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A family of Snail-related zinc finger proteins regulates two distinct and parallel mechanisms that mediate *Drosophila* neuroblast asymmetric divisions

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Three *snail* family genes *snail*, *escargot* and *worniu*, encode related zinc finger transcription factors that mediate *Drosophila* central nervous system (CNS) development. We show that simultaneous removal of all three genes causes defective neuroblast asymmetric divisions; *inscuteable* transcription/translation is delayed/suppressed in the segmented CNS. Furthermore, defects in localization of cell fate determinants and orientation of the mitotic spindle in dividing neuroblasts are much stronger than those associated with *inscuteable* loss of function. In *inscuteable* neuroblasts, cell fate determinants are mislocalized during prophase and metaphase, yet during anaphase and telophase the great majority of mutant neuroblasts localize these determinants as cortical crescents overlying one of the spindle poles. This phenomenon, known as 'telophase rescue', does not occur in the absence of the *snail* family genes; moreover, in contrast to *inscuteable* mutants, mitotic spindle orientation is completely randomized. Our data provide further evidence for the existence of two distinct asymmetry-controlling mechanisms in neuroblasts both of which require *snail* family gene function: an *inscuteable*-dependent mechanism that functions throughout mitosis and an *inscuteable*-independent mechanism that acts during anaphase/telophase.

Keywords: *insc* expression/neuroblast asymmetry/*snail* family genes/telophase rescue

Introduction

The segmented *Drosophila* embryonic central nervous system (CNS) is derived from a specialized epithelial layer, the neuroectoderm (Campos-Ortega and Hartenstein, 1985; Doe, 1992; Goodman and Doe, 1993). Neural stem cells, neuroblasts (NBs), delaminate from the epithelial layer and divide asymmetrically to produce two daughter cells with different sizes. The large apical cell retains NB identity and continues to undergo successive asymmetric divisions. The small basal/lateral cell is the ganglion mother cell (GMC) and divides terminally to produce two neuron/glial cells (for a review see Lu *et al*., 2000). Both epithelial cells and NBs are polarized. In wild-type embryos, NB polarity required for asymmetric division is inherited from epithelial cells. Previous studies have suggested that the inheritance of this apical–basal polarity from epithelial cells is mediated through Bazooka (Baz) (Kuchinke *et al*., 1998; Schober *et al*., 1999; Wodarz *et al*., 1999). Baz is apically localized in the epithelial cells. When NBs delaminate from the epithelial layer, Baz and Inscuteable (Insc) (Kraut and Campos-Ortega, 1996; Kraut *et al*., 1996) are first concentrated in the apical stalk, maintaining the apical–basal polarity cue for NBs. Partner-of-Inscuteable (Pins) joins Baz and Insc, forming an apically localized functional complex before the NB enters mitosis (Schaefer *et al*., 2000; Yu *et al*., 2000). It has been reported that *Drosophila* homologue of atypical protein kinase C (DaPKC) binds to Baz, and *Drosophila* G-protein α-subunit binds to Pins, and these molecules might also be involved in asymmetric NB divisions (Schaefer *et al*., 2000; Wodarz *et al*., 2000).

The apical complex controls the basal localization of cell fate determinants such as Prospero (Pros) (Doe *et al*., 1991; Vaessin *et al*., 1991; Matsuzaki *et al*., 1992) and Numb (Uemura *et al*., 1989), and orients the mitotic spindle along the apical–basal axis for NB divisions (Kraut *et al*., 1996; Schober *et al*., 1999; Wodarz *et al*., 1999; Schaefer *et al*., 2000; Yu *et al*., 2000). Both Pros and Numb are segregated preferentially into the GMC daughter cell (Hirata *et al*., 1995; Knoblich *et al*., 1995; Spana and Doe, 1995; Spana *et al*., 1995). Miranda (Mir) and Partner-of-Numb (Pon), the two adaptor proteins that always co-localize with Pros (Mir) and Numb (Pon), respectively, are also segregated into the GMCs in mitosis (Ikeshima-Kataoka *et al*., 1997; Shen *et al*., 1997, 1998; Lu *et al*., 1998, 1999; Schuldt *et al*., 1998).

In mutants that disrupt apical complex formation/maintenance, cell fate determinants Pros and Numb are mislocalized in NBs and spindle orientation is affected (Kraut *et al*., 1996; Schaefer *et al*., 2000; Yu *et al*., 2000). It has been suggested that, in addition to the Insc/apical complex-dependent mechanism, there exists another Insc-independent asymmetry-controlling mechanism (Schober *et al*., 1999; Wodarz *et al*., 1999; Peng *et al*., 2000) mainly based on two observations. First, in *insc* null embryos, the basal cell fate determinants such as Pros and Numb are localized randomly only in the early phases of mitosis, at and prior to metaphase. Mutant NBs will redistribute these basal proteins to the basal/lateral cortex from where the future GMCs are formed in late mitotic phases (anaphase onwards); this phenomenon has been referred to as 'telophase rescue' (Peng *et al*., 2000). Consequently, most mutant GMCs inherit, at least in part, the basal cell fate determinants and adopt correct GMC identity. Secondly, spindle misorientation occurs at relatively low frequency in *insc* NBs. It has been reported that NBs, similarly to cells in the procephalic neurogenic region (mitotic domain 9) (Foe, 1989), rotate their mitotic spindles 90° in metaphase (Kaltschmidt *et al*., 2000). The major difference between the asymmetric divisions of these two types of neural stem cells is that *insc* is

absolutely required for the spindle reorientation in mitotic domain 9 cells (Kraut *et al*., 1996), but appears partially dispensable in the segmented CNS. These findings suggest that when *insc* function is removed, a second Insc-independent asymmetry-controlling mechanism can compensate effectively for the functions of Insc in the NBs of the segmented CNS. To our knowledge, no mutations affecting the postulated Insc-independent asymmetry-controlling mechanism have been reported.

The *snail* (*sna*) mutant was identified in a genetic screen for genes involved in larval pattern formation (Nusslein-Volhard *et al*., 1984). The *sna* gene encodes a zinc finger DNA-binding transcription factor (Boulay *et al*., 1987; Ip *et al*., 1992; Kasai *et al*., 1992; Mauhin *et al*., 1993) and plays a critical role in mesoderm formation (Leptin, 1991). Before gastrulation, Sna defines the presumptive mesoderm and establishes the boundary between mesoderm and neuroectoderm by directly repressing the expression of neuroectodermal genes *rhomboid* and *single-minded* in the mesoderm (Kosman *et al*., 1991; Ip *et al*., 1992). In embryos homozygous for *sna* loss-of-function mutations, gastrulation does not occur and mesoderm formation is abolished (Grau *et al*., 1984; Nusslein-Volhard *et al*., 1984). Cells that normally form mesoderm will adopt a new cell fate and become part of the neuroectoderm (Rao *et al*., 1991).

It has been shown that *sna* and *escargot* (*esg*) (Whiteley *et al*., 1992) together with a third member of the *sna* gene family, *worniu* (*wor*), which also encodes a related transcription factor with Zn finger sequences, are involved in CNS development (Ashraf *et al*., 1999). Sna, Esg and Wor show highly homologous protein sequences and are all expressed in NBs during neurogenesis. Similarly to the Sna and Esg regulation in wing disc development (Fuse *et al*., 1996), Sna, Esg and Wor show functional redundancy during CNS development. It has been observed that in deficiencies that simultaneously remove *sna*, *esg* and *wor*, a set of GMC markers was not detected in the embryonic CNS although NB formation appeared to be normal. Ectopic expression of any one of the *sna* family genes alone in these deficiencies effectively rescued early embryonic CNS defects. It was suggested that Sna family proteins had essential functions during CNS development around the time of GMC formation (Ashraf *et al*., 1999).

We conducted a screen of the Bloomington deficiency kit looking for defects in Insc localization/expression in deficiency homozygous embryos. Our results indicate that deficiencies of the 35B–D region, uncovering all three *sna* family genes, showed severe NB asymmetry defects. We report here that the specific removal of the three Sna family proteins results in the down-regulation of *insc* transcription and translation in the NBs of the segmented embryonic CNS. Moreover, our analyses reveal the existence of two distinct asymmetry-controlling mechanisms, an Insc-dependent and an Insc-independent mechanism, both of which require the function of the *sna* family genes.

