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Inscuteable and Staufen Mediate Asymmetric Localization and Segregation of *prospero* RNA during Drosophila Neuroblast Cell Divisions

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Summary

When neuroblasts divide, *inscuteable* acts to coordinate protein localization and mitotic spindle orientation, ensuring that asymmetrically localized determinants like Prospero partition into one progeny. *staufen* encodes a dsRNA-binding protein implicated in mRNA transport in oocytes. We demonstrate that *prospero* RNA is also asymmetrically localized and partitioned during neuroblast cell divisions, a process requiring both *inscuteable* and *staufen.* Inscuteable and Staufen interact and colocalize with *prospero* RNA on the apical cortex of interphase neuroblasts. Staufen binds *prospero* RNA in its 3′UTR. Our findings suggest that Inscuteable nucleates an apical complex and is required for protein localization, spindle orientation, and RNA localization. Stau, as one component of this complex, is required only for RNA localization. Hence *staufen* also acts zygotically, downstream of *inscuteable*, to effect aspects of neuroblast asymmetry.

Introduction

The asymmetric cell division (reviewed in Horvitz and Herskowitz, 1992) in which one progenitor divides to produce two progeny that adopt distinct cellular identities is utilized in a variety of developmental contexts (Jan and Jan, 1995) and organisms (Sternberg and Horvitz, 1984; Chenn and McConnell, 1995) to generate cellular diversity. The generation of asymmetric sibling cell fates can be mediated extrinsically, for example through Notch signaling (Artavanis-Tsakonas et al., 1995); intrinsically, through asymmetrically localized and segregated protein determinants (Doe and Spana, 1995; Campos-Ortega, 1996); or both mechanisms can work in conjunction (Guo et al., 1996; Spana and Doe, 1996).

The Drosophila embryonic CNS consists of repeats of a basic building block, the hemineuromere, comprised of ~300 neurons (Bossing et al., 1996). The majority of these cells are derived from ~30 neural progenitor cells, called neuroblasts (NBs). NBs undergo repeated asymmetric cell divisions, budding off a series of ganglion mother cells (GMCs) from their basal cortex. Each GMC in turn divides once to give rise to two neurons (or glia). NB cell divisions are asymmetric since the progeny NB and GMC are not only different in morphology but also show different capacities for cell division and different patterns of gene expression. NBs, therefore, provide an excellent model for understanding asymmetric cell divisions.

Two proteins, Numb (Nb) (Uemera et al., 1989) and Prospero (Pros) (Doe et al., 1991; Vaessin et al., 1991; Matsuzaki et al., 1992), which can act as determinants of cell fate, are asymmetrically localized on the basal cortex of NBs during mitosis, allowing them to be selectively partitioned to the GMC progeny when NBs divide (Rhyu et al., 1994; Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995). Although Nb acts as a determinant in sibling cell fate decisions during the development of the external sensory organs (Rhyu et al., 1994; Guo et al., 1996) and for the sibling neurons derived from the CNS MP2 precursor (Spana et al., 1995), it is unclear whether it plays a role in mediating GMC cell fate. Pros, on the other hand, is required in GMCs to activate genes required to specify GMC identity (Doe et al., 1991) and to repress genes that are normally expressed in NBs (Vaessin et al., 1991). *pros* is transcribed in all NBs and encodes a homeobox protein; however, Pros protein is found only in the GMC nuclei (Vaessin et al., 1991; Matsuzaki et al., 1992). In NBs, Pros is associated with the cellular cortex as an apical crescent in late interphase and as a basal cortical crescent during mitosis (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995).

For asymmetrically localized determinants to be partitioned into just one of the NB progeny, the processes of mitotic spindle orientation and protein localization must be coordinated. In wild-type NBs there is a tight correlation between the basal localization of protein determinants like Pros and Nb and the apical/basal orientation of the mitotic spindle, ensuring that the protein determinants are inherited by the more basally localized progeny. However, since the correlation between these processes can be dissociated by drug treatment, they are independent processes (Knoblich et al., 1995). *inscuteable* (*insc*) encodes a protein that localizes as an apical cortical crescent in NBs (Kraut and Campos-Ortega, 1996). It is not only required for correct protein localization and mitotic spindle orientation, but also for their correlation; in the absence of *insc*, Pros and Nb are either localized throughout the NB cortex or form crescents that localize to random positions on the cortex during mitosis; the apical/basal orientation of the mitotic apparatus as well as the correlation between the position of the protein crescent and the division plane are also disrupted (Kraut et al., 1996).

The localization of mRNAs as a means of localizing proteins to specific regions within a cell is a mechanism that is used in a variety of organisms and cell types (St. Johnston, 1995). The importance of this mechanism has been emphasized in particular by the genetic analysis of the Drosophila body pattern (St. Johnston and Nüsslein-Volhard, 1992). For example, the localization of *oskar* (*osk*) mRNA to the posterior pole of the oocyte defines the site of accumulation of both Osk protein and the posterior determinant Nanos necessary for abdominal development (Ephrussi et al., 1991; Kim-Ha et al., 1991;

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Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991); the localization of *bicoid* (*bcd*) mRNA to the anterior pole enables the formation of a Bcd morphogen gradient necessary for the formation of the head and thorax (Driever and Nüsslein-Volhard, 1988).

