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The Snail protein family regulates neuroblast expression of *inscuteable* and *string*, genes involved in asymmetry and cell division in *Drosophila*

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SUMMARY

Delaminated neuroblasts in *Drosophila* function as stem cells during embryonic central nervous system development. They go through repeated asymmetric divisions to generate multiple ganglion mother cells, which divide only once more to produce postmitotic neurons. Snail, a zinc-finger transcriptional repressor, is a pan-neural protein, based on its extensive expression in neuroblasts. Previous results have demonstrated that Snail and related proteins, Worniu and Escargot, have redundant and essential functions in the nervous system. We show that the Snail family of proteins control central nervous system development by regulating genes involved in asymmetry and cell division of neuroblasts. In mutant embryos that have the three genes deleted, the expression of *inscuteable* is significantly lowered, while the expression of other genes that participate in asymmetric division, including *miranda*, *staufen* and *prospero*, appears normal. The deletion mutants also have much reduced expression of *string*, suggesting that a key component that drives neuroblast cell division is abnormal. Consistent with the gene expression defects, the mutant embryos lose the asymmetric localization of *prospero* RNA in neuroblasts and lose the staining of Prospero protein that is normally present in ganglion mother cells. Simultaneous expression of *inscuteable* and *string* in the *snail* family deletion mutant efficiently restores Prospero expression in ganglion mother cells, demonstrating that the two genes are key targets of Snail in neuroblasts. Mutation of the dCtBP co-repressor interaction motifs in the Snail protein leads to reduction of the Snail function in central nervous system. These results suggest that the Snail family of proteins control both asymmetry and cell division of neuroblasts by activating, probably indirectly, the expression of *inscuteable* and *string*.

Key words: Snail, Neuroblasts, Asymmetry, Repressor, Cell division, *Drosophila*

INTRODUCTION

The Snail protein family contains a key signature of four to six conserved zinc fingers. These proteins function as transcriptional regulators during embryonic development, cancer formation and apoptosis (Hemavathy et al., 2000; Manzanares et al., 2001). *Drosophila* Snail was the first member identified and shown to be a repressor essential for mesoderm development (Grau et al., 1984; Nusslein-Volhard et al., 1984; Boulay et al., 1987; Kosman et al., 1991; Leptin, 1991; Ip et al., 1992; Hemavathy et al., 1997). Expression pattern analyses revealed that Snail is also present in embryonic wing disc primodia and neuroblasts (Alberga et al., 1991; Kosman et al., 1991; Leptin, 1991; Ip et al., 1994). Snail acts redundantly with Escargot, another member of this zinc-finger protein family, to control wing disc development (Whiteley et al., 1992; Hayashi et al., 1993; Fuse et al., 1996). However, single and double mutants of *snail* and *escargot* do not have a significant phenotype in the developing central nervous system (CNS) (Ashraf et al., 1999).

The absence of a CNS phenotype in the null mutants of *snail* is due to the redundant function provided by *escargot* and *worniu*, the third member of the protein family. These three genes are clustered in 35D1 region of the second chromosome. In deletion mutants that uncover these three genes, the ventral nerve cord is severely underdeveloped, as revealed by analysis of multiple neuronal markers (Ashraf et al., 1999). Some of the early CNS markers affected include *fushi tarazu* (*ftz*) and *even-skipped* (*eve*). *ftz* is initially expressed in many ganglion mother cells (GMCs) and later in many neurons (Goodman and Doe, 1993). In the deletion mutants, *ftz* expression in GMCs is almost abolished, and such defect can be rescued efficiently by transgenic expression of *snail*, *worniu* or *escargot* (Ashraf et al., 1999).

During CNS development, clusters of cells in the neuroectoderm receive instructions from proneural genes to become competent to form neuroblasts (Campos-Ortega, 1993). These proneural genes include the *achaete-scute* complex, *ventral nervous system defective* (*vnd*), *intermediate neuroblast defective* (*ind*) and *muscle segment homeobox* (*msh*)

(Isshiki et al., 1997; Campos-Ortega, 1998; Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998). Through the process of lateral inhibition, which involves Notch-Delta signaling, one of the cells in each cluster is selected to become neuroblast and delaminates from the ectoderm (Bhat, 1998; Rooke and Xu, 1998). Delaminated neuroblasts have stem cell property, whereby each goes through repeated asymmetric cell divisions to generate multiple GMCs (Lu et al., 2000). Many genes that participate in neuroblast asymmetric division have been identified. For example, *bazooka* functions in the neuroectoderm to help polarize the cells (Schober et al., 1999; Wodarz et al., 1999). During or soon after delamination, genes such as *inscuteable*, *miranda* and *staufen* are expressed (Ikeshima-Kataoka et al., 1997; Li et al., 1997; Shen et al., 1997). One of the functions of these genes is to control the subcellular localization within the neuroblasts of *prospero* mRNA and Prospero protein, which are segregated preferentially into GMCs after cell division. Prospero is a key factor in determining GMC fate, regulating the expression of neural genes such as *ftz* and the single round of cell division that produces postmitotic neurons (Doe et al., 1991; Vaessin et al., 1991; Li and Vaessin, 2000). As *snail* and *worniu* have extensive expression in neuroblasts, and GMC and neuronal marker expression is defective in mutants that have the *snail* family locus deleted, we surmised that Snail family of proteins may function at a regulatory step in neuroblast or GMC development (Ashraf et al., 1999).

We show here that the absence of *ftz* and *eve* in GMCs and neurons is probably due to misregulation of early steps of neuroblast function. The *snail* family deletion mutant embryos exhibit normal early neuroblast delamination. The delaminated neuroblasts, however, have significantly lower level of *inscuteable* RNA expression. The expression of other genes involved in asymmetric division, including *miranda*, *staufen* and *prospero* appears to be normal. Consistent with the defect of *inscuteable* expression, the asymmetric localization of *prospero* RNA is disrupted and the strong Prospero protein staining in GMCs, normally a result of asymmetric division, is lost. All of these defects can be rescued by transgenic expression of Snail, Worniu or Escargot. Thus, the establishment of neuroblast asymmetry is partially dependent on the Snail family of proteins. We have also observed that the phenotype of loss of Prospero protein staining in GMCs is more severe in the *snail* family genes deletion mutants than in the *inscuteable* mutants. Therefore, Snail family may have functions in addition to the regulation of *inscuteable*. Accordingly, we have found that expression of neuroblast-specific *string* RNA and *string* promoter-*lacZ* reporters (Lehman et al., 1999) are also dependent on Snail protein family. The Prospero expression in GMCs of *snail* family mutant embryos can be rescued by transgenic expression of *inscuteable* and *string*, suggesting that activation of *inscuteable* and *string* are key functions of Snail in neuroblasts. We further demonstrate that the co-repressor interaction motifs (Nibu et al., 1998a; Nibu et al., 1998b) of Snail are essential for rescue of the CNS phenotypes, indicating that Snail probably acts as a repressor in the CNS and activates target genes indirectly. Together, the results support the idea that both neuroblast cell division and asymmetry are regulated by the Snail family of proteins, perhaps by repression of a yet to be identified target gene that normally functions to suppress *inscuteable* and *string* transcription.