Results

Df(2L)TE35BC-3 embryos show defective NB asymmetry

We have screened a collection of ~170 deficiencies representing ~70% of the fly genome from the Bloomington Stock Center, to identify possible asymmetry defects in NB divisions during early neurogenesis with anti-Pros and anti-Insc. Initially, one deficiency, Df(2L)TE35D-24 (34F5–35E1), was identified, which showed mislocalized Pros crescents and the absence of Insc expression in dividing NBs. Subsequent analyses of available deficiencies from this region (Ashburner *et al*., 1999) identified three additional deficiencies, Df(2L)osp29 (35B3–E6), Df(2L)A48 (35B2–35D5) and Df(2L)TE35BC-3 (35B10–35D2), all of which exhibited an identical phenotype (Figure 1). We illustrate these defects using Df(2L)osp29 and Df(2L)TE35BC-3 homo-

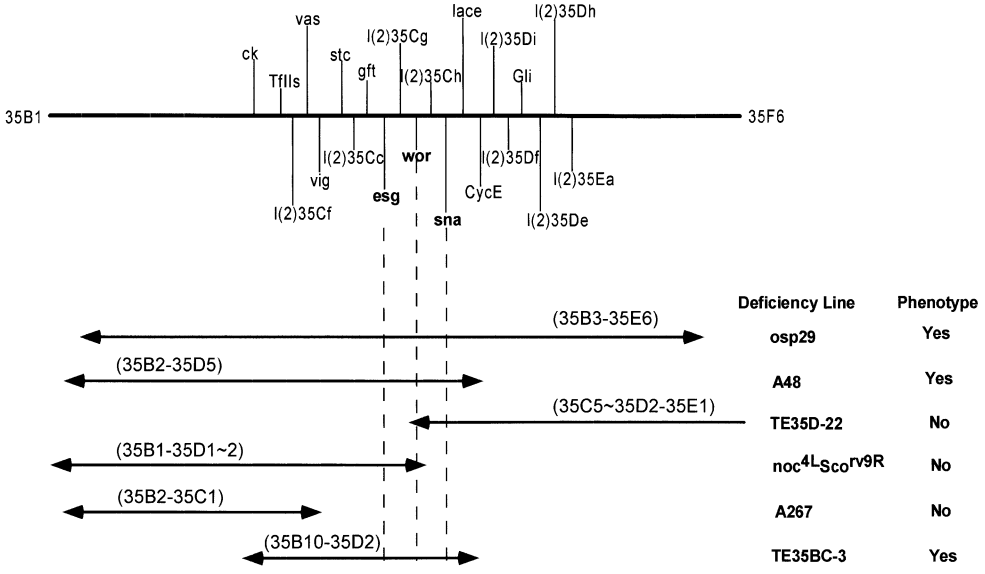

Fig. 1. Schematic representation of the 35B–D genomic region. Some of the deficiencies used to map the asymmetry phenotypes along with their cytological breakpoints are given. The positions of *sna*, *wor* and *esg* and other lethal complementation groups are shown in the diagram. Double-ended arrows represent the extent of the deficiencies.

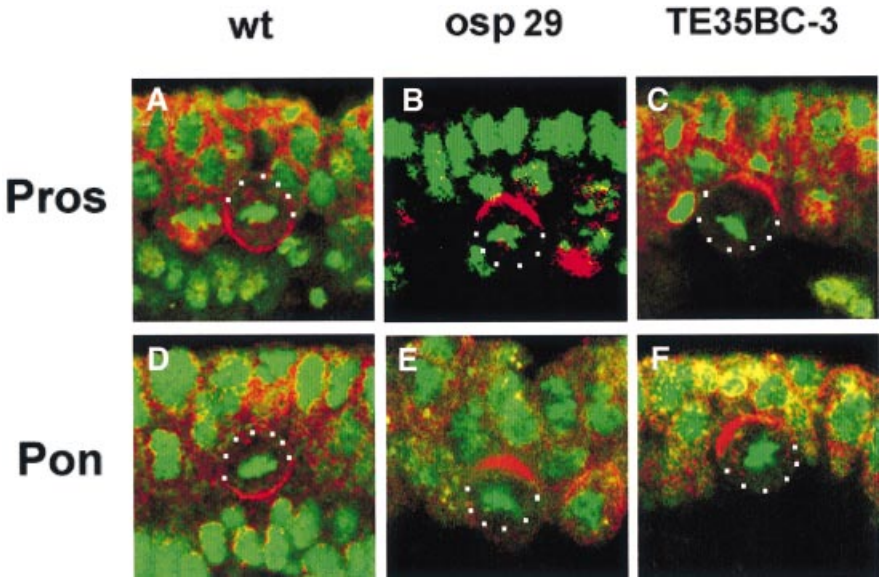

Fig. 2. Localization of cell fate determinants is defective in deficiency lines Df(2L)osp29 and Df(2L)TE35BC-3. Confocal images of dividing NBs double-labelled with anti-Pros (red, A–C) or anti-Pon (red, D–F) and DNA staining to indicate the condensed chromosomes (green). Note that in wild-type embryos (**A** and **D**), Pros and Pon form basal crescents in dividing neuroblasts, while in the deficiency lines, although the crescents are formed, they fail to move to the basal cortex (**B**, **C**, **E** and **F**). Apical is up. NBs are outlined with white dots.

zygotes. In Df(2L)osp29 embryos, Pros (Figure 2B) is no longer basally localized during NB division as seen in the wild-type embryos (Figure 2A). Since Mir is an adaptor protein for Pros localization, it is not surprising to find that Mir is also misplaced (data not shown). Basal localization of Numb and its adaptor protein Pon are also affected in Df(2L)osp29 embryos; anti-Pon antibody staining, as well as the staining with anti-Numb (data not shown), shows that the localization of the Pon/Numb crescent is no longer basal but is misplaced in mutant embryos (Figure 2E).

Df(2L)TE35BC-3 was the smallest deficiency available that exhibited these defects (Figure 2C and F). Df(2L)TE35BC-3 uncovers ~30 known and predicted genes according to the database from the *Drosophila* Genome Project, including the *sna* family genes *sna*, *esg* and *wor*. Two observations suggest that the observed defects in protein localization cannot be caused by mutation of a single gene. First, Df(2L)TE35D-22 (35C5–35D2–35E1) and Df(2L)noc^{4L}Sco^{rv9R} (35B1–35D1–2) are deficiencies with a small overlap (Ashraf *et al*., 1999), which together uncover 35B1–35E1, so one would expect homozygotes of one or both deficiencies to exhibit protein localization defects; yet neither of these two deficiencies when homozygous showed any protein localization defects (data not shown). Secondly, we have analysed the available lethal complementation groups in the region uncovered by Df(2L)TE35BC-3 and failed to identify the mislocalization of the Mir/Pros and Pon/Numb phenotype in any of the available mutants. These results support the notion that the observed Mir/Pros and Pon/Numb localization defects are probably multigenic in nature.

Removal of snail family genes affects Mir/Pros and Pon/Numb basal localization in mitotic NBs

It has been reported that CNS development is abnormal in Df(2L)osp29 embryos due to deletion of Sna family proteins (Ashraf *et al*., 1999). Both Sna and Wor are expressed strongly in all NBs (Figure 3A and B), including those in the procephalic region, during early neurogenesis (Alberga *et al*., 1991; Ashraf *et al*., 1999). The expression of Esg is also seen in NBs (Yagi *et al*., 1997; Ashraf *et al*., 1999) and other tissues, as visualized with anti-Esg immunostaining (Figure 3C). Expression of Esg can be detected in the midline cells as well as GMCs during embryonic development. The functions of these three genes are overlapping; the early CNS defects are detected only when all three genes are removed simultaneously (Ashraf *et al*., 1999). In order to test whether the defects of localization of Mir/Pros and Pon/Numb seen in Df(2L)TE35BC-3 embryos are due to the absence of the three *sna* family genes, we examined the localization of Mir/Pros and Pon/Numb in embryos single mutant for *sna*, *esg* or *wor*, a double mutant for *sna/esg* (made by recombination; Fuse *et al*., 1996) and deletions that removed *sna/wor* or *esg/wor*, as well as embryos double mutant for *sna/esg* and further subjected to *wor* double-stranded RNA (RNAi; Fire *et al*., 1998; Kennerdell and Carthew, 1998) treatment. In single and double mutant embryos, both Mir/Pros and Pon/Numb form normal basal crescents in mitotic NBs (data not shown). Only the *sna/esg* double mutant embryos that have been injected with *wor* RNAi reproduce the phenotype found in Df(2L)TE35BC-3 embryos (Figure 3D and E).