The localization of *bcd* mRNA to the anterior pole is a complex multistep process requiring *stau* for the final step (St. Johnston et al., 1989; 1991); a 400 nt region of the *bcd* 3′UTR can apparently associate with Stau to prevent diffusion of the RNA once it is released from anterior cortex (Ferrandon et al., 1994). *stau* is also required for the anterior-to-posterior transport of *osk* mRNA in the oocyte (reviewed St. Johnston, 1995). Consistent with a direct role in mRNA transport, Stau contains five copies of a dsRNA-binding motif, binds to dsRNA in vitro (St. Johnston et al., 1992), and colocalizes with *osk* mRNA to the posterior and *bcd* mRNA to the anterior of the egg (St. Johnston et al., 1991; Ferrandon et al., 1994). Upon injection of *bcd* 3′UTR, the RNA recruits Stau protein into particles that can move in a microtubule-dependent manner (Ferrandon et al., 1994). These observations support a direct role for Stau in the transport of mRNA species for generating the molecular asymmetry that presages the A/P body pattern.

To examine whether the asymmetric NB cell divisions incorporate asymmetric RNA localization as part of its overall strategy, we examined the distribution of *pros* and *insc* RNAs during the NB cell cycle. In this paper we show that *insc*, in addition to its previously defined roles of coordinating mitotic spindle orientation and protein localization, is also required for asymmetric *pros* RNA localization and segregation; this process is *stau* dependent. We show that Insc can interact with Stau, and the two proteins colocalize on the apical cortex of NBs during late interphase; a region of Stau distinct from its Insc interacting domain can bind *pros* 3′UTR in vitro. We suggest that Insc nucleates a multicomponent apical complex and is required for protein localization, RNA localization, and spindle orientation during mitosis. Stau, as one component of this complex, is required only for the basal localization of *pros* RNA.

Results

The localization of *inscuteable* and *prospero* RNAs in Neuroblasts Is Asymmetric and Cell Cycle Dependent

Insc and Pros proteins are localized as crescents on the apical/basal regions of the NB cell cortex in a cell cycle–dependent manner (see Introduction). Is this asymmetric localization of the proteins also reflected at the level of their respective RNAs? To visualize the location of the RNAs at various stages of the NB cell cycle, wild-type stage 10 and 11 embryos were hybridized against single-stranded RNA probes (see Experimental Procedures), and the embryos were doubly labeled with a DNA stain (see Experimental Procedures) to follow the progression of NBs through the cell cycle. The results show that these RNAs, like the proteins they encode, are also asymmetrically localized. Moreover, the localization of the RNAs is cell cycle dependent and parallels the localization of the respective proteins they encode. Hence, in the case of *pros*, both the Pros protein (not shown; see Kraut et al., 1996) and *pros* RNA are localized primarily as crescents to the apical cortex during late interphase (Figure 1A) (although in the case of the RNA, we also see some cases in which the localization is apical but apparently cytoplasmic rather than cortical; see Discussion); both Pros protein (not shown) and *pros* RNA are localized as basal cortical crescents from prophase to telophase (Figures 1B and 1C); when the NBs ultimately divide, the RNA (along with the protein) remains cortical and segregates preferentially into the more basally located daughter cell, the GMC (arrow in Figure 1C).

The *insc* RNA, like the Insc protein, is also localized to the apical cortex starting at interphase (Figures 2A and 2E). Although Insc protein remains apically localized until anaphase (Kraut et al., 1996), *insc* RNA is localized to the cytoplasm during mitosis (Figures 2B and 2F). So in contrast to *pros* RNA, which is apparently associated primarily with the cortex throughout the NB cell cycle, the *insc* RNA is cortical only during interphase.

The Change in the Localization of *pros* RNA from the Apical to the Basal Cortex of Neuroblasts Requires *insc*

Since *insc* is required for the localization of Pros (and Nb) protein and for orientation of the mitotic spindle, is it possible that the RNA localization is also *insc* dependent? We followed *pros* RNA localization in NBs at various stages of the cell cycle in *insc* mutant embryos. A striking difference in *pros* RNA localization is observed between wild-type and mutant NBs (Figure 1). In wild-type NBs, there is a dramatic change in the *pros* RNA localization from the apical cortex to the basal cortex between late interphase and prophase (compare Figure 1A to Figure 1B) that mirrors the change in the Pros protein localization (not shown; see Kraut et al., 1996); *pros* RNA remains on the basal cortex until it preferentially segregates to the GMC at telophase (Figure 1C). In *insc* mutant NBs, this change in the localization of the *pros* RNA does not occur during the interphase-to-prophase transition, and *pros* RNA remains primarily on the apical cortex during mitosis (Figures 1D–1F). Hence, whereas the initial apical localization of *pros* RNA during interphase does not depend on *insc* function, the basal localization during mitosis does. Our results therefore indicate that in addition to its requirement for correct spindle orientation and protein localization, *insc* also plays a role in RNA localization.

Interaction between Inscuteable and Staufen Proteins

Insc protein is known to be localized to the apical cortex of NBs from interphase to metaphase (Kraut et al., 1996). Hence, it is unlikely that Insc is involved directly in the basal localization of *pros* RNA, since when the *pros* RNA is basally localized, Insc protein is on the opposite side of the cell. To begin addressing the question of what, if any, other protein(s) might be involved in mediating the *insc*-dependent localization of *pros* RNA, a yeast two-hybrid screen was conducted. The full-length, N-terminal, C-terminal, and middle portions of the *insc* coding region were used separately as baits to screen Drosophila embryonic library to identify molecules that