MATERIALS AND METHODS

Drosophila stocks and genetics

Fly stocks were maintained at 25°C using standard cornmeal-yeast-agar medium. The *y* w^{67} stock was used for all P-element-mediated transformation. The transformation constructs were co-injected with the Δ2-3 transposase helper plasmid. Genetic crosses that established the rescue lines were performed as described previously (Ashraf et al., 1999). Briefly, the individual rescue transgenes on the third chromosome were crossed with the *osp29* mutant chromosome and stable lines were established. Chromosomes that contain two transgenes were obtained by meiotic recombination. The recombined third chromosome containing two transgenes was then crossed with the *osp29* chromosome. The *string-lacZ* 5.3 transgene is located on the third chromosome and was crossed to the deletion mutant background by genetic crosses. The *string-lacZ* 6.4 and UAS-*string* transgenes are located on the second chromosome and were recombined with the *osp29* mutant chromosome. Female flies transheterozygous for *osp29* and *string transgenes* were collected and mated with a second chromosome balancer line (*y w*; *BcElp*/*CyO*) males. Male offspring that had red eyes and curly wings were collected and mated with *y w*; *osp29*/*CyO* and sna^{HG31}/*CyO* females to test for lethality over the mutant chromosomes. The crosses that produced no straight wing progeny were further tested by examining morphological defects that are similar to *snail* mutants and by in situ RNA staining for *lacZ* or *string*, confirming the presence of transgenes. $Df(1)sc^{B57}$, $vnd^{\Delta 38}$, $ind^{\Delta 79.3}$, ind^{RR108}, $msh^{\Delta 68}$ and $insc^{P72}$ mutant alleles were used in gene expression analyses (Kraut et al., 1996; Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998). UAS-*string* (Neufeld et al., 1998) was obtained from Bloomington stock center. The deficiency lines have been described in detail elsewhere (Ashraf et al., 1999).

Plasmids

snail cDNA fragments with dCtBP-binding site mutations were isolated from pSK(+)snail M1, M2 or M12 (Nibu et al., 1998a; Nibu et al., 1998b) by digestion with *Asc*I. The fragments were blunted with T4 polymerase and cloned into the *Kpn*I and *Xba*I (both blunted) sites of pCaSpeR-Snailp vector (Ashraf et al., 1999). This vector contain 2.8 kb of the *snail* promoter. The generation of transgenic rescue constructs of *snail*, *worniu* and *escargot* were described previously (Ashraf et al., 1999). pSnailpGal4 plasmid was constructed by cloning the Gal4 fragment (*Kpn*I/*Xba*I) from pGATN into the same sites of pCaSpeR-Snailp vector.

Embryo RNA in situ hybridization and immunohistochemical staining

RNA in situ hybridization was performed as described previously (Hemavathy et al., 1997). Antibody staining was performed essentially as previously described (Ashraf et al., 1999). For localization of Snail in CNS, affinity purified polyclonal antibody (guinea pig) was used at 1:5 dilution. The Prospero monoclonal antibody was used at 1:1 and Hb polyclonal antibody (guinea pig) at 1:400. The secondary antibodies were obtained from the Jackson Laboratory and used at 1:1000 for anti-rabbit, 1:400 for anti-mouse and 1:1000 dilutions for anti-guinea pig. Anti-phosphorylated H3 antibodies (rabbit) were purchased from Upstate Biotechnology and used at 1:200 dilution. Embryo sectioning was performed by embedding the stained embryos in Epon plastic (Hemavathy et al., 1997). The plastic embedded embryos were cut as sections of 3 μm thickness.

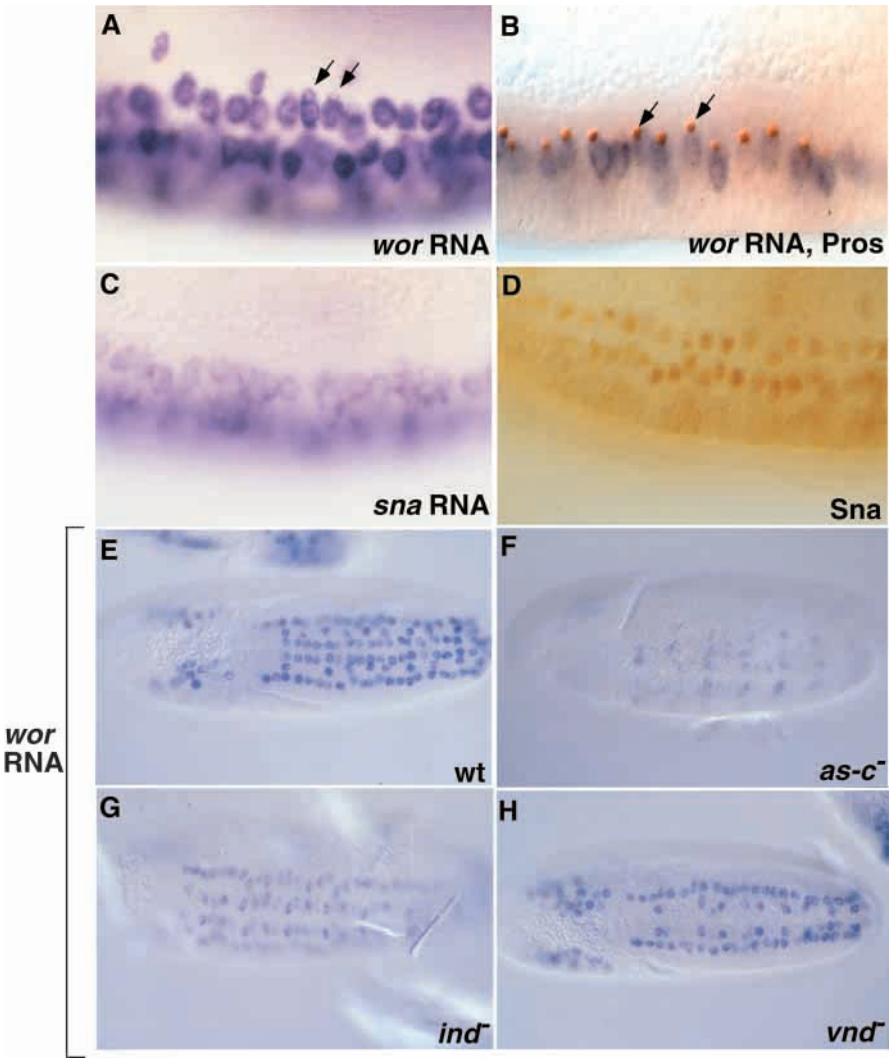


Fig. 1. Expression of *snail* and *worniu* in early CNS. (A,B,E-H) RNA in situ hybridization of *worniu*. (C) RNA in situ of *snail*. (D) Antibody staining for Snail. (B) Double staining with Prospero antibody. (A-E) Wild-type embryos; the genotypes of the embryos in F-H are as indicated. The orientation of the embryos in this and following figures is anterior towards the left. (A-D) Lateral views; (E-H) Dorsal-ventral views. All the embryos are approximately at stage 9. *wor* RNA is expressed extensively in neuroblasts but in only a small number of GMCs, indicated by arrows (A). At a similar stage, many GMCs have formed, as indicated by the Prospero protein staining (arrows, B). (C,D) *sna* RNA and protein expression is also restricted predominantly to neuroblasts. (E-H) *wor* RNA expression is defective in embryos mutant for different pro-neural genes.

RESULTS

Expression and regulation of Snail and Worniu in early CNS

Both *snail* and *worniu* have extensive expression in neuroblasts, while that of *escargot* is transient and sparse. Furthermore, based on genetic analysis, *snail* and *worniu* have more important role than *escargot* in the regulation of CNS development (Ashraf et al., 1999). As loss of *ftz* expression in GMCs was the earliest CNS defect observed, we carefully examined the expression of *snail* and *worniu* in GMCs. A better understanding of the patterns should help us to predict their possible functions in either neuroblasts or GMCs. In situ hybridization revealed that *worniu* RNA, in contrast to the extensive expression in neuroblasts, is present in only a small number of GMCs (Fig. 1A, compare with 1B). Even in later staged embryos, when there should be multiple GMCs surrounding each neuroblast, we could detect the staining in no more than one small cell next to each neuroblast (data not shown). The limited staining in the GMCs is probably due to the segregation of some RNA from the parental neuroblast. Once the GMC is formed, the active transcription of *worniu* probably ceases. We also examined the protein and RNA expression of *snail*. The results showed that there is also very limited expression of *snail* in GMCs. We rarely detected *snail* RNA-containing GMCs next to neuroblasts (data not shown). Consistent with RNA expression, antibody staining revealed that the protein is predominantly in the neuroblasts (Fig. 1C,D).