In wild-type embryos, NBs are located between the ectoderm and mesoderm. The Df(2L)TE35BC-3 embryos lack mesoderm. Therefore, it is possible that correct NB asymmetry requires signal(s) from the mesoderm, and the asymmetry defects seen in Df(2L)TE35BC-3 could be due simply to the absence of mesoderm in these embryos. We believe this is unlikely since NB asymmetry is intact in *sna* embryos, which lack mesoderm and share the abnormal morphology of Df(2L)TE35BC-3 embryos. Furthermore, the partial rescue of mesoderm in Df(2L)TE35BC-3

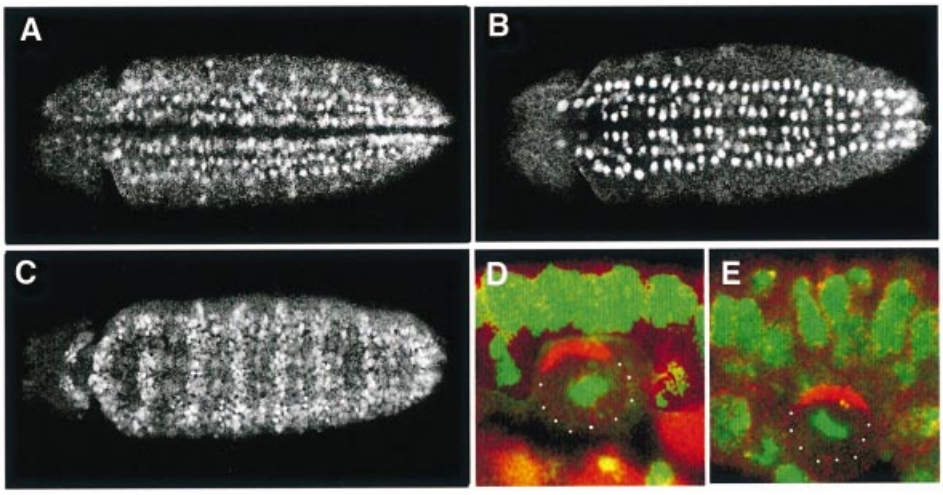

Fig. 3. Sna family proteins are expressed in NBs and required for basal localization of Pros and Pon in dividing NBs. Confocal images of ventral views of stage 10 embryos stained with anti-Sna (**A**), anti-Wor (**B**) and anti-Esg (**C**). Anterior is left. (**D** and **E**) Double-labelled images of *sna/esg* double mutant embryos treated with *wor* RNAi. The NBs are stained with anti-Pros (red, D) and anti-Pon (red, E); DNA staining is green. Note that both Pros and Pon are misplaced in the *sna/esg* double mutant embryos with *wor* RNAi treatment. Apical is up.

embryos by ectopic expression (Brand *et al*., 1993) of the Sna protein driven by *twist-gal4* does not reverse the asymmetry defects (data not shown). Thus, we conclude that mislocalization of Mir/Pros and Pon/Numb in Df(2L)TE35BC-3 embryos is due to the absence of all three *sna* family genes. Based on this conclusion, Df(2L)TE35BC-3 is referred to as *sna/esg/wor* deficient and was used in subsequent studies.

The apical complex is disrupted in sna/esg/wor-deficient embryos

In wild-type embryos, Baz, Insc and Pins form a complex that is localized to the apical cortex of the dividing NBs (Schober *et al*., 1999; Wodarz *et al*., 1999; Schaefer *et al*., 2000; Yu *et al*., 2000). The apical complex is required for the asymmetric distribution of cell fate determinants such as Pros and Numb to the basal cortex of NBs and coordinates the orientation of the mitotic spindle along the apical–basal axis of the NB. In embryos deficient for the *sna* family genes, Mir/Pros and Pon/Numb are no longer concentrated to the basal cortex of mitotic NBs, indicating defects in NB asymmetry. It is possible that the asymmetry defects seen in *sna/esg/wor*-deficient NBs are due to the alteration of Insc expression. Anti-Insc staining indicates that Insc protein is indeed undetectable in the segmented CNS of *sna/esg/wor*-deficient embryos (Figure 4B and D). Although the signal intensity in the procephalic region is comparable to that in the wild-type controls, the number of cells with anti-Insc staining appears to be decreased (Figure 4B). This altered expression of Insc in the mutant embryos suggests that the mislocalization of Mir/Pros and Pon/Numb in *sna/esg/wor*-deficient embryos is, at least in part, due to a lack of Insc protein expression in dividing NBs (see below). As expected, Baz protein levels are low and undetectable in the great majority of mutant NBs (data not shown). The lack of easily detectable Baz in NBs is probably due to the instability of the protein when Insc is absent (Schober *et al*., 1999; Wodarz *et al*., 1999; Yu *et al*., 2000) since the *baz* mRNA levels remain unchanged in *sna/esg/wor* NBs (data not shown). Pins protein localization is also affected in *sna/esg/wor*-deficient embryos (data not shown).

The down-regulation of Insc protein in NBs is also dependent on the simultaneous loss of *sna*, *esg* and *wor* functions. Insc expression in double mutant embryos of *sna/esg* was similar to that of wild-type embryos (Figure 4E). In *sna/esg* double mutant embryos, further removal of the third member of *sna* gene family, *wor*, with RNAi leads to the total loss of Insc protein expression (Figure 4F). Moreover, ectopic expression of any one of the *sna* family genes under the control of an early neural driver *sca-gal4* in *sna* family gene mutant embryos largely restores the Insc expression in NBs (*sna* 79%, $n = 43$; *esg* 64%, $n = 64$ and *wor* 44%, $n = 50$), further indicating that Insc expression is indeed regulated by the Sna family proteins.

Dual regulation of insc expression in the segmented CNS by Sna family proteins

We further examined *insc* transcript levels in the *sna/esg/wor*-deficient embryos. In wild-type stage 9–10 embryos, *insc* RNA is expressed prominently in NBs of the segmented CNS and in the procephalic region (Figure 4G and I) (Kraut and Campos-Ortega, 1996). The transcript level is maintained in the segmented CNS and procephalic NBs throughout embryogenesis. In *sna/esg/wor*-deficient embryos, RNA *in situ* hybridization data indicate that the *insc* RNA is absent in the segmented CNS at stages 9–10 but is detectable in the procephalic NBs (Figure 4H and J). This suppression of *insc* RNA transcription in the segmented CNS of *sna/esg/wor*-deficient embryos provides evidence that the Sna family proteins are essential for *insc* mRNA transcription during early neurogenesis (stage 9–10). The suppression of *insc* transcription in the segmented CNS is transient and *insc* RNA can be detected, at a lower level, in late stage 11 embryos (Figure 4L). However, Insc protein in the segmented CNS of *sna/esg/wor*-deficient embryos remains undetectable at late