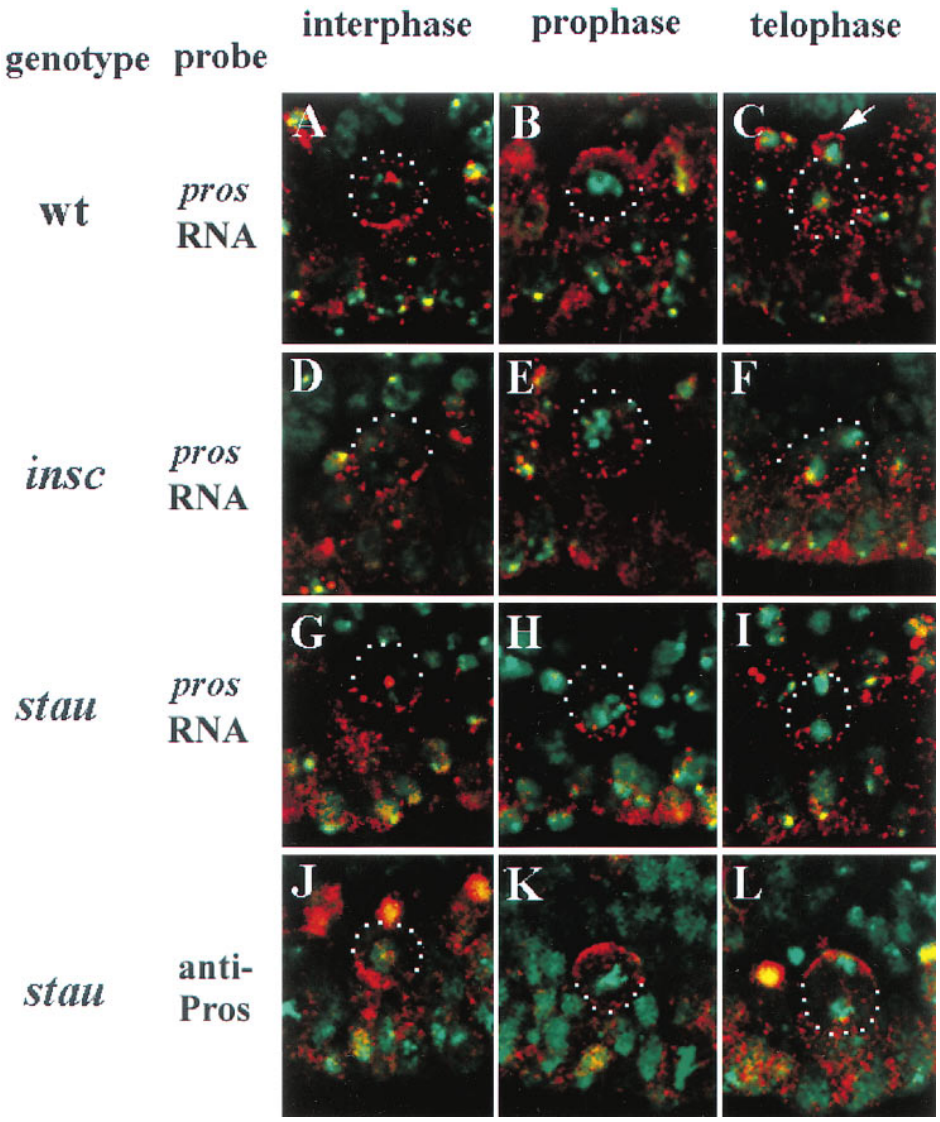


Figure 1. In NBs, the Change in *pros* RNA Localization from the Apical Cortex during Late Interphase to the Basal Cortex during Mitosis Requires Both *insc* and *stau*

In wild-type embryos, localization of *pros* RNA is cell cycle dependent (A-C). *pros* RNA (red) is apically localized during interphase (A) and becomes basally localized throughout mitosis starting at prophase (B). During telophase, the cortically localized *pros* RNA is preferentially partitioned into the GMC (C). This cell cycle–dependent localization of the RNA is identical to that previously described for the Pros protein (not shown). In both *insc* (D–F) and *stau* (G–I) embryos, the apical localization of *pros* RNA in interphase mutant NBs is unchanged (D and G); however, unlike the *pros* RNA in wild-type NBs, the *pros* RNA remains on the apical cortex of mutant NBs during prophase (E and H) and telophase (F and I); no obvious enrichment of the *pros* RNA is observed at the basal cortex from which the future GMC is formed (F and I). In *stau* embryos, asymmetric Pros protein (red) localization and segregation occurs normally in NBs (J–L); the apical localization of Pros during late interphase (J) and its basal localization during (mitosis) prophase (K) and telophase (L) in *stau* mutant NBs are identical to those previously described for wild-type NBs (data not shown). DNA staining is shown in green. White dots indicate the boundary of the cell. Apical is toward the bottom.

can potentially interact with Insc (see Experimental Procedures). Three classes of positive clones were identified from screening approximately 2×10^6 colonies with the Insc C-terminal bait. Sequencing revealed that one class encodes Staufen, a dsRNA-binding protein that has been implicated in mRNA transport in the oocyte (see Introduction). Since a role for Stau in *pros* RNA localization can be easily rationalized, we proceeded to further analyze the Insc/Stau molecular interaction.

Deletion mapping analyses using the yeast two-hybrid assay (see Experimental Procedures) indicate that LexA fusion proteins containing the C-terminal region of Insc can interact with Stau (Figure 3A). Furthermore the C-terminal 108 amino acid residues of Insc are sufficient to confer this interaction with Stau; interestingly, addition of residues 664–750 appears to inhibit the interaction mediated by the C-terminal 108 residues, and this inhibition can in turn be relieved by the further addition of residues 570–663 (compare YI-3, YI-4, and YI-5 in Figure 2A). Since the bait YI-3 containing the C-terminal

end of Insc interacts strongly with Stau, it was used for mapping the regions of Stau that are necessary to interact with Insc. As shown in Figure 3B, the C-terminal region (residues 769–1026, YS-3) of Stau, when expressed as a fusion protein, confers specific interaction with Insc. Further deletions from either the N-terminal or the C-terminal ends of the YS-3 region of Stau (YS-4, YS-5, or YS-6) disrupt the interaction.

