wg\) expression during the time Wg is required for NB4-2 formation and specification. Since the loss of NB4-2 lineage in \(slp\) mutants is not parasegment-specific and the expression of \(slp\) in NB4-2 and its precursor neuroectodermal cells is lost in \(wg\) mutants, it must be that \(slp\) genes are downstream of \(wg\) in these CNS cells.

The Wg signal regulates the specification of NB4-2 identity via the Arm and Pan (K. B., unpublished results). The Arm-Pan must activate certain downstream target gene(s), presumably transcription factors, and these transcription factors then initiate a program that mediates the formation and specification of NB4-2. Our results indicate that \(slp\) genes function as downstream targets of the \(wg\) signaling, which regulate both the NB4-2 formation as well as the identity specification during neurogenesis. This conclusion is based on the following facts. First, the loss of function effect for \(slp\) genes has the same effect as the loss of function for \(wg\) on NB4-2 lineage (Fig. 1). Second, the loss of \(wg\) activity in row 5 cells in the CNS leads to a loss of \(slp\) expression from the Wg-receiving NB4-2 and its precursor cell (Fig. 3). Third, the loss of NB4-2 in \(wg\) mutants can be rescued by the expression of \(slp\) genes from a heterologous promoter during the time when \(wg\) is known to be required for the process (Fig. 4). We acknowledge that the \(slp\) genes might be either the direct targets of the Wg-signaling pathway (i.e. Arm-Pan complex directly activating \(slp\) genes), or instead there are additional genes inbetween \(pan\) and the \(slp\) genes. While this issue has not been resolved here, the rescue of the NB4-2 lineage defect in \(wg\) mutants by expressing \(slp\) genes from a heterologous promoter reveals that the Wg-signaling pathway must ultimately activate \(slp\) genes, and the \(slp\) genes then regulate the formation and specification of NB4-2.

**Gooseberry and Sloppy paired negatively interact with each other during neurogenesis**

Previously, we showed that the specification of NB4-2 identity in row 4 and NB5-3 in row 5 requires a complex signaling cascade between row 6/7 and row 5, and between row 5 and row 4 (Bhat, 1996; Bhat and Schedl, 1997; reviewed in Bhat, 1999). One of the major findings of these previous studies is that Gsb blocks the Wg signal from specifying NB4-2 identity. For example, in embryos mutant for the segment polarity gene \(ptc\), Gsb is ectopically expressed in row 4 cells. The ectopic Gsb present in row 4 cells in \(ptc\) mutants prevents the paracrine \(wg\) from specifying NB4-2 identity in row 4. Thus, while the NB4-2 identity is not specified in \(ptc\) mutants, it is normally specified in \(ptc, gsb\) double mutants in a \(wg\)-dependent manner (Bhat, 1996; see also Duman-Scheel et al., 1997). In row 5, the expression of \(gsb\) is uncoupled from the repression of \(Ptc\) and this prevents the specification of NB4-2 identity by autocrine Wg to NB5-3. Thus, in \(gsb\) mutants, NB5-3 is specified as NB4-2 in a \(wg\) dependent manner (Bhat, 1996; see also Duman-Scheel et al., 1997). Similarly, in \(en, inv\) mutants, Gsb is lost from NB5-3 and its precursor neuroectodermal cells. This leads to the transformation of NB5-3 to NB4-2 in a \(wg\)-dependent manner (Bhat and Schedl, 1997). Moreover, ectopic expression of \(gsb\) at high levels blocks the specification of NB4-2 to a cell (see Zhang et al., 1993; Li and Noll, 1994; Bhat, 1996; Bhat and Schedl, 1997).

The analysis of mutants between \(gsb\) and \(slp\) or \(en, inv\) and \(slp\) presented in this study indicates that the way Gsb prevents the specification of NB4-2 identity (in \(ptc\) mutants) and NB4-2 specification to NB5-3 in wild type is by blocking \(slp\) genes. Thus, while NB5-3 is transformed into NB4-2 in \(gsb\) or \(en, inv\) mutants, such transformation is prevented in \(slp, gsb\) or \(slp, en, inv\) mutant embryos. Furthermore, whereas the NB4-2 identity is normally specified in \(ptc, gsb\) double mutants, it is not so in \(ptc, gsb, slp\) mutants (data not shown).

The finding that Gsb negatively interacts with Slp and prevent Slp from specifying NB4-2 identity is further supported by the result that Slp and Gsb exhibit a dose-dependent effect on NB5-3 and NB4-2 specification. That is, in wild type both \(slp\) and \(gsb\) genes are expressed in NB5-3 and its precursor cells; however, NB5-3 is not re-specified as NB4-2 by Slp. However, overexpression of \(slp\) respecifies NB5-3 as NB4-2 without affecting the levels of Gsb in NB5-3 and its precursor cells. This indicates that Slp overrides the inhibitory effects of Gsb when present at high levels and specifies NB4- identity. However, a simultaneous overexpression of \(gsb\) can suppress the respecification of NB5-3 as NB4-2 by an increased level of Slp. Moreover, the observation that inappropriate (location and level) expression

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**Fig. 8.** The interaction of \(wg\), \(slp\) and \(gsb\) during neurogenesis. \(Wg\) is expressed only in row 5 cells and signals to rows 4 and 6 (paracrine) and to row 5 (autocrine). The \(slp\) genes are expressed in rows 4 and 5, whereas \(gsb\) is expressed in row 5. The \(Wg\)-signaling pathway controls the expression of \(slp\) in row 4 (either directly or indirectly), and the \(slp\) genes in turn promote NB4-2 formation and its identity specification. Since the loss of function of \(wg\) on \(slp\) expression in row 5 is less severe than on row 4, the expression of \(slp\) genes must be partially controlled by the \(Wg\)-signaling pathway in row 5 cells. In row 5, although \(slp\) genes are expressed, they do not specify NB4-2 identity to any of the row 5 cells, such as NB5-3. The Gsb protein blocks the ability of Slp to specify NB4-2 identity to row 5 cells.
of slp genes respecifies additional neuroblasts as NB4-2 (as many as three neuroblasts), but this can be blocked by a simultaneous overexpression of gsb, further lends support to the contention that the two negatively interact with each other during neurogenesis. While the genetic experiments indicate that Gsb and Slp negatively interact with each other during NB4-2 specification, they do not reveal the precise mode of interaction between Gsb and Slp. That is, this interaction could be a direct protein-protein interaction, or involves activation or repression of intermediate genes.

Respecification versus fate transformation: are they different?

The issue of respecification versus fate transformation, whether or not these two events are one and the same, requires some examination. For example, in gsb mutants, the NB5-3 behaves as NB4-2 (i.e. it generates a RP2/sib lineage), indicating that at high levels is able to respecify the neuroblast as NB4-2, overriding NB5-3 genes (gsb and genes downstream of gsb). One can also argue that the expression of Gsb or Runt may be permissive to NB4-2 specification and that transformation versus respecification is just a matter of definition. The fact that gsb is able to block slp from specifying NB4-2 identity, but in a dose-dependent manner, argues that the two may be different.

We would like to thank Ken Cadigan for the slp mutant, the hs-slp lines and anti-Slp1 antibody and Bob Holmgren for the Gsb-d antibody. We also thank Chris Doe for the hkb983 line, Peter Gergen for the Runt antibody and Manfred Frasch for the Eve antibody. Comments from members of the Bhat laboratory were very useful. This work is supported by a grant from NIH (ROI GM58237).

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