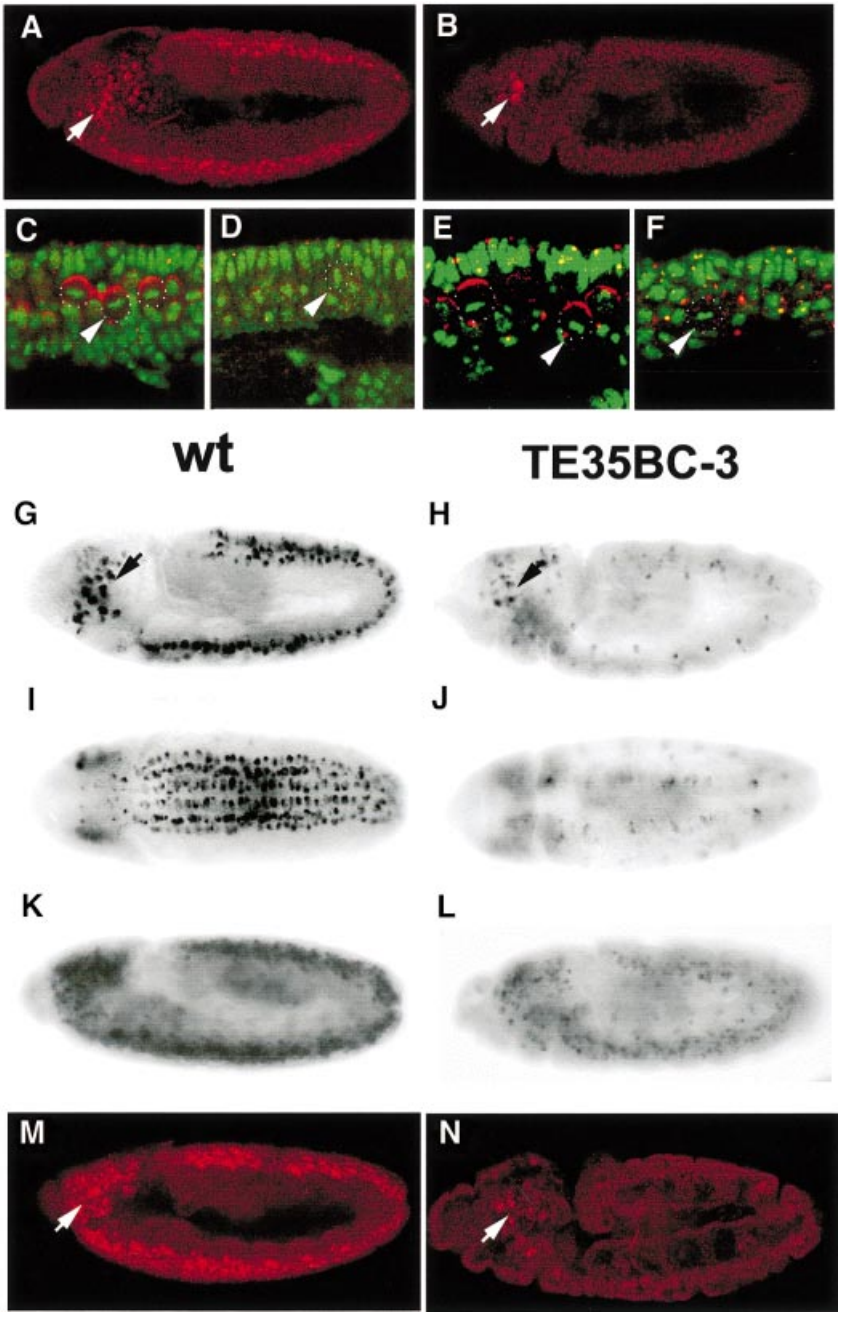

Fig. 4. Dual regulation of *insc* expression by Sna family proteins. Lateral views of stage 10 wild-type (**A**) and Df(2L)TE35BC-3 (*sna/esg/wor*-deficient) (**B**) embryos stained with anti-Insc (red). Insc protein level is undetectable in *sna/esg/wor*-deficient NBs (**D**) and NBs in *sna/esg* double mutant embryos treated with *wor* RNAi (**F**), compared with Insc expression in NBs of wild-type (**C**) and *sna/esg* double mutant embryos (**E**). DNA is green. (G–L) RNA *in situ* hybridization images showing *insc* transcripts in wild-type embryos (stage 10, lateral and ventral views, **G** and **I**; and stage 11, **K**) and in *sna/esg/wor*-deficient embryos at the same developmental stages (stage 10, **H** and **J**; and stage 11, **L**). Insc protein expression is maintained in wild-type stage 11 embryos (**M**) but is undetectable in stage 11 *sna/esg/wor*-deficient embryos (**N**) even though *insc* transcript levels partially recover (L). Anterior is left, apical is up. Arrows indicate NBs in the procephalic region where Insc is normally expressed; NBs in the segmented CNS are indicated by arrowheads and outlined with white dots.

stage 11 when the *insc* RNA levels partially recover by an unknown mechanism (Figure 4N). It is obvious that translation of *insc* RNA in late stage 11 embryos is inhibited in the segmented CNS of embryos deficient for *sna/esg/wor*. Although the inhibition mechanism is unknown, we believe that the *insc* 5′- and/or 3′-untranslated regions (UTRs) are involved since Insc protein (Kraut *et al*., 1996) can be ectopically expressed in *sna/esg/wor*-deficient embryos from a uas-*insc* transgene in which the 5′- and 3′-UTRs have been partially removed (see below). Considering that the Sna family proteins are localized to nuclei, it is unlikely that they interact directly with 5′- and/or 3′-UTRs of *insc* RNA. Presumably other genes regulated by the Sna family proteins mediate the observed translational effect.

The observation of delayed and decreased *insc* mRNA transcription and the inhibition of Insc protein synthesis in the segmented CNS of *sna/esg/wor*-deficient embryos

suggests the dual regulation of *insc* expression by the Sna family proteins at both transcriptional (stage 9–10) and translational (stage 11 onwards) levels. This dual regulation mechanism is prominent in the segmented CNS but *insc* RNA and protein expression in the procephalic region is only partially affected in *sna/esg/wor*-deficient embryos. The mechanism that enables the partial restoration of *insc* transcription in NBs of the segmented CNS at late stage 11 in the absence of *sna* family gene function remains to be identified.

Insc-dependent and -independent asymmetry-controlling mechanisms are abolished in sna/esg/wor-deficient embryos

In $insc^{22}$ (Burchard *et al*., 1995) mutant NBs, in which the apical complex required for correct asymmetric division is abolished, basal components such as Mir/Pros and Pon/Numb often form random crescents, sometimes broad and loose, from prophase to metaphase; however, Pros/Mir and Pon/Numb can eventually be redistributed to the 'budding site' of the future GMCs, although sometimes not as exclusively as seen in wild-type embryos, at anaphase and telophase (Figure 5B and E) even when the spindle is misorientated (Schober *et al*., 1999; Wodarz *et al*., 1999). Consequently, the great majority of all GMCs inherit, at least in part, cell fate determinants such as Pros (100%; 50/50) and adopt correct GMC fate. This phenomenon, referred to as 'telophase rescue' (Peng *et al*., 2000), does not occur in NBs lacking the three *sna* family genes. For example, in *sna/esg/wor*-deficient NBs, basal proteins Mir/Pros and Pon/Numb form a randomly localized crescent in dividing NBs but, unlike in *insc* embryos, these proteins are not redistributed at anaphase/telophase to the region of the cortex that gives rise to the GMC. Consequently, the great majority of the GMCs do not inherit the basal proteins Mir/Pros (Pros 90%; 45/50) and Pon/Numb (94%; 60/64) (Figure 5C and F) and thus lose their GMC identities (Broadus *et al*., 1998). This finding explains why GMCs were not specified correctly in Df(2L)osp29 embryos as previously reported (Ashraf *et al*., 1999).

Furthermore, it is known that the mitotic spindle in NBs rotates 90° during metaphase so that it is realigned along the apical–basal (A/B) axis of the embryos (Kaltschmidt *et al*., 2000); in *insc* mutants, this spindle rotation during metaphase occurs only in a small proportion (~20%) of NBs; nevertheless, even some of these NBs are able to reorient spindles late in mitosis (Kaltschmidt *et al*., 2000). We measured the NB spindle orientation during anaphase or telophase in wild-type and mutant embryos and categorized them into four equal quadrants depending on the angle that the spindle forms with the A/B axis (Figure 5G–J). Based on the spindle orientation in wild-type embryos, we consider all spindles with an angle >45° relative to the A/B axis (groups III and IV) during late mitosis to be misorientated. The misoriented spindles in $insc^{22}$ mutant embryos are limited (Figure 5H, 10%); the great majority of NBs (90%) have their spindles oriented within 45° of the A/B axis (groups I and II), compared with 100% in wild-type NBs. In contrast to wild-type and *insc* NBs, in *sna/esg/wor*-deficient NBs spindle orientation is completely randomized with almost equal distribution for each of the four quadrants (Figure 5I) (compare with $insc^{22}$ in Figure 5H and wild-type in Figure 5G). Moreover, a small number of NBs (10%; 11/110) completely reverse their polarity, giving rise to a small apical GMC (Figure 5C; right NB), which has never been reported in any known asymmetry mutant.

These observations indicate that removal of Insc alone has only a limited effect on NB asymmetric divisions in terms of basal protein localization and spindle orientation late in mitosis, suggesting that the Insc-dependent mechanism is not the only apparatus that controls the asymmetric divisions in NBs. It appears that an Insc-independent mechanism exists that functions in parallel to coordinate the asymmetry events at later stages (anaphase onwards) of mitosis. This Insc-independent asymmetry-controlling mechanism, which is responsible for the 'telophase rescue' phenomenon and for prevention of random spindle orientation in $insc^{22}$ embryos, is destroyed upon removal of the three *sna* family genes. However, one might argue that the severe asymmetry defects seen in the absence of the *sna* family genes might be artefactual, caused by the combination of loss of *insc* expression and the absence of the mesoderm. We can eliminate this possibility because in *insc/sna* double mutant embryos, which lack both *insc* and the mesoderm, NBs exhibit phenotypes that are indistinguishable from those seen in the *insc* single mutant (data not shown). We therefore conclude that in the absence of the *sna* family genes, both the Insc-dependent and -independent asymmetry-controlling mechanisms are destroyed, leading to asymmetry defects that are more severe than those seen in *insc* single mutants.