In vitro binding assays (see Experimental Procedures) using GST-insc fusion proteins containing different portions of Insc (Figure 4A) indicate that fusion proteins containing its C-terminal end binds to the ^{35}S-labeled full-length Stau protein (Figure 4B, In-1, In-3, and In-4). These results derived from in vitro binding assays are in good agreement with those obtained from the yeast two-hybrid assays, strongly suggesting that there is a direct interaction between Stau and Insc and that the C-terminal regions of both Insc and Stau are required for this interaction. However, possibly due to the low proportion of cells that express Insc, the transient nature of the association and/or the low solubility of Stau (Daniel St. Johnston, personal communication), we were unable to demonstrate coimmunoprecipitation of the two proteins from wild-type embryonic extracts.

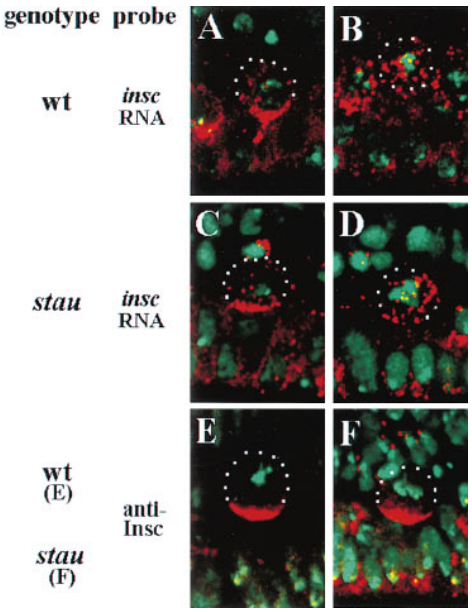


Figure 2. The Asymmetric Localization of *insc* RNA and Protein Seen in Wild-Type NBs Is Not Altered in *stau* Embryos

The *insc* RNA localization pattern is identical for wild-type (A and B) and *stau* (C and D) NBs at similar stages of the cell cycle. *insc* RNA (red) is localized on the apical cortex of both wild-type (A) and *stau* (C) interphase NBs; the wild-type NB in (A) has not completed delamination, and *insc* RNA can be seen in the stalk that extends toward the apical surface. During prophase (B and D) and throughout mitosis (not shown), the apical RNA (red) is delocalized and redistributed to the cytoplasm of both wild-type (B) and *stau* (D) NBs. Anti-Insc staining shows that the Insc protein (red) localizes as an apical crescent in prophase NBs in both wild-type (E) and *stau* (F) embryos (and Insc protein localization is identical in wild-type and *stau* NBs throughout mitosis, data not shown). DNA staining is shown in green. White dots indicate the boundaries of the NBs. Apical is toward the bottom.

Staufen and Inscuteable Proteins Are Both Cortically Localized in Neuroblasts

If the protein/protein interactions seen in vitro and in the yeast two-hybrid sytem reflect a genuine in vivo interaction, one would expect Stau and Insc to be colocalized at some stage during development. Available data suggest that this may be the case since Insc is expressed like a neural precursor gene, in all neural progenitor cells (Kraut and Campos-Ortega, 1996), and St. Johnston et al. (1991) have shown that zygotic Stau protein expression occurs during the development of the embryonic CNS. Using serum that specifically recognizes Stau (see Experimental Procedures), we have confirmed that Stau protein is expressed in the NBs and GMCs in the segmented CNS (Figure 5), and additionally in cells in the procephalic region that are also known to express Insc (data not shown). Stau protein is expressed at high levels in GMCs; newly formed GMCs show only weak staining, suggesting that Stau protein is synthesized de novo in GMCs (data not shown).

We focused in detail on the localization of Stau protein in NBs and compared it to the Insc protein localization. Stau protein is always seen associated with the NB cortex. During interphase, Stau protein distribution in the majority of interphase NBs (81%) resembles an apical cortical gradient with high levels of the protein associated with the apical region and lower levels throughout the rest of the (lateral and basal) cortex (Figures 5A and 5E). In a minority of the interphase NBs (19%), Stau protein appears to be cortical without a concentration bias toward the apical side (Figures 5B and 5E). The relative proportion of the NBs showing the two types of staining patterns does not appear to change between interphase and prophase in wild-type (Figure 5E). However, during later stages of mitosis (metaphase–telophase), the proportion of NBs with Stau protein preferentially enriched on their apical cortex decreases dramatically, and the majority of cells show general cortical distribution that is not obviously biased toward any one region. Our results therefore indicate that Stau and Insc proteins largely colocalize on the apical cortex during late interphase and prophase, consistent at least with the notion that the two proteins do interact in vivo. However, during the period when there is a dramatic change in the localization of *pros* RNA between interphase (apical) and prophase (basal), there is no obvious change in the distribution of Stau protein (see Discussion).