We then examined whether the neuroblast expression of *snail* and *worniu* is regulated by proneural genes. Such a result would place the *snail* family in the well established genetic hierarchy that controls early neuroblast differentiation. The *scute*^{B57} deletion mutant uncovers the three pro-neural genes: *achaete*, *scute* and *lethal of scute*. In this mutant, the expression of *worniu* in neuroblasts was significantly reduced (Fig. 1F). Only a few neuroblasts within each segment exhibited staining, and the expression level was substantially lower than in the wild type. The expression of *worniu* is also regulated by *vnd* and *ind*, such that in these mutant embryos the whole ventral and intermediate columns of staining were missing (Fig. 1G,H). In the *msh*$^{\Delta 68}$ mutant, no abnormal expression of *worniu* was detected (data not shown). Previous results have shown that the neuroblast expression of *snail* is slightly affected in *achaete-scute* and *vnd* mutants but is not affected in a *daughterless* mutant (Ip et al., 1994; Skeath et al., 1994). In *ind* and *msh* mutants, we observed Snail protein expression in many neuroblasts but the spatial pattern was rather disorganized (data not shown). In summary, most of the proneural genes tested have profound effects on the expression of *worniu*, and have detectable but lesser effects on that of *snail*. The predominant expression of *snail* and *worniu* in neuroblasts and their regulation by proneural genes suggest that the *snail* family genes may have important functions within neuroblasts.

inscuteable expression is regulated by Snail family of proteins

In mutants containing deletions that uncover *escargot*, *worniu* and *snail*, many early neuroblast markers are normal, but *ftz*

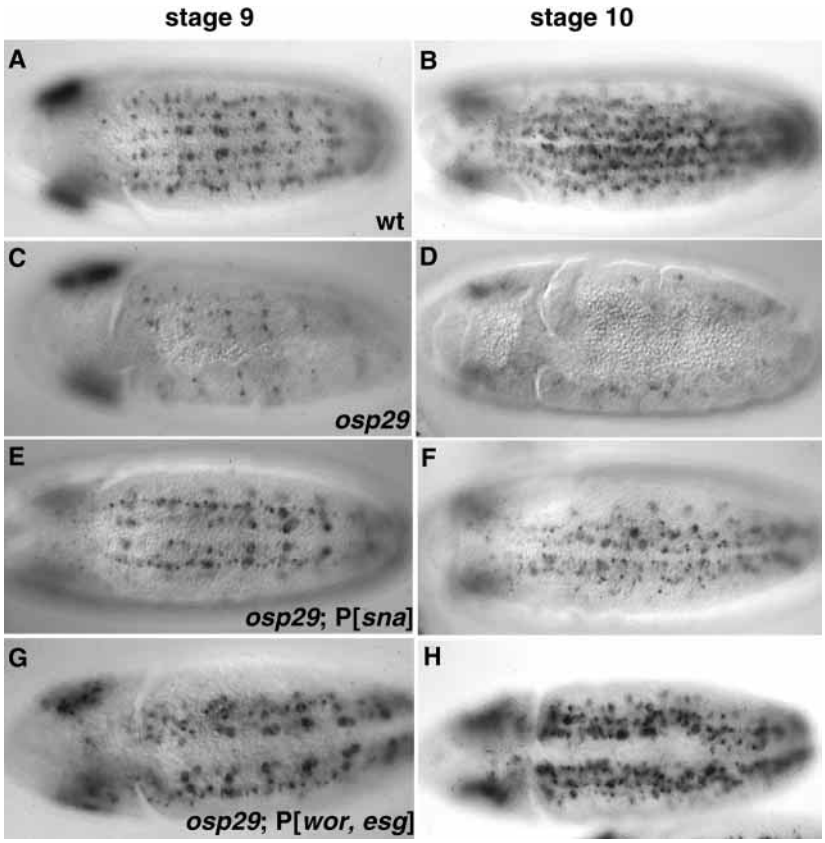


Fig. 2. *inscuteable* expression is dependent on the Snail family of proteins. (A,C,E,G) Stage 9 embryos. (B,D,F,H) Stage 10 embryos. All embryos are ventral views. RNA in situ hybridization reveals the mRNA expression of *inscuteable* is significantly lower in *osp29* mutant (C,D) than in wild-type embryos (A,B). Note that some localized mRNA is still present in *osp29* embryos. (E-H) Loss of *inscuteable* expression can be efficiently rescued in embryos expressing transgenic Snail family of proteins. (E,F) Embryos with *P*[*snail*] transgene; (G,H) embryos with *P*[*wor, esg*] transgenes. The *P*[*wor, esg*] was generated by recombination of the two individual transgenes (Ashraf et al., 1999), with each under the control of 2.8 kb *snail* promoter, including the neuroblast expression element (Ip et al., 1992; Ip et al., 1994). For this and following figures, some of the mutant embryos shown have morphological defects that are due to the requirement of Snail in gastrulation. The morphological phenotype is used whenever possible to identify embryos that harbor the mutation. The gastrulation phenotype has no direct consequence on the expression of CNS markers. This is based on the observations that rescue of morphological defect by *snail* driven by mesoderm promoter alone cannot rescue the CNS defect, and *worniu* and *escargot* transgenes can rescue CNS defect but not gastrulation defect (e.g. see G,H).

expression in GMCs is abnormal (Ashraf et al., 1999). The regulation of *ftz* depends on Prospero, a homeodomain protein that controls GMC fate (Doe et al., 1991; Vaessin et al., 1991; Li and Vaessin, 2000). Prospero protein and mRNA are preferentially segregated to GMCs from the neuroblast through the process of asymmetric division (Lu et al., 2000). Genes that are involved in asymmetric segregation of Prospero include *inscuteable*, *miranda* and *staufen*. Therefore, we examined the expression of these possible Snail family target genes in neuroblasts.

We used mutant embryos collected from deficiency strains that uncovers the 35D1 chromosomal region including the *snail* family genes [for detail descriptions of the *Df(2L)osp29* and other deletions, see Ashraf et al. (Ashraf et al., 1999)]. In wild-type embryos, the expression of *inscuteable* can be detected in delaminating neuroblasts. After delamination, many neuroblasts show localization of the *inscuteable* RNA (Fig. 2A,B) (Li et al., 1997). Embryos homozygous for the *osp29* deletion, however, had significantly lower level of the RNA and the staining was detected in a much smaller number of neuroblasts (Fig. 2C,D). Transgenic copies of *snail*, *worniu* or *escargot* efficiently rescued the expression of *inscuteable* RNA (Fig. 2E-H), demonstrating that it is the uncovering of the *snail* family of genes in the deletion that causes the phenotype. The rescue transgenes were under the control of the 2.8 kb *snail* promoter, which contains the neuroblast expression element (Ip et al., 1992; Ip et al., 1994; Ashraf et al., 1999). A 1.6 kb *snail* promoter construct (Ip et al., 1992) that contains the mesoderm element but lacks the CNS element could not rescue the defect (data not shown), demonstrating that expression of the transgenes within neuroblasts is essential for the function.