Ectopic expression of Insc rescues asymmetry defects in sna/esg/wor-deficient embryos

The existence of two distinct asymmetry-controlling mechanisms in wild-type NBs raises an interesting issue: how do these two mechanisms work in concert to mediate asymmetric divisions? Since embryos deficient for the *sna* family genes lack both mechanisms, we reasoned that by restoring the Insc-dependent mechanism in these embryos we should be able to assess the consequences of missing just the *insc*-independent mechanism. Ectopic expression of full-length Insc protein with an early neural driver *sca-gal4* in NBs of *sna* family gene mutant embryos shows complete rescue of the protein localization defects described earlier. The apical complex forms normally, as indicated by the formation of apical Insc (Figure 6C) as well as Pins and Baz crescents (data not shown). The defects in basal protein localization are also completely rescued; Mir/Pros (100%, 54/54) and Pon/Numb (100%; 45/45) form tight basal crescents in mitotic NBs (Figure 6F and I). These results suggest that, with respect to protein localization, Insc protein is the only component missing in the Insc-dependent asymmetry machinery, and replacement of Insc through ectopic expression is sufficient to restore wild-type localization of the apical and basal components. Furthermore, it indicates that the Insc-independent mechanism is cryptic with respect to protein localization since it is dispensable when the Insc-dependent mechanism is in place. Either mechanism alone is able to distribute basal proteins to the cortex of the future GMC 'budding site' with clear temporal and efficiency differences: the Insc-dependent mechanism localizes basal proteins starting in late prophase in the form of tight

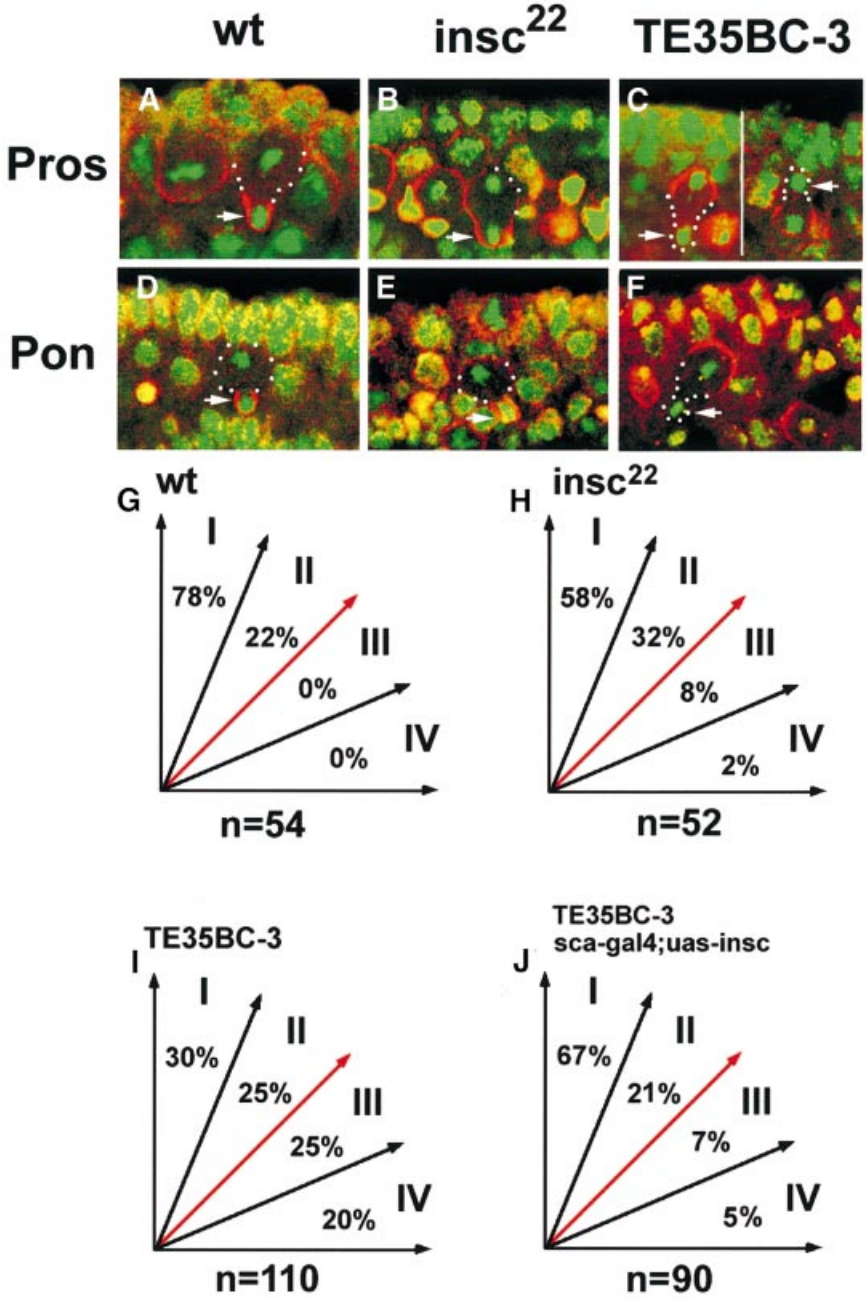

Fig. 5. Comparisons of asymmetry defects between *insc*^{22} and Df(2L)TE35BC-3 (*sna/esg/wor*-deficient) embryos. Confocal images of telophase NBs of wild-type (**A** and **D**), *insc*^{22} (**B** and **E**) and *sna/esg/wor*-deficient (**C** and **F**) embryos labelled with anti-Pros (red; A–C) or anti-Pon (red; D–F) and DNA (green). The future GMCs are indicated (arrows). The mitotic spindle orientation is sampled during late anaphase or telophase when the spindle positions are finalized in dividing NBs. Mitotic spindle orientations of NB populations are grouped arbitrarily into four sectors, depending on the angle the spindle forms with respect to the A/B axis; summary diagrams are given for wild-type (**G**), *insc*^{22} (**H**), *sna/esg/wor*-deficient (**I**) and *sna/esg/wor*-deficient embryos ectopically expressing Insc protein (**J**). The number of total NBs examined is given under each diagram. The apparent discrepancy of NB spindle orientation data in *insc* NBs between this study and an earlier report (Kraut *et al*., 1996) is most probably due to the time of sampling. We measured the NB spindle orientations in late anaphase and telophase.

crescents, while the Insc-independent mechanism is only able to redistribute, sometimes partially, mislocalized basal proteins late in mitosis (telophase rescue).

The spindle misorientation phenotype in *sna* family gene mutant embryos is also largely corrected by ectopic Insc expression. However, unlike protein localization, the rescue of mitotic spindle orientation is incomplete; the population of NBs with misoriented spindles (groups III and IV) drops from 45% to only 12% (compare Figure 5I and J). These data suggest that both the Insc-dependent and -independent mechanisms are required for correct spindle orientation in wild-type embryos since ~10% of the mitotic spindles are misoriented in anaphase/telophase NBs defective for either mechanism. However, a complete randomization of spindle orientation is seen when both mechanisms are absent.

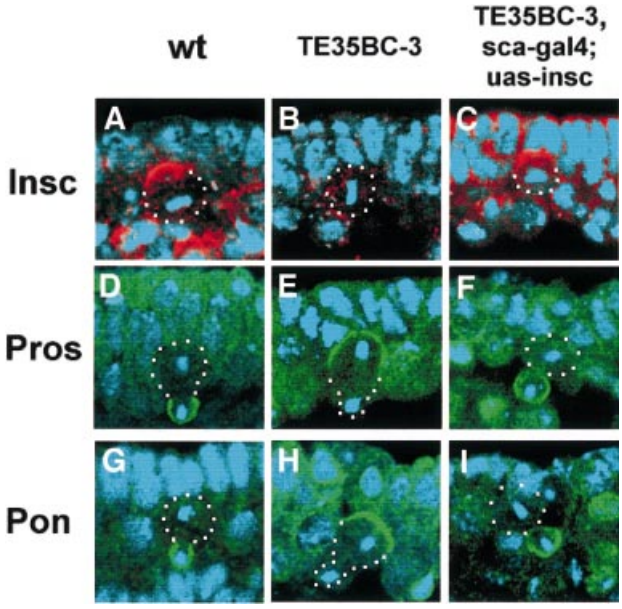

Fig. 6. Ectopic expression of Insc in Df(2L)TE35BC-3 (*sna/esg/wor*-deficient) embryos rescues asymmetry defects. Confocal images of metaphase NBs of wild-type (**A**), *sna/esg/wor*-deficient (**B**) and *sna/esg/wor*-deficient embryos with ectopically expressed Insc (**C**), double-labelled with anti-Insc (red) and DNA (blue). Images of telophase NBs of wild-type (**D** and **G**), *sna/esg/wor*-deficient (**E** and **H**) and *sna/esg/wor*-deficient embryos with ectopically expressed Insc (**F** and **I**) labelled with anti-Pros (green) or anti-Pon (green) and DNA (blue). Apical is up and telophase NBs are outlined with white dots. The future GMCs are indicated (arrows).