Staufen Is Required for the Basal Localization of *pros* RNA during Mitosis

Since Stau is known to be an RNA-binding protein and plays a crucial role in RNA localization in the oocyte, an obvious question is whether *stau* might also affect *pros* RNA localization in the NBs. RNA in situ experiments demonstrate that in animals that lack both maternal and zygotic *stau*, *pros* RNA is localized to the apical cortex during interphase; however, the change in *pros* RNA localization from the apical cortex at interphase to the basal cortex at prophase fails to occur (Figures 1G and 1H). Similar results have been obtained in embryos lacking only the zygotic *stau* (data not shown). That only the

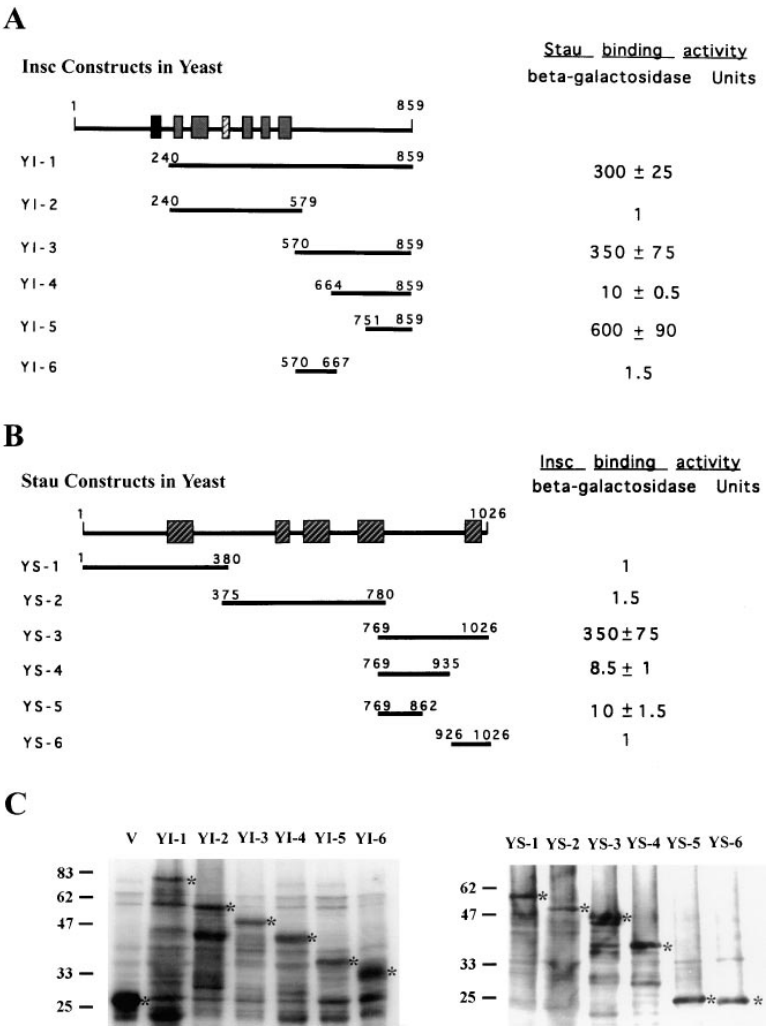


Figure 3. Delineating Domains in Insc and Stau That Interact with Each Other by the Yeast Two-Hybrid Assay

(A) Schematic diagram illustrating serial deletions of Insc and their corresponding binding activity with Stau. YI-1–YI-6, Insc deletions that were fused in-frame with LexA domain in the PEG202 MYC vector. The β-galactosidase units for each fusion protein represents five independent experiments. The five shaded boxes represent regions that share homology with ankyrin repeats. The hatched box indicates the nuclear localization signal sequence. The position of the putative WW domain is shown in the closed box.

(B) Mapping the region in Stau that interacts with Insc. YS-1–YS-6 represent different regions of Stau that were fused in-frame with the B42 activation domain and HA epitope in JG4-5 vector. β-galactosidase units for each fusion protein were obtained from four independent experiments.

(C) Western blot analysis showing the expression level of all of the LexA-Insc fusion constructs (left panel) by using a monoclonal antibody against the MYC epitope; expression level of JG4-5-Stau fusion proteins (right panel) using a monoclonal antibody against the HA epitope. The lane labeled (V) contained LexA fusion alone. Asterisks indicate the positions of the different fusion proteins.

zygotic *stau* is required in this process is consistent with previous observations (St. Johnston et al., 1991) that maternal Stau protein becomes undetectable in somatic tissues by the time of neurogenesis. In the *stau* mutant NBs, the *pros* RNA remains primarily on the apical cortex during mitosis, and this phenotype is indistinguishable from that seen in *insc* mutants (compare Figures 1D–1F with 1G–1I). These results indicate that the apical cortical localization of *pros* RNA during interphase requires neither *insc* nor *stau* function; however, the basal cortical localization requires both *insc* and *stau* functions.

stau Acts Downstream of *insc* and Is Required Neither for Protein Localization Nor for Mitotic Spindle Orientation in Neuroblasts

Since both *stau* and *insc* appear to be involved in the process of localizing *pros* RNA to the basal cortex, what is their hierarchical relationship? Two lines of evidence indicate that *stau* acts downstream of *insc*. First, the defects seen in the *insc* mutant NB are more pleiotropic than those seen in embryos lacking *stau*. *insc* mutant NBs exhibit defects not only in RNA localization but also show defective mitotic spindle orientation, and protein localization (see Kraut et al., 1996). In contrast, the total absence of *stau* function affects only *pros* RNA localization (Figure 1). The apical/basal orientation of the mitotic spindles of NBs as well as those of the mitotic domain 9 cells in the procephalic region that is disrupted in *insc* mutant embryos (see Kraut et al., 1996) remains apical/basal in *stau* mutants (see Figures 1C, 1F, 1I, and 1L and data not shown); similarly the localization of Pros protein to the apical cortex in interphase and to the basal cortex of these cells during mitosis, which we have previously shown is defective in *insc* mutant embryos (Kraut et al., 1996), is unaffected in *stau* mutants (Figures 1J–1L). These observations indicate that *stau* is involved in only a subset of the processes that require *insc*.