In contrast to that of *inscuteable*, the *miranda* RNA pattern and level were very similar in wild-type and *osp29* embryos (Fig. 3A,B), suggesting that the RNA expression of *miranda* is independent of Snail. The Miranda protein was also present in the mutants, and some localization was detectable but less prominent when compared with that of wild type (Fig. 3C,D). The *staufen* RNA is expressed ubiquitously, with enhanced expression in neuroblasts of wild-type embryos. The overall RNA level of *staufen* also appeared normal in the mutant (data not shown). The *prospero* RNA was similarly detectable in the mutant although expression was delayed; in stage 9 mutant embryos, the level was slightly lower, but in older mutant embryos the staining was stronger and similar to that of wild type (Fig. 3F,H). However, one defect we observed was the loss of localization of the *prospero* RNA (Fig. 3H). Such localization occurs in wild-type neuroblasts just before mitosis (Fig. 3G), and is dependent on Inscuteable and Staufen (Li et al., 1997). Therefore, the localization defect is consistent with the reduction in *inscuteable* expression. The subcellular localization defect of *prospero* was rescued in the presence of transgenic *snail* family (Fig. 3I), demonstrating that the localization phenotype in the *osp29* mutant was caused by loss of *snail* family genes.

Transition from neuroblast to GMC is defective in the absence of Snail family

The segregation of Prospero protein into GMCs from neuroblasts is a critical event during asymmetric cell division. As *inscuteable* plays a role in the segregation of *prospero* gene products into GMCs, we examined whether there is Prospero protein in GMCs of mutant embryos. Prospero protein staining

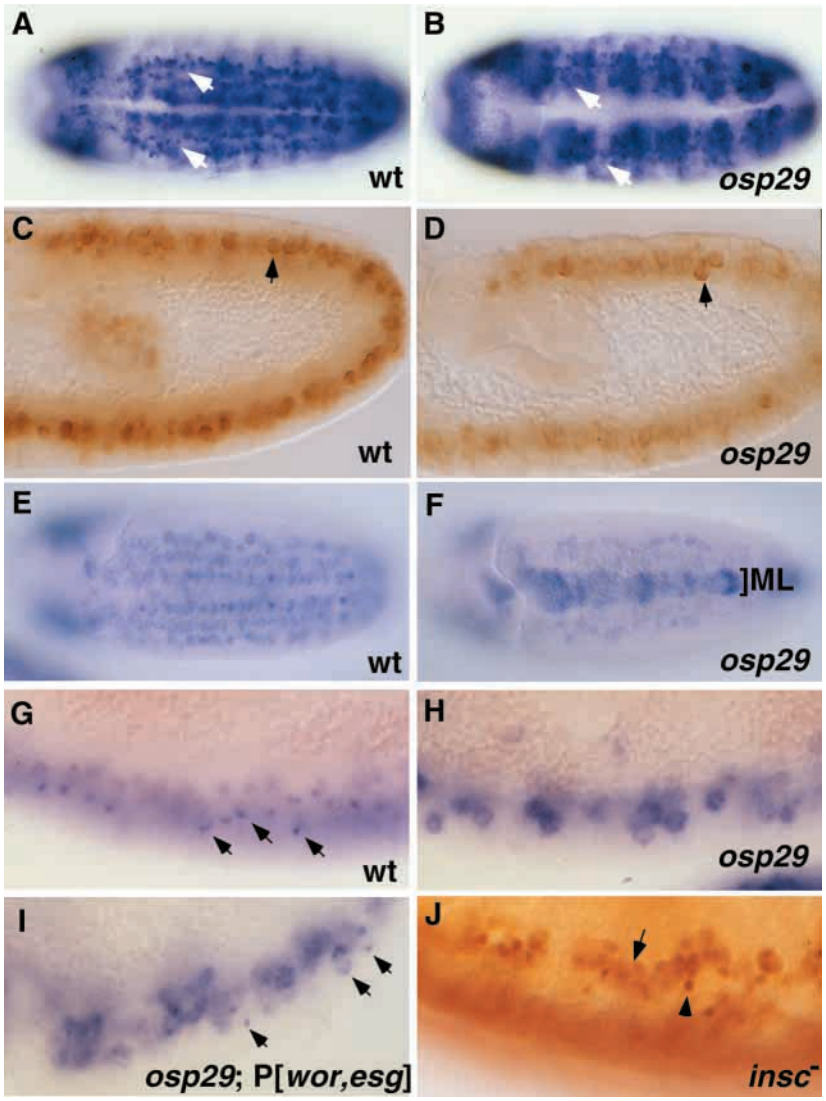


Fig. 3. Defective *prospero* mRNA localization in the deletion mutant. (A,B,E,F) Ventral views of embryos at low magnification. (C,D) Sagittal views of embryos at medium magnification. (G-J) Sagittal views of embryos at high magnification. (A,B) RNA in situ hybridization for *miranda* mRNA expression shows normal *miranda* mRNA expression and subcellular localization in wild-type and *osp29* mutant embryos (arrows indicate cells with RNA localization). (C,D) The Miranda protein is detectable, although less abundant and in fewer cells, in the deletion mutant (arrows indicate cells with subcellular localization). (E,F) The expression of *prospero* mRNA is detectable in the neuroblasts of mutant embryos albeit slightly lower in early stage; the intense midline (ML) staining in the mutant is probably due to expansion of midline cell fate in the absence of Snail in the blastoderm. Older mutant embryos (H) accumulate higher levels of *prospero* RNA, similar to that of wild type (G). The apparently lower level in the mutant in earlier stages may also be due to the loss of subcellular localization (compare G with H, arrows indicate localization). This localization defect can be rescued (arrows) in the presence of *P*[*wor*, *esg*] transgenes (I). In *inscuteable* mutant embryos (J), the Prospero protein expression is clearly seen in some GMCs (arrowhead) and neuroblast (arrow) nuclei. This phenotype is different from Prospero protein pattern in *osp29* embryos (compare with Fig. 4B).

can be easily detected in many wild type GMC nuclei (Fig. 4A). The staining was largely absent in the deletion that uncovers the *snail* family locus (Fig. 4B); only a few cells with the size of normal GMCs had clear nuclear staining. A band of cells along the midline also had Prospero staining (Fig. 4B, bracket), but these cells probably represent an expansion of the midline (see also Fig. 3F for clear staining of RNA). It