Discussion

Removal of the sna family genes causes defective NB asymmetric divisions

We have demonstrated that the underlying cause for the asymmetry defects associated with some deficiencies uncovering the 35B–D region of the genome, e.g. Df(2L)TE35BC-3, is the simultaneous loss of three members of the *sna* gene family, *sna*, *esg* and *wor*. All available lethal complementation groups uncovered by Df(2L)TE35BC-3, all deficiencies that remove only two out of the three *sna* family members and a *sna/esg* double mutant (Fuse *et al*., 1996) generated from recombination did not show any defects in any aspect of NB asymmetric division; only embryos double mutant for *sna/esg*, and further subjected to *wor* RNAi, reproduced the asymmetry defects seen in the deficiencies. These data indicate that the defects in *sna/esg/wor*-deficient embryos are caused by the simultaneous functional loss of all three *sna* family genes. The observation that the ectopic expression of *sna*, *esg* or *wor* in the segmented CNS of *sna/esg/wor*-deficient embryos reverses the asymmetry phenotypes further supports this conclusion. These conclusions are in agreement with an earlier study reporting that the *sna* family genes are required for CNS development (Ashraf *et al*., 1999).

Two parallel asymmetry mechanisms

It has been observed that in *insc* embryos, cell fate determinants such as Pros and Numb are mislocalized early during mitosis; however, in anaphase and telophase, the effect termed 'telophase rescue' causes the misplaced crescents to redistribute and overlie one spindle pole, enabling the basal cell fate determinants to segregate, exclusively or partially, to the GMCs. The *insc* loss-of-function alleles $insc^{22}$, $insc^{P49}$ and $insc^{P72}$ (Burchard *et al*., 1995; Kraut and Campos-Ortega, 1996) all show telophase rescue. In this study, we find that essentially all NBs in *insc* embryos can redistribute Pros and Numb, at least partially, into GMCs. Our observations and previous studies (Schober *et al*., 1999; Wodarz *et al*., 1999; Peng *et al*., 2000) suggest the existence of a second asymmetry-controlling mechanism that does not require *insc* functions, which operates late in mitosis to coordinate protein localization with spindle orientation. These observations explain why *insc* mutants have minimal effect on GMC cell fate. The Insc-independent mechanism corrects the earlier errors caused by absence of Insc during anaphase/telophase, thereby enabling cell fate determinants to be inherited by the GMC. This mechanism is apparently less efficient, as shown by the fact that in some *insc* NBs, normally basal components form a broad and loose crescent and are only partially sequestered into GMCs. Furthermore, our observation that mitotic spindle orientation is only mildly affected in *insc* NBs is also consistent with an Insc-independent compensatory mechanism.

Our analysis of NB divisions in embryos deficient for the three *sna* family genes provides further support for the existence of an Insc-independent mechanism. In these embryos, the Insc-dependent mechanism is clearly abolished; both the transcription and the translation of *insc* are suppressed in the mutant NBs. In addition, telophase rescue no longer occurs; the normally basally localized components are misplaced in mitotic NBs and not redistributed to the future GMCs even at anaphase/telophase. Moreover, the spindle orientation in embryos deficient for the *sna* family genes becomes randomized; ~45% of NBs exhibit misoriented spindles with an angle >45° with respect to the A/B axis at anaphase/telophase, which is not seen in wild-type NBs and is at a much higher frequency than that seen in $insc^{22}$ NBs. Thus, NBs deficient for the *sna* family genes show two defects that are not seen in *insc* NB: (i) the absence of telophase rescue; and (ii) randomization of the spindle orientation late in mitosis. These observations indicate that both the Insc-dependent and -independent mechanisms require the *sna* family genes.

These two mechanisms can apparently function independently. In *insc* NBs, the Insc-independent mechanism functions in the absence of the Insc-dependent mechanism to correct the earlier (prophase to metaphase) asymmetry defects during anaphase/telophase. In *sna/esg/wor*-deficient NBs that have been forced to express Insc, the Insc-dependent mechanism can act in the absence of the Insc-independent mechanism to mediate the localization of the basal components from prophase to telophase, obviating the requirement for telophase rescue; however, although the Insc-dependent mechanism can reduce the extent of the mitotic spindle orientation defects seen in the *sna/esg/wor* NBs, it does not restore wild-type spindle orientation. Therefore, it appears that both mechanisms are required and act in concert to mediate mitotic spindle orientation. However, with respect to localization of the basal components, the effects of the Insc-independent mechan-

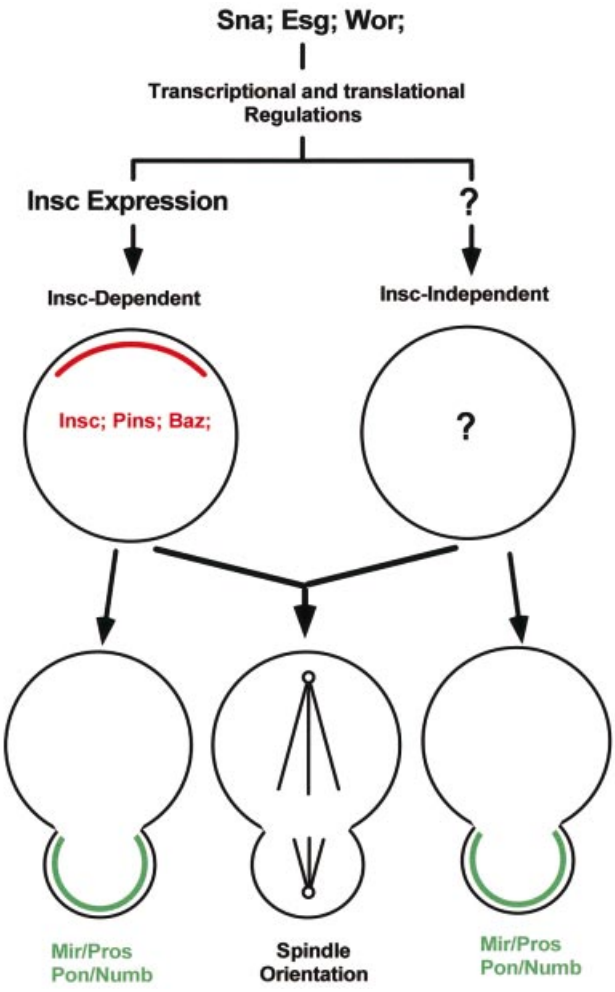

Fig. 7. Relationship between the two independent asymmetry-controlling mechanisms in wild-type NBs. Sna family proteins are required for both the Insc-dependent and -independent mechanisms, most probably through transcriptional and translational regulation. Without Sna family protein functions, Insc is absent and the apical complex is not formed in NBs. Sna family proteins are also required for the Insc-independent mechanism, whose components are yet to be established. With respect to protein localization (telophase rescue), the Insc-independent mechanism is cryptic since its effects can only be observed when the Insc-dependent mechanism is absent. However, both mechanisms contribute towards the A/B orientation of the NB mitotic spindle.

ism are only visible when the Insc-dependent mechanism is absent. Figure 7 summarizes the role of the Sna family proteins and the relationship between these two asymmetry-controlling mechanisms.