Second, consistent with the idea that *stau* acts downstream of *insc*, Stau protein localization appears to be affected in *insc* mutants, albeit in a subtle way. In *insc* mutant embryos, the proportion of NBs showing a higher concentration of Stau protein on their apical cortex as compared to NBs that show more uniform cortical distribution is significantly reduced during interphase and prophase (see Figure 5E). Hence, the overall asymmetry and the relative preference of the Stau protein for the apical region of the NB cortex is reduced in *insc* mutants.

In contrast, neither the localization of *insc* RNA nor that of the Insc protein is altered in embryos that lack both maternal and zygotic *stau*; the apical cortical *insc* RNA localization during late interphase (Figures 2A and 2C) and the cytoplasmic localization during mitosis (Figures 2B and 2D) are seen in both wild-type and *stau* mutant NBs; similarly the previously described apical cortical Insc protein crescents seen in wild-type NBs from interphase to metaphase are unaffected in *stau* mutants (Figures 2E and 2F). Taken together, these data indicate that *stau* acts downstream of *insc*.

Staufen Binds to a Probe Containing the 3′UTR of *pros* RNA

Since Stau is known to be a dsRNA-binding protein and its function is required for the posterior localization of *osk* RNA and the anterior localization of *bcd* RNA in the egg, one possible mechanism by which it might act to mediate *pros* RNA localization is by binding *pros* RNA directly. Consistent with this notion, *pros* RNA has a large 3′UTR where most of the localization motifs have been found for a number of specifically localized mRNAs. To test this possibility, we made fusion proteins containing various portions of the *stau* coding region (Figures 6A and 6C). As shown in Figure 6B, a ^{32}P-labeled RNA probe containing the 3′UTR of *pros* binds to GST-stau fusion proteins (see Experimental Procedures) that contain the third putative double-stranded RNA binding domain of Stau (S-2, S-3, and S-7). Further deletions of either the first 20 amino acid residues from the N terminus or the last 15 amino acid residues from the C terminus of this domain abolish its ability to bind. In contrast, Stau fusion proteins show little binding affinity toward the coding region of *pros* RNA (data not shown). These results suggest that the Stau binding to *pros* RNA is specific to the 3′UTR; this may in turn mediate the *insc*-regulated *pros* RNA localization. Since the region of Stau (residues 565–661) that is capable of binding *pros* 3′UTR lies amino terminal to and is distinct from the the region of Stau that is required to interact with the Insc protein, these results are consistent with the notion that Stau is capable of interacting simultaneously with both Insc protein and *pros* RNA.

Discussion

inscuteable Mediates Several Processes Associated with Asymmetric Neuroblast Cell Divisions

To aid the discussion, the localization of *pros* RNA, Pros protein, *insc* RNA, Insc protein, and Stau protein during interphase, prophase, and telophase in wild-type and mutant NBs is schematically summarized (Figure 7A). We have previously shown that *insc* is required for two processes associated with the NB asymmetric cell division—localization of protein determinants (Pros and Nb) and mitotic spindle orientation (Kraut et al., 1996). These processes are normally tightly correlated (see Rhyu and Knoblich, 1995) with protein determinants localized to the basal cortex during mitosis and the mitotic spindle oriented along the apical/basal axis to ensure that localized determinants are asymmetrically inherited only by the more basally located progeny. We have rationalized (Kraut et al., 1996) that the correlation of mitotic spindle orientation and Nb and Pros protein localization occur because both processes are utilizing the same positional information that is either provided or interpreted by *insc*; nevertheless, they represent independent processes because in the absence of *insc* or through drug treatment (Knoblich et al., 1995), the tight correlation for these processes can be decoupled. Here, we have shown that *insc* is also required to localize RNA (*pros*) to the basal cortex during mitosis and facilitate its asymmetric segregation with the more basal progeny cell. Moreover, we can demonstrate that all three of the processes mediated by *insc*, RNA and protein localization as well as mitotic spindle orientation, are independent of each other and that their normally tight correlation can be decoupled through the loss of *insc*.

Since Pros protein localization and mitotic spindle orientation during NB mitosis are apparently normal in embryos that lack both maternal and zygotic *stau, stau* appears to be involved in only one of the three *insc*-mediated processes, that of *pros* RNA localization. Moreover, the loss of *insc* causes a decrease in the

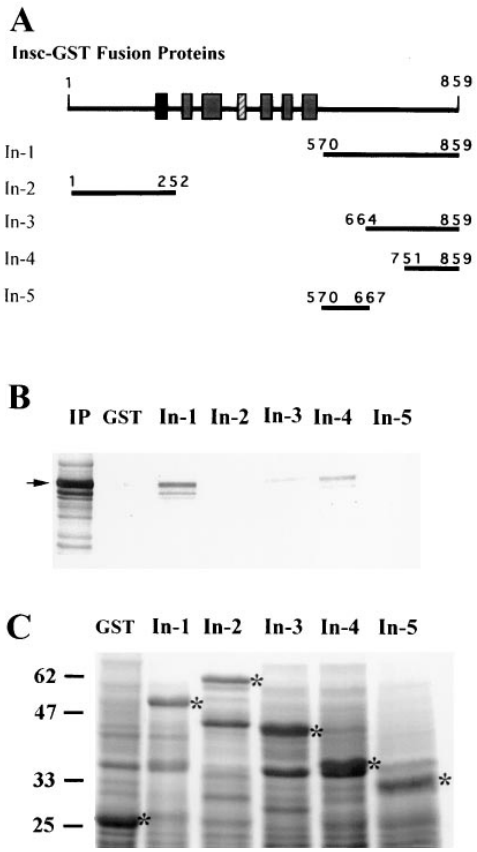
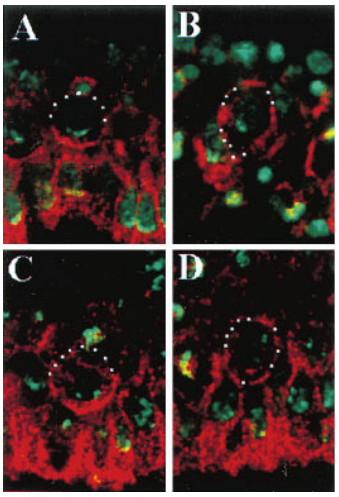