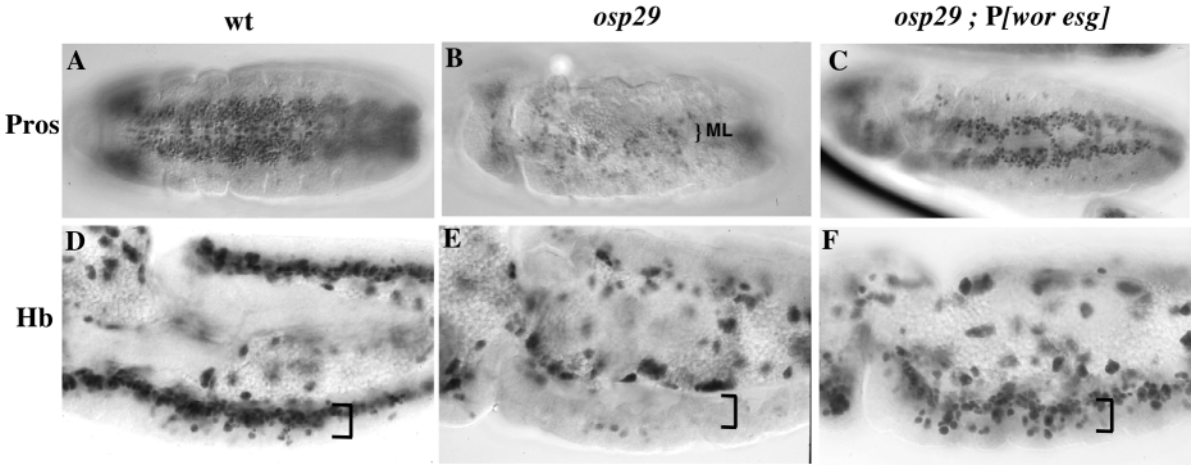


Fig. 4. GMC formation is defective in the absence of Snail family of proteins. All embryos are approximately at stage 11. (A-C) Ventral view. (D-F) Sagittal views of embryos. (A,D) Wild type; (B,E) *Df(2L)osp29* mutant; (C,F) *osp29* with *P*[*wor, esg*] transgenes. The Prospero protein staining is largely absent in the deletion mutant (B). More cells show staining in the midline (ML), probably owing to the derepression of midline determinants in the absence of Snail (see also Fig. 3F). Transgenes of *worniu* and *escargot* rescued Prospero expression efficiently (C). Similar results are also observed in the presence of *snail* transgene (data not shown). Hunchback (Hb) at this stage is present in many GMCs (D). The brackets in D-F indicate where Hb-positive GMCs are seen in wild-type and rescued embryos but absent in *osp29* mutant embryos.

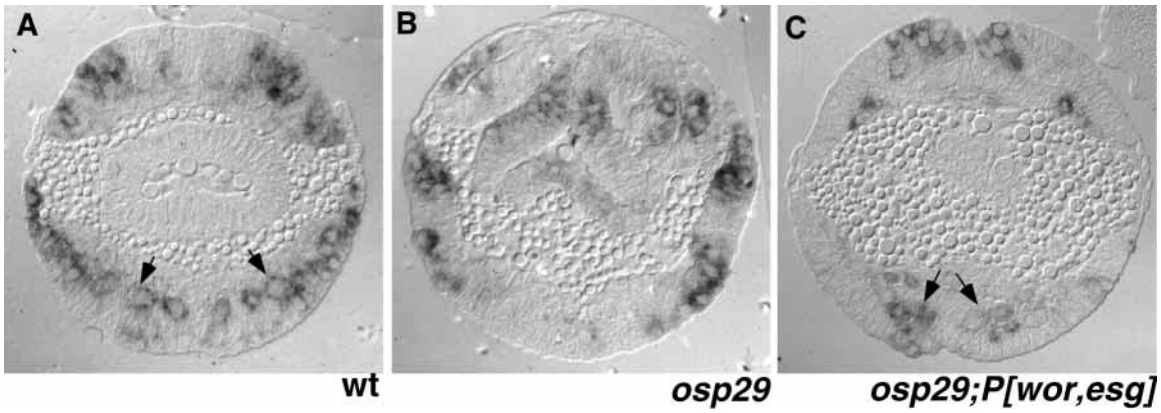
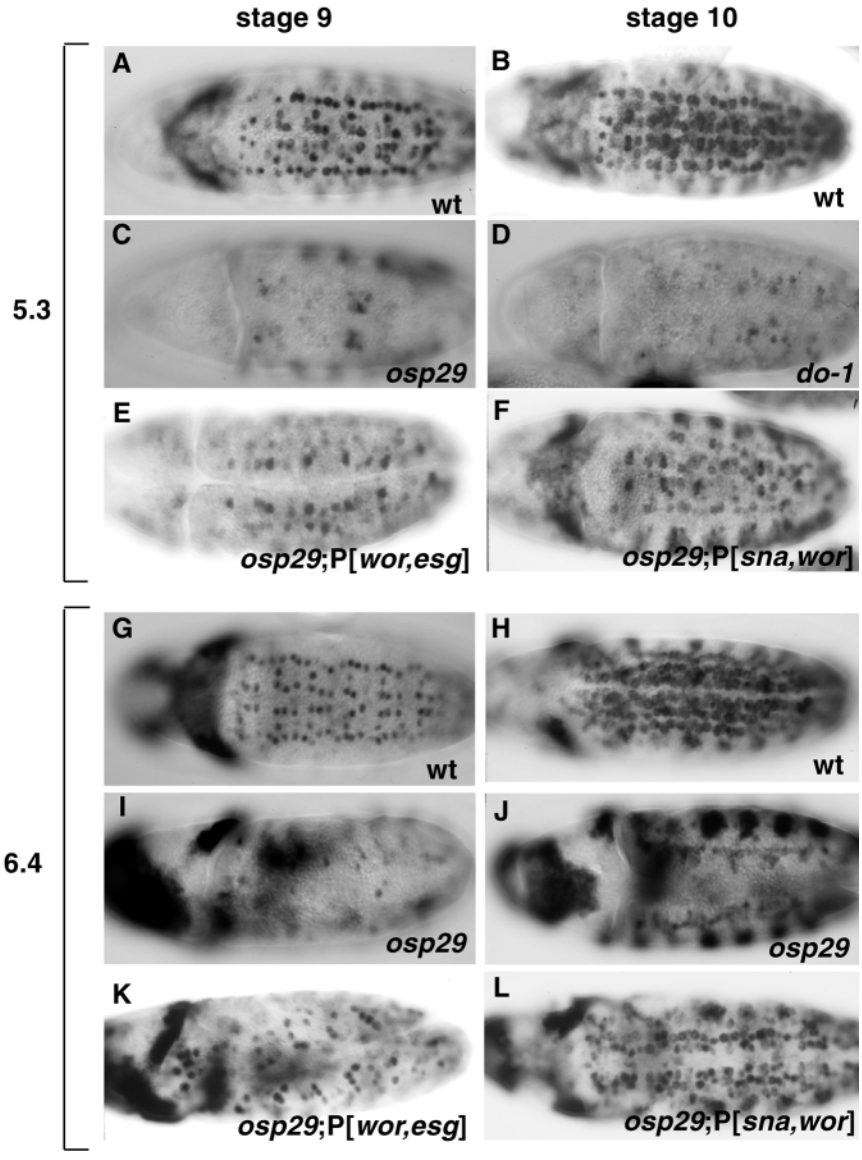


Fig. 5. Snail family of proteins regulate *string* expression in neuroblasts. RNA in situ hybridization was carried out using antisense *string* probe on wild-type (A), *osp29* mutant (B) and *P[wor, esg]*-carrying mutant (C) embryos. After in situ hybridization, the embryos were embedded in Epon plastic and 3 μm sections were cut and representative sections are shown here. The arrows in panels A and C indicate RNA expression of *string* in neuroblasts. Staining is also seen in ectodermal cells. The neuroblast layer is located between the ectoderm and the mesoderm (A). The *osp29* mutant embryos also have more folding, indicating gastrulation defects. Nonetheless, the ectodermal staining is clear but the neuroblast staining is largely absent (B). The transgenes can partially rescue expression of *string* in the neuroblasts (C).



has been well documented that in all *snail* mutants there is derepression of the mid-line determinant *single-minded* in the blastoderm stage embryo (Nambu et al., 1990; Kosman et al., 1991; Kasai et al., 1992).

To determine whether there are defects within GMCs in addition to loss of Prospero, we examined the expression of Hunchback (Kosman et al., 1998), which is present transiently in early neuroblasts and later in many GMCs (Fig. 4D). In the deletion mutant, the Hunchback protein in GMCs was also absent (Fig. 4E, bracket), while staining in cells surrounding the amnioserosa appeared normal. Transgenes of *snail*, *worniu* and *escargot* rescued the staining of Prospero and Hunchback (Fig. 4C,F; data not shown), indicating that these GMC determinants are downstream of the Snail family. The results also suggest that the regulation of *ftz* by the Snail family is indirect, probably through an earlier event such as segregation of Prospero from neuroblast to GMC.