Components of the asymmetry machinery in NBs

For the Insc-dependent mechanism, three components have been identified: Baz, Insc and Pins (Kraut *et al*., 1996; Schober *et al*., 1999; Wodarz *et al*., 1999; Schaefer *et al*., 2000; Yu *et al*., 2000) are known to form an apically localized functional complex. The function of this complex requires the participation of all members. Insc appears to be the only component of the Insc-dependent mechanism missing in *sna/esg/wor*-deficient embryos since ectopic expression of Insc restores its function. Little information is available on the components of the Insc-independent mechanism. Other members of asymmetry machinery identified so far in NBs are the basal components such as Mir/Pros, Pon/Numb, Stau and *pros* RNA (Rhyu *et al*., 1994; Hirata *et al*., 1995; Knoblich *et al*., 1995; Spana and Doe, 1995; Spana *et al*., 1995; Li *et al*., 1997; Broadus *et al*., 1998; Schuldt *et al*., 1998). These downstream components are controlled and coordinated by both Insc-dependent and -independent mechanisms.

Transcriptional and translational suppression of insc expression in sna/esg/wor-deficient embryos

In embryos deficient for the *sna* family genes, one of the major defects is the absence of Insc protein expression in the segmented CNS. RNA *in situ* hybridization indicates that the *insc* RNA transcripts are not detected in NBs of stage 9–10 embryos. Even in late stage 11 embryos when the *insc* RNA levels partially recover, Insc protein is never seen in the segmented CNS, indicating that the down-regulation of *insc* occurs at both the transcriptional and translational levels. In the procephalic region of these *sna/esg/wor*-deficient embryos, Insc expression is only partially affected. The 5′- and/or 3′-UTRs of the *insc* transcript appear to play an important role in the translational regulation of Insc expression. This is supported by two observations. First, Insc protein can be detected in *sna/esg/wor* embryos following ectopic expression of a cDNA construct containing the complete *insc* coding region but with the 5′- and 3′-UTRs partially removed. Secondly, transcripts derived from *lacZ* driven by a 1.2 kb *insc* 5′ CNS promoter sequence (our unpublished data) are not subjected to this translational repression in *sna/esg/wor* embryos, although their expression pattern is identical to that of Insc in the CNS. Given that the Sna family proteins are localized to nuclei, it is unlikely that they play a direct role in translational regulation. Other unknown intermediates must be involved.

To summarize, our data indicate that the *sna* family genes mediate two distinct asymmetry mechanisms that control wild-type NB asymmetric divisions, an Insc-dependent and an Insc-independent mechanism. These mechanisms act in parallel to effect NB asymmetric divisions. We have shown that *insc* expression in NBs of the segmented CNS is regulated by Sna family proteins at both the transcriptional and translational levels. With respect to protein localization, the Insc-dependent mechanism acts from prophase to telophase to localize the basal components; the Insc-independent mechanism acts during anaphase/telophase to mediate the process of ‘telophase rescue’ and its effects can only be seen when the Insc-dependent mechanism is absent. Both mechanisms contribute towards A/B orientation of the mitotic spindle.

Materials and methods

Drosophila stocks

The deficiency kit, Df(2L)osp29 and wor^{1} were kindly provided by the Bloomington Stock Center. The deficiencies Df(2L)TE35D-22, Df(2L)TE35BC-3 and all lethal complementation group stocks used in this study were a kind gift of John Roote (Ashburner *et al*., 1999). Df(2L)noc^{4L}-$scorv^{9R}$ was a gift from Tony Ip. The sna^{1}, esg^{G66B}, sna^{1}/esg^{G66B}; uas-*sna* and uas-*esg* stocks were a kind gift from Shigeo Hayashi.

twist-gal4 was a kind gift of Michael Bate and *sca-gal4* driver was obtained from Chris Doe.

Full-length *wor* cDNA was amplified from a 4–8 h embryonic cDNA library (Brown and Kafatos, 1988) with specific PCR primers (5′-primer GAATTCATGCTGATTTCAACAGATGAAGG and 3′-primer GGATCCTTAATAAATGGCCGGTGGTTGC) and subcloned into the Puast vector (Brand and Perrimon, 1993) for germline transformation.

Fusion protein and generation of anti-Wor antibody

Full-length Wor (amino acids 1–525) and N-terminal Wor (amino acids 1–305) were subcloned into the pGEX 4T-1 (Pharmacia) vector. Both glutathione *S*-transferase fusion proteins were purified and used to immunize mice essentially as described (Yu *et al*., 2000) using standard protocols.

Immunochemistry and microscopy

Embryos were collected and fixed accordingly to Yu *et al*. (2000). Rabbit anti-Insc (1:1000), rabbit anti-Pins (1:1000), rabbit anti-Baz (1:500; from F.Matsuzaki), rabbit anti-Pon (1:500; from Y.N.Jan), rabbit anti-Numb (1:500; from Y.N.Jan), rabbit anti-Mir (1:1000; from F.Matsuzaki), rabbit anti-β-gal (Cappel), mouse anti-Pros (1:2; from C.Q.Doe), mouse anti-Sna (1SN-5G6 and 2SN-5H6, 1:500; from Audrey Alberga and Geoff Richards), mouse anti-Esg (1:500; from Shigeo Hayashi) and mouse anti-Wor (1:1000) were used in this study. Cy3- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were obtained from Jackson Laboratories. Stained embryos were mounted in DNA mounting medium (Lundell and Hirsh, 1994) and analysed with laser scanning confocal microscopy (Bio-Rad MRC 1024). RNA *in situ* hybridization experiments were carried out essentially as described in Tear *et al*. (1996).

Double-stranded RNA interference

The *Pst*I fragment of *wor* (bases 57–597) was subcloned into pKS-ds-T7, a pBluescript (Stratagene)-derived vector with two T7 polymerase sites. The *wor* fragment was released with the T7 site on both ends by *Asc*I digestion and was used for *in vitro* transcription with the RiboMAX™ kit (Promega). Double-stranded RNA injection was performed as described in Fire *et al*. (1998) and Kennerdell and Carthew (1998). After injection, embryos were aged to stage 10 at 20°C in a moist chamber then fixed and stained with antibodies.

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References

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

Ashburner,M. *et al*. (1999) An exploration of the sequence of a 2.9-Mb region of the genome of *Drosophila melanogaster*: the Adh region. *Genetics*, **153**, 179–219.

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

Boulay,J.L., Dennefeld,C. and Alberga,A. (1987) The *Drosophila* developmental gene *snail* encodes a protein with nucleic acid binding fingers. *Nature*, **330**, 395–398.

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

Broadus,J., Fuerstenberg,S. and Doe,C.Q. (1998) Staufen-dependent localization of *prospero* mRNA contributes to neuroblast daughter-cell fate. *Nature*, **391**, 792–795.

Brown,N.H. and Kafatos,F.C. (1988) Functional *Drosophila* cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.*, **203**, 425–432.

Burchard,S., Paululat,A., Hinz,U. and Renkawitz-Pohl,R. (1995) The mutant *not enough muscles* (*nem*) reveals reduction of the *Drosophila* embryonic muscle pattern. *J. Cell Sci.*, **108**, 1443–1454.

Campos-Ortega,J.A. and Hartenstein,V. (1985) *The Embryonic Development of Drosophila melanogaster*. Spinger-Verlag, Berlin, Germany.

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

Doe,C.Q., Chu-LaGraff,Q., Wright,D.M. and Scott,M.P. (1991) The *prospero* gene specifies cell fates in the *Drosophila* central nervous system. *Cell*, **65**, 451–464.

Fire,A., Xu S., Montgomery,M.K., Kostas,S.A., Driver,S.E. and Mello,C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, **391**, 806–811.

Foe,V.E. (1989) Mitotic domains reveal early commitment of cells in *Drosophila* embryos. *Development*, **107**, 1–22.

Fuse,N., Hirose,S. and Hayashi,S. (1996) Determination of wing cell fate by the *escargot* and *snail* genes in *Drosophila*. *Development*, **122**, 1059–1067.

Goodman,C.S. and Doe,C.Q. (1993) Embryonic development of the *Drosophila* central nervous system. In Bate,M. and Martinez-Arias,A. (eds), *The Development of Drosophila melanogaster.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1091–1131.

Grau,Y., Carteret,G. and Simpson,P. (1984) Mutation and chromosomal rearrangements affecting the expression of *snail*, a gene involved in embryonic patterning in *Drosophila melanogaster*. *Genetics*, **108**, 347–360.

Hirata,J., Nakagoshi,H., Nabeshima,Y. and Matsuzaki,F. (1995) Asymmetric segregation of a homeoprotein, prospero, during cell division in neural and endodermal development. *Nature*, **377**, 627–630.

Ikeshima-Kataoka,H., Skeath,J.B., Nabeshima,Y., Doe,C.Q. and Matsuzaki,F. (1997) Miranda directs Prospero to a daughter cell during *Drosophila* asymmetric divisions. *Nature*, **390**, 625–629.