Figure 4. Direct Interactions between Stau and Insc Using a GST Binding Assay

(A) Schematic diagram showing the different GST-Insc fusion proteins used.

(B) In vitro interaction between the ^{35}S-labeled full-length Stau protein and the C-terminal region of Insc. The arrow indicates the position of the in vitro-translated full-length Stau protein. The lane labeled (IP) contained 20% of the total input in each binding reaction.

(C) SDS–PAGE gel showing the expression levels of GST-Insc fusion proteins used as starting material. See Experimental Procedures.



E **Distribution of Stau Protein in Neuroblasts**

		interphase	prophase	metaphase	anaphase	telophase
WT	total NBs observed	243	94	43	13	23
	apical gradient	198	77	27	1	2
	cortical	45	17	16	12	21
	% apical gradient	81	82	63	8	9

		interphase	prophase	metaphase	anaphase	telophase
insc	total NBs observed	186	91	54	15	11
	apical gradient	67	49	21	4	0
	cortical	119	42	33	11	11
	% apical gradient	36	54	39	27	0

Figure 5. Stau Protein Distribution in Wild-Type and *insc* NBs

Stau protein (red), detected by anti-Stau antibody staining, is associated with the NB cortex, but its distribution within the cellular cortex is cell cycle dependent. In wild-type embryos, the majority (81%) of the interphase NBs exhibit a higher concentration of Stau on their apical cortex than elsewhere in the cortex (A), and we refer to this as "apical gradient." In the rest of the NBs, this preferential apical distribution is not obvious and staining is seen throughout the NB cortex (B), and we refer to this as "cortical." During prophase, the proportion of the NBs containing a Stau apical gradient remains unchanged (82%). As wild-type mitosis continues, this asymmetry in the Stau protein distribution disappears and most of the dividing NBs have Stau throughout their cortex. At no time during wild-type mitosis do we see a significant number of NBs showing a preferential distribution of Stau on the basal cortex. In *insc* embryos, Stau apical gradient is detected during interphase ([C], red), but at a much reduced frequency (36%); the majority (64%) of the NBs do not contain an obvious Stau gradient and show cortical localization (D). This ratio varies during mitosis, but from prophase to telophase, the majority of the *insc* mutant NBs have Stau protein throughout their cortex. DNA staining is shown in green. White dots indicate the boundary of the cell. Apical is toward the bottom.

propensity for Stau to associate with the apical cortex during interphase, whereas loss of *stau* does not affect the localization of either Insc protein or *insc* RNA. These results suggest that *insc* acts upstream of *stau* and has a more general role in mediating the NB asymmetric cell division (see Figure 7B). However, whereas little is known about the mechanistic details of how Insc might mediate spindle orientation and protein localization, its interaction with Stau and what is known about the function of *stau* in the oocyte suggest possible mechanisms by which *insc* may be involved in mediating *pros* RNA localization (see below).

It is interesting to note that there appears to be redundancy in at least one of the processes mediated by *insc*. *pros* normally acts in GMC nuclei to repress the expression of genes that are normally expressed in NBs and to promote the expression of genes that are needed to establish GMC identity (see Introduction). Therefore, its activity must be suppressed in the NB; this is presumably accomplished by tightly regulating its translation and sequestering the protein on the cortex to prevent its entry into the nucleus. But why express *pros* in the NBs in the first place? We speculate that this might be due, in part, to temporal considerations. *pros* encodes a large transcription unit, and the time required to produce the transcript has been estimated to be ~20 minutes (Spana and Doe, 1995). This would represent a long delay if *pros* were to be expressed de novo in the GMCs. Under normal circumstances, *insc*-mediated processes cause both *pros* protein and RNA to segregate into the GMC progeny, presumably to ensure that some protein and the means to translate more are both available early in the GMC cell cycle. However, in *stau* mutants where *pros* RNA does not (but Pros protein does) segregate asymmetrically to the GMC progeny, we have not detected obvious cell fate changes in the nervous system (X. Y., unpublished data). This may be because the changes are subtle and have therefore escaped detection; alternatively, it could mean that the segregation of *pros* RNA to the GMC is redundant and can be compensated by Pros protein segregation and/or by the de novo synthesis of *pros* RNA later in the GMC cell cycle.