If the misregulation of *inscuteable* in the

Fig. 6. *string-lacZ* reporter expression is regulated by the Snail protein family. RNA in situ hybridization using an antisense *lacZ* probe reveals *string* promoter-*lacZ* reporter expression. Two different *string-lacZ* reporters were used. (A-F) Embryos expressing *string 5.3-lacZ*. (G-L) embryos expressing *string 6.4-lacZ*. (A,B,G,H) Wild-type embryos. (C,I,J) *osp29* embryos. (D) Another deletion *do-1* embryo (Ashraf et al., 1999). (E,F,K,L) *osp29* embryos carrying the indicated rescue transgenes. In the deletion mutants, the *lacZ* expression is almost abolished. A single copy of each of the indicated transgenes was sufficient to confer a clear rescue of the reporter expression.

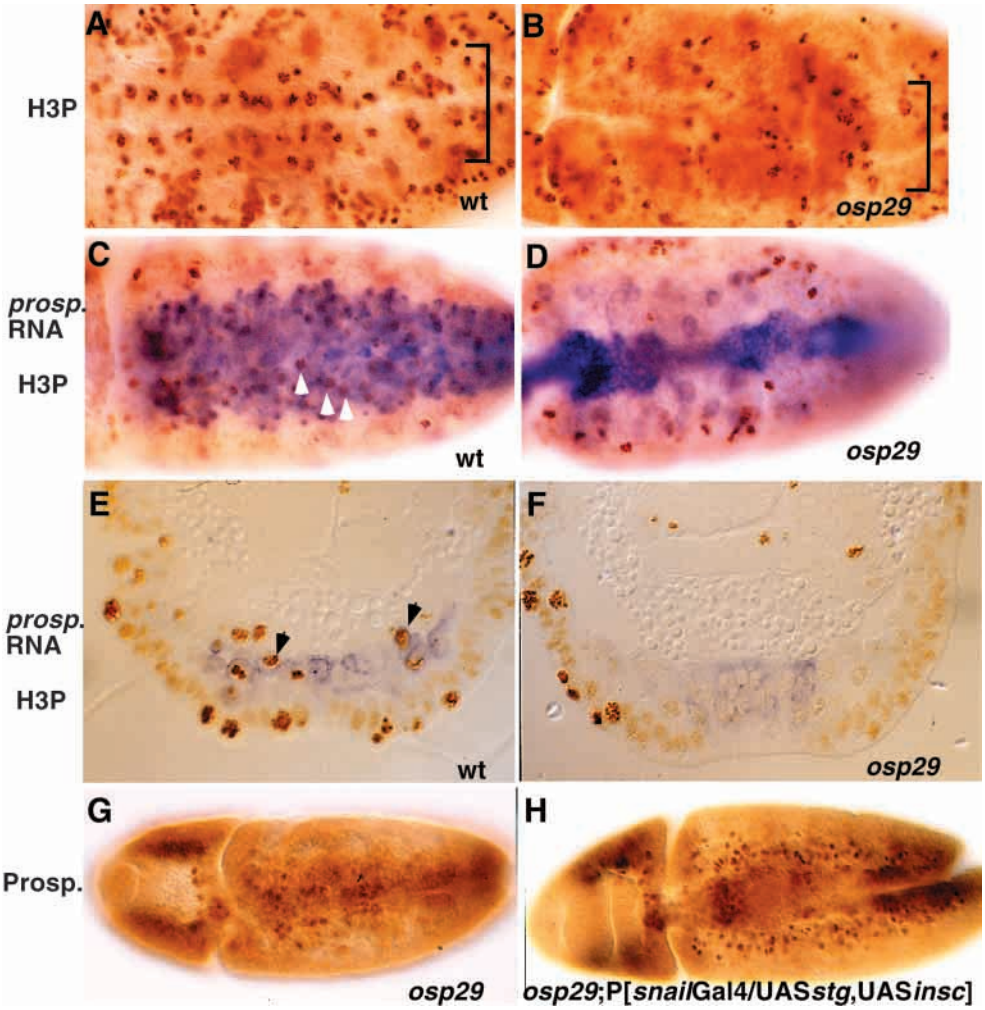


Fig. 7. Cell division defects and rescue of Prospero GMC expression. (A-F) Embryos stained for phosphorylated-histone H3 (brown). (C-F) Embryos also stained for *prospero* RNA (blue). (A-D) Ventral views of embryos shown at medium magnification; (E-F) sections of similar embryos shown at high magnification. The brackets in A,B indicate the ventral neurogenic region where most neuroblasts are located. There is clear staining of phosphorylated H3 in several cells in wild-type embryo (A), indicating mitosis. Much less staining is seen in the *osp29* mutant embryo (B). Similar embryos double stained for *prospero* RNA showing the neuroblast layer (C,D). The arrowheads indicate some of the phosphorylated-H3 staining. Sections of similar embryos shown in E,F indicate that the neuroblast cell layer with *prospero* RNA staining also show mitosis occurring in wild-type embryos, but very rarely in *osp29* embryos. The results suggest that the deletion mutants undergo less mitosis. (G,H) Staining of Prospero protein in GMCs. The expression of Prospero protein can be detected in many GMC nuclei along the two sides of the expanded midline in an embryo containing the *string* and *inscuteable* transgenes (H). The embryo containing the transgenes still has expanded midline, indicating the lack of Snail activity in blastoderm.

deletion mutant is the cause of the loss of Prospero and *ftz* expression in GMCs, the expression of *inscuteable* should correct the defects even in the absence of Snail family of proteins. We crossed a line carrying an *inscuteable* transgenic construct driven by the 2.8 kb *snail* promoter into the *osp29* deletion genetic background. However, the rescue of Prospero expression in GMCs was variable and not nearly as strong as those embryos expressing the *snail* family transgenes (data not shown, but see Fig. 7). This suggests that *inscuteable* may not be the only important target gene of Snail. Another line of evidence supporting the idea of an additional target gene comes from the comparison of the phenotypes in *osp29* and *inscuteable* mutant embryos. In *inscuteable* mutants, the Prospero crescent is formed but the mitotic spindle rotation is randomized (Kraut et al., 1996; Li et al., 1997). As a result, the Prospero protein frequently is present both in neuroblasts and GMCs (Fig. 3J). This phenotype is less severe than the almost total loss of Prospero GMC staining in *osp29* deletion mutant (Fig. 4B). Therefore, we surmised that in addition to the misregulation of *inscuteable*, there may be other defects that lead to the more severe phenotype in the deletion mutants.

Control of the cell cycle regulator *string*

One possibility that may explain the more severe phenotype in *snail* family deletion mutants is additional defects in cell division. Neuroblasts are arrested at the G2/M transition at the embryonic cell cycle 14 (Edgar and O'Farrell, 1990; Edgar, 1995). After delamination, a pulse of *string* (which encodes a Cdc25 phosphatase homolog) expression in neuroblasts drives the cells to enter mitosis. We examined the expression of *string* RNA in whole-mount mutant embryos, but the result was ambiguous, owing to the dynamic, high level expression in ectoderm and other tissues, which obscures the signal in the neuroblast cell layer (data not shown) (Edgar et al., 1994). We therefore used tissue sectioning in order to better view the expression of *string* in neuroblasts. The sections clearly showed expression of *string* RNA in wild-type neuroblasts at stage 9 embryos (Fig. 5A). There are consistently three to four neuroblasts on each side of the midline that exhibit staining. This neuroblast expression appeared very faint in the *osp29* mutant embryos (Fig. 5B), and most sections did not show staining in neuroblasts while expression in ectoderm appeared normal. The presence of *wor* and *esg* transgenes in the deletion mutant background led to accumulation of *string* RNA in some neuroblasts (Fig. 5C), suggesting a positive role for Snail family in regulating *string* expression.