Ip,Y.T., Park,R.E., Kosman,D., Bier,E. and Levine,M. (1992) The dorsal gradient morphogen regulates stripes of *rhomboid* expression in the presumptive neuroectoderm of the *Drosophila* embryo. *Genes Dev.*, **6**, 1728–1739.

Kaltschmidt,J.A., Davidson,C.M., Brown,N.H. and Brand,A.H. (2000) Rotation and asymmetry of the mitotic spindle direct asymmetric cell division in the developing central nervous system. *Nature Cell Biol.*, **2**, 7–12.

Kasai,Y., Nambu,J.R., Lieberman,P.M. and Crews,S.T. (1992) Dorsal–ventral patterning in *Drosophila*: DNA binding of snail protein to the single-minded gene. *Proc. Natl Acad. Sci. USA*, **89**, 3414–3418.

Kennerdell,J.R. and Carthew,R.W. (1998) Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the *wingless* pathway. *Cell*, **95**, 1017–1026.

Knoblich,J.A., Jan,L.Y. and Jan,Y.N. (1995) Localisation of *numb* and *prospero* reveals a novel mechanism for asymmetric protein segregation during mitosis. *Nature*, **377**, 624–627.

Kosman,D., Ip,Y.T., Levine,M. and Arora,K. (1991) Establishment of the mesoderm–neuroectoderm boundary in the *Drosophila* embryo. *Science*, **254**, 118–122.

Kraut,R. and Campos-Ortega,J.A. (1996) *inscuteable*, a neural precursor gene of *Drosophila*, encodes a candidate for a cytoskeleton adaptor protein. *Dev. Biol.*, **174**, 65–81.

Kraut,R., Chia,W., Jan,L.Y., Jan,Y.N. and Knoblich,J.A. (1996) Role of *inscuteable* in orienting asymmetric cell divisions in *Drosophila*. *Nature*, **383**, 50–55.

Kuchinke,U., Grawe,F. and Knust,E. (1998) Control of spindle orientation by the Par-3-related PDZ-domain protein Bazooka. *Curr. Biol.*, **8**, 1357–1365.

Leptin,M., (1991) *twist* and *snail* as positive and negative regulators during *Drosophila* mesoderm development. *Genes Dev.*, **5**, 1568–1576.

Li,P., Yang,X., Wasser,M., Cai,Y. and Chia,W. (1997) Inscuteable and Staufen mediate asymmetric localization and segregation of prospero RNA during *Drosophila* neuroblast cell divisions. *Cell*, **90**, 437–447.

Lu,B., Rothenberg,M., Jan,L.Y. and Jan,Y.N. (1998) Partner of Numb, a novel protein that colocalizes with Numb during mitosis, directs Numb asymmetric localizations in *Drosophila* neural and muscle progenitors. *Cell*, **95**, 225–235.

Lu,B., Ackerman,L., Jan,L.Y. and Jan,Y.N. (1999) Modes of protein

movement that lead to the asymmetric localization of Partner of Numb during *Drosophila* neuroblast division. *Mol. Cell*, **4**, 1–20.

Lu,B., Jan,L.Y. and Jan,Y.N. (2000) Control of cell divisions in the nervous system: symmetry and asymmetry. *Annu. Rev. Neurosci.*, **23**, 531–556.

Lundell,M.J. and Hirsh,J. (1994) A new visible light DNA fluorochrome for confocal microscopy. *Biotechniques*, **16**, 434–440.

Matsuzaki,F., Koizumi,K., Hama,C., Yoshioka,T. and Nabeshima,Y. (1992) Cloning of the *Drosophila prospero* gene and its expression in ganglion mother cells. *Biochem. Biophys. Res. Commun.*, **182**, 1326–1332.

Mauhin,V., Lutz,Y., Dennfeld,C. and Alberga,A. (1993) Definition of the DNA-binding site repertoire for the *Drosophila* transcription factor Snail. *Nucleic Acids Res.*, **21**, 3951–3957.

Nusslein-Volhard,C., Wieschaus,E. and Kluding,H. (1984) Mutations affecting the pattern of the larval cuticule in *Drosophila melanogaster*: I. Zygotic loci on the second chromosome. *Wilhelm Roux's Arch. Dev. Biol.*, **193**, 267–282.

Peng,C.Y., Manning,L., Alberson,R. and Doe,C. (2000) The tumour-supressor genes *lgl* and *dlg* regulate basal protein targeting in *Drosophila* neuroblasts. *Nature*, **408**, 596–600.

Rao,Y., Vaessin,H., Jan,L.Y. and Jan,Y.N. (1991) Neuroectoderm in *Drosophila* embryos is dependent on the mesoderm for positioning but not for formation. *Genes Dev.*, **5**, 1577–1588.

Rhyu,M.S., Yan,L.Y. and Jan,Y.N. (1994) Asymmetric distribution of Numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell*, **76**, 477–491.

Schaefer,M., Shevchenko,A., Shevchenko,A. and Knoblich,J.A. (2000) A protein complex containing Inscuteable and the Gα-binding protein Pins orients asymmetric cell division in *Drosophila*. *Curr. Biol.*, **10**, 353–362.

Schober,M., Schaefer,M. and Knoblich,J. (1999) Bazooka recruits Inscuteable to orient asymmetric cell divisions in *Drosophila* neuroblasts. *Nature*, **402**, 548–551.

Schuldt,A.J., Adams,J., Davidson,C., Micklem,D., Haseloff,J., St Johnston,D. and Brand,A.H. (1998) Miranda mediates asymmetric protein and RNA localization in the developing nervous system. *Genes Dev.*, **12**, 1847–1857.

Shen,C.P., Jan,L.Y. and Jan,Y.N. (1997) Miranda is required for the asymmetric localization of Prospero during mitosis in *Drosophila*. *Cell*, **90**, 449–458.

Shen,C.P., Knoblich,J.A., Chan,Y.M., Jiang,M.M., Jan,L.Y. and Jan,Y.N. (1998) Miranda as a multidomain adapter linking apically localised Inscuteable and basally localized Staufen and Prospero during asymmetric cell division in *Drosophila*. *Genes Dev.*, **12**, 1837–1846.

Spana,E. and Doe,C.Q. (1995) The *prospero* transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in *Drosophila*. *Development*, **121**, 3187–3195.

Spana,E. and Doe,C.Q. (1996) Numb antagonises Notch signaling to specify sibling neuron cell fate. *Neuron*, **17**, 21–26.

Spana,E., Kopczynski,C., Goodman,C.S. and Doe,C.Q. (1995) Asymmetric localization of Numb autonomously determines sibling neuron identity in the *Drosophila* CNS. *Development*, **121**, 3489–3494.

Tear,G., Harris,R., Sutaria,S., Kilomanski,K., Goodman,C.S. and Seeger,M.A. (1996) *Commissureless* controls growth cone guidance across the CNS midline in *Drosophila* and encodes a novel membrane protein. *Neuron*, **16**, 501–514.

Thummel,C.S., Boulet,A.M. and Lipshitz,H.D. (1988) Vectors for *Drosophila* P-element-mediated transformation and tissue culture transfection. *Gene*, **74**, 445–456.

Uemura,T., Shepherd,S., Ackerman,L., Jan,L.Y. and Jan,Y.N. (1989) *numb*, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell*, **58**, 349–360.

Vaessin,H., Grell,E., Wolff,E., Bier,E., Jan,L.Y. and Jan,Y.N. (1991) *prospero* is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in *Drosophila*. *Cell*, **67**, 941–953.

Whiteley,M., Noguchi,P.D., Sensabaugh,S.M., Odenwald,W.F. and Kassis,J.A. (1992) The *Drosophila* gene *escargot* encodes a zinc finger motif found in *snail*-related genes. *Mech. Dev.*, **36**, 117–127.

Wodarz,A., Ramrath,A., Kuchinke,U. and Knust,E. (1999) Bazooka provides an apical cue for Inscuteable localization in *Drosophila* neuroblasts. *Nature*, **402**, 544–547.

Wodarz,A., Ramrath,A., Grimm,A. and Knust,E. (2000) *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J. Cell Biol.*, **150**, 1361–1374.

Yagi,Y. and Hayashi,S. (1997) Role of the *Drosophila* EGF receptor in determination of the dorsoventral domains of *escargot* expression during primary neurogenesis. *Genes Cells*, **2**, 41–53.

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

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