We also used *string* promoter-*lacZ* reporters that have more specific expression in neuroblasts to confirm the above observation. Promoter analysis demonstrated that many modular regulatory elements are present in a 50 kb region of

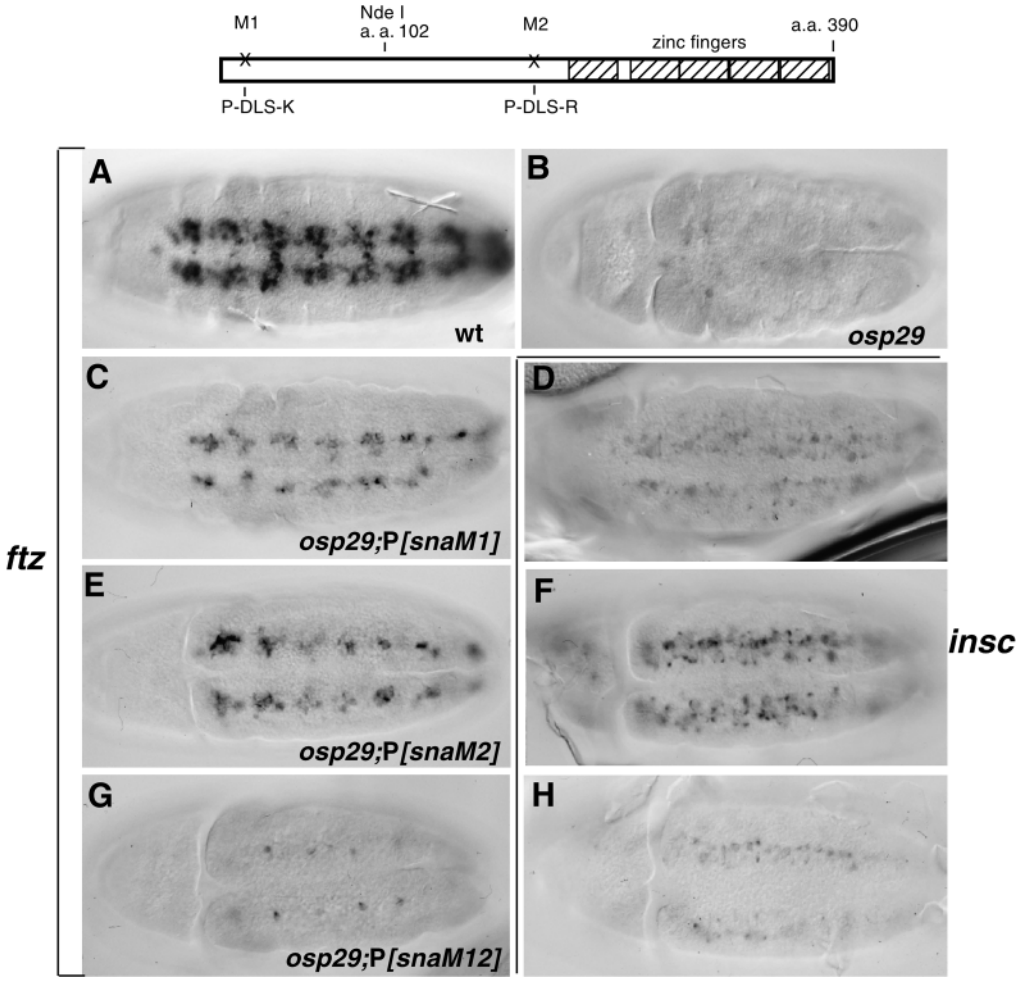


Fig. 8. Snail function in neuroblasts requires the dCtBP co-repressor interaction motif. The schematic shows the protein structure of Snail. The embryos show RNA in situ hybridization for *ftz* (A-C,E,G) and *inscuteable* (D,F,H). All the embryos are *osp29* mutants, except in A (wild type). (C,D) Embryos expressing a *P*[*snail*] transgene in which the N-terminal dCtBP interaction motif is mutated (M1). (E,F) Embryos containing the M2 mutant; (G,H) Embryos containing the M12 double mutant. There is much lower rescue of *ftz* and *inscuteable* in the absence of both dCtBP interaction motifs.

the *string* locus (Lehman et al., 1999). The *string* 6.4 line contains a 6.4 kb genomic fragment which is originally located approximately 10 kb from the transcription start site of *string*. The *string* 5.3 line contains 5.3 kb of DNA, which is originally located approximately 20 kb from the transcription start site. Both lines have strong expression in early and late neuroblasts (Fig. 6A,B,G,H) (Lehman et al., 1999). In *osp29* deletion mutant, the *lacZ* expression of *string* 5.3 was almost undetectable (Fig. 6C,D). The *string* 6.4 *lacZ* staining was also largely abolished (Fig. 6I,J). The expression of *string* 5.3 was partially rescued and the *string* 6.4 was efficiently rescued by *snail* family transgenes, even when the transgenes were heterozygous (Fig. 6E,F,K,L). The results obtained together demonstrate that transcription activation of *string* is at least partly under the control of Snail family of proteins.

If regulation of *string* is an important downstream event of Snail family of proteins, then cell division of neuroblasts should be affected in the absence of these proteins. We therefore examined the mitotic process by staining for phosphorylated histone H3, which reveals condensed chromosomes. In wild-type embryos, although the neuroblasts do not exhibit highly synchronized mitosis, anti-phosphoH3 staining can be detected in multiple cells (Fig. 7A). In the *osp29* mutant embryos, such staining was consistently reduced (Fig. 7B). The use of *prospero* RNA to mark the neuroblast layer and the use of tissue sectioning (Fig. 7C-F) provided further support for the idea that the mutant embryos had reduced mitosis in neuroblasts.

We further demonstrate by genetic rescue experiments that the severe CNS defects are likely due to a combination of loss of *inscuteable* and *string* expression. Similar to the results obtained for *inscuteable*, transgenic expression of *string* alone had weak and variable effect in the rescue of Prospero expression in GMCs (data not shown). When both *inscuteable* and *string* were simultaneously expressed in neuroblasts of *osp29* mutants using the UAS-Gal4 system, clear staining of Prospero in many cells resembling GMCs was observed (Fig. 7H). The staining was particularly apparent alongside the expanded midline, characteristic of mutant embryos with no Snail function in early mesoderm. The results support the idea that both *inscuteable* and *string* are relevant targets of the Snail family.

Snail function in neuroblasts requires the dCtBP co-repressor interaction motifs

A clearly demonstrated in vivo function of Snail is transcriptional repression. The repression function is mediated through the recruitment of dCtBP (*Drosophila* C-terminal binding protein), which acts as a co-repressor for Snail to regulate target genes such as *rhomboid*, *lethal of scute* and *single-minded* (Nibu et al., 1998a; Nibu et al., 1998b). There are two conserved P-DLS-R/K motifs in Snail (Fig. 8), as well as in Worniu and Escargot, and they have been shown to be critical for recruiting dCtBP (Nibu et al., 1998a; Nibu et al., 1998b; Ashraf et al., 1999; Hemavathy et al., 2000). Mutations of these motifs abolish the repressor function of Snail in the blastoderm. To gain insight into the molecular mechanism of how Snail regulates CNS development, we introduced into the *osp29* deletion background transgenic copies of *snail* which had the dCtBP interaction motifs mutated. M1 contains the N-

terminal motif mutation and M2 contains the C-terminal motif mutation (Fig. 8). The expression of *inscuteable* and *ftz* was examined. The assay showed that the double mutant (M12) lost most of the ability to rescue (Fig. 8G,H), and M1 had lost some ability to rescue (Fig. 8C,D). However, M2 functioned quite efficiently, closer to that of the wild-type protein, to rescue *inscuteable* and *ftz* expression (Fig. 8E,F, compare with Fig. 2F for *inscuteable* and with Ashraf et al. (Ashraf et al., 1999) for *ftz*). These results demonstrate that the dCtBP interaction motifs are essential for the Snail function in the CNS, consistent with the idea that Snail acts as a repressor in neuroblasts to regulate gene expression. Thus, the activation of *inscuteable* and *string* by the Snail family may be indirect.

DISCUSSION

We have demonstrated that the Snail family of proteins function within neuroblasts to control CNS development through regulation of two determinants in the asymmetry and cell division pathways (Fig. 9). One of the downstream events is the regulation of *inscuteable* expression. In the absence of Snail family of proteins, *inscuteable* RNA level is substantially reduced. The mutant embryos also lose the asymmetric localization of *prospero* RNA within the neuroblasts. Inscuteable is required to anchor Staufen, which in turn binds and localizes *prospero* RNA (Li et al., 1997). Thus, it is conceivable that Snail family regulates the expression of *inscuteable*, which is essential for the asymmetric localization and segregation of *prospero* gene products. The loss of *prospero* gene products in GMCs is probably responsible for the loss of *ftz* expression we observed previously (Ashraf et al., 1999). A recent publication also reported that in deletions that uncover the *snail* family genes, the apical crescent of Prospero protein is formed but the basal localization is absent (Cai et al., 2001). This loss of basal accumulation of Prospero, as well as the randomization of spindle rotation, has been shown to be caused by the misregulation of *inscuteable*, owing to the loss of the Snail family (Cai et al., 2001). Our observations are consistent with and complementary to those conclusions.

While the loss of *inscuteable* may help to explain the localization defects of *prospero* gene products, the regulation of *inscuteable* is not the only function of Snail family of proteins. Together with Miranda and Staufen, Inscuteable helps to localize *prospero* RNA and Prospero protein (Li et al., 1997; Shen et al., 1997; Lu et al., 2000). Inscuteable is also required for spindle orientation (Kraut et al., 1996; Kaltschmidt et al., 2000). Previous reports have shown that in *inscuteable* mutants, the apical crescent of Prospero is still formed, but the transport to basal side is much delayed. In the meantime, the spindle rotation during metaphase is randomized. As a result, Prospero protein sometimes segregated correctly into GMCs. In the *osp29* deletion mutant embryos, however, Prospero protein staining in GMCs is largely absent. Thus, the phenotype of deleting *snail* family is much more severe than that of *inscuteable*. As the expression of *miranda* appears normal, additional target genes are probably regulated by the Snail family.

Transcriptional regulation of *string* is a key event in controlling embryonic cell cycle 14-16 (Edgar, 1995). At this stage, the maternal and zygotic supplies of other cell cycle regulators drive all postmitotic cells through S phase. Maternal String, however, has been depleted or degraded during early divisions. Thus, the embryonic cells including neuroblasts are arrested at G2/M transition. Once the neuroblasts have delaminated, a pulse of zygotic *string* transcription leads to the activation of M phase and the cells go through mitosis. We have shown that the expression of *string* is defective specifically in the neuroblasts, and such defect can be partially rescued by the Snail family of proteins. We also find that two neuroblast-specific regulatory elements of *string* are at least partially dependent on Snail family for activity. The results strongly indicate a positive involvement of Snail family of proteins. The regulation of *string* predicts a neuroblast cell division defect, and staining for phosphorylated H3 in the *osp29* deletion supports such an interpretation. Most importantly, while transgenic expression of either *inscuteable* or *string* did not rescue Prospero expression in GMCs substantially, the combination of both transgenes consistently rescued Prospero staining in GMCs in the *snail* family deletion background. Our results support the idea that both *inscuteable* and *string* are important downstream targets, and that Snail family has an important role in modulating the asymmetry and cell division of neuroblasts.

Although *inscuteable* and *string* are at present the two most

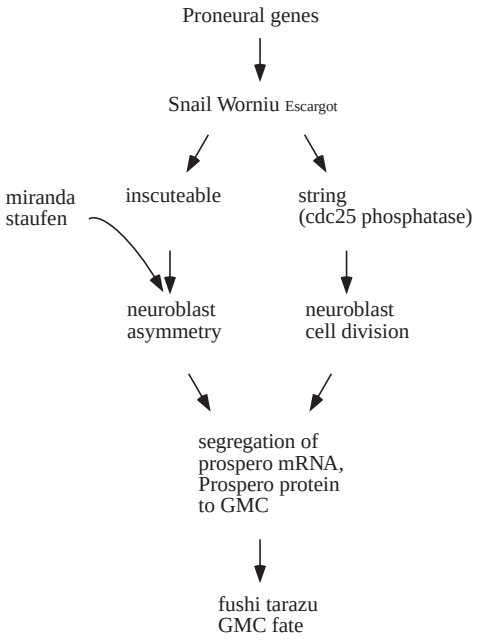


Fig. 9. Snail family of proteins play an essential role in neuroblast development. Many pro-neural genes control the expression of *snail* and *worniu* in neuroblasts. Snail and Worniu, and to a lesser extent Escargot, normally function to regulate the asymmetry and cell division of neuroblasts by controlling the expression of *inscuteable* and *string*, respectively. The correct expression of these two genes is required for proper segregation of Prospero into GMC, where Prospero functions as a crucial factor for cell fate determination. The arrows indicate genetic hierarchy, not necessarily direct regulation. The Snail family of proteins likely function as repressors. Thus, the regulation of *inscuteable* and *string* may be through the repression of another repressor, leading to gene activation. It is also possible that the Snail family of proteins can directly activate the expression of these two genes.

proximal downstream targets, there is no evidence that they are direct targets of the Snail family transcription factors in neuroblasts. Published results support the theory that Snail is a transcriptional repressor. Snail, Worniu and Escargot all contain two dCtBP-binding motifs (Ashraf et al., 1999), and mutations of the dCtBP interaction motifs in Snail abolish the repressor activity at blastoderm stage (Nibu et al., 1998a). Thus, our results of dCtBP binding motif mutants of Snail suggest that Snail family of proteins function as repressors in neuroblasts. We attempted to examine the expression of some neural markers in *dCtBP* mutant embryos, but the severe morphological defects in post-gastrulation stages precluded a conclusive interpretation [dCtBP also functions as co-repressor for segmentation determinants such as Krüppel and Knirp (Nibu et al., 1998b; Keller et al., 2000)]. Although we favor the role of Snail family of proteins as repressors, it is formally possible that they can activate target gene expression. First, repression of known target genes by Snail in the early embryos is not sufficient to explain the gastrulation phenotype associated with the *snail* mutants (Hemavathy et al., 1997). Second, dCtBP can act as an anti-repressor by antagonizing the Groucho co-repressor function when binding the Hairy repressor (Phippen et al., 2000). Third, *snail* family genes are expressed at approximately the same time as that of *inscuteable* and *string*, leaving very little time for the transcription and translation of an intermediate regulator. Therefore, it is also possible that the Snail-dCtBP interaction can lead to direct activation of *inscuteable* and *string*. An analysis of the promoters of *inscuteable* and *string* and the associated proteins will shed some light on the regulatory mechanism.

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