Two Independent Mechanisms Mediate the Localization of *pros* RNA Apically during Interphase and Basally during Mitosis

During interphase, *pros* RNA is localized to the apical cortex; however, the position of *pros* RNA changes as NBs enter mitosis. Starting at prophase and for the duration of the NB mitosis, *pros* RNA is localized to the basal cortex (where Pros protein is also localized) and segregates (with Pros protein) to the more basal progeny cell, the GMC. There is some precedence for RNAs that are ultimately localized in a particular subcellular compartment to be transiently localized in a different compartment en route to its final destination. For example, in the developing oocyte, incoming RNAs from the nurse cells can be localized to several different sites as the polarity of microtubule cytoskeleton changes during the maturation of the oocyte at these early stages (e.g., see Theurkauf et al., 1992; Clark et al., 1994). Our data suggest that two independent mechanisms control the apical and basal localizations of *pros* RNA. The apical localization of *pros* RNA during interphase occurs independent of *insc* and *stau*; in contrast, the basal localization of *pros* RNA is dependent on both. It is possible that other RNAs contain localization signals that, when read in the context of an interphase NB, will take them

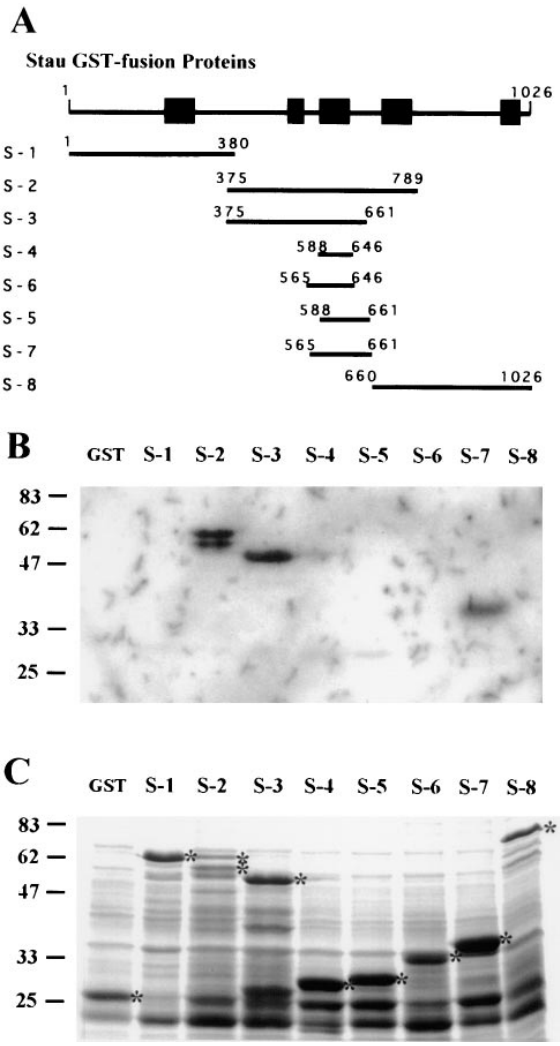


Figure 6. Direct Interaction between Stau and the 3′ Untranslated Region of *prospero*

(A) Schematic diagram showing the different regions of Stau that were ligated into pGEX4T-1 to generate Stau-GST fusion proteins used for this experiment. Closed boxes represent the five regions on Stau that share high homology with other double-stranded RNA-binding proteins.

(B) GST-Stau fusion proteins that contain the third RNA-binding domain (S-2, S-3, and S-7) bind to the ^{32}P-labeled Pros 3′UTR probe.

(C) SDS–PAGE gel showing the level of protein expression for the various Stau-GST fusion proteins used.

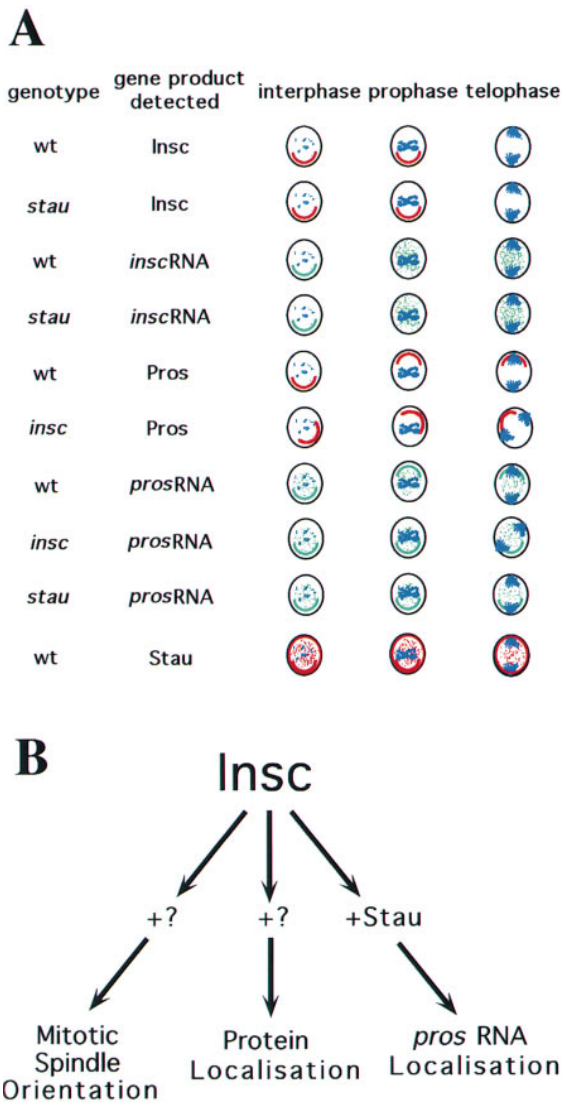


Figure 7. Schematic Summaries

(A) Summary of the localizations of *insc* RNA, Insc protein, *pros* RNA, Pros protein, and Stau protein during late interphase, prophase, and telophase of wild-type and mutant NBs. Apical is toward the bottom. Protein is represented in red, DNA in blue, and RNA in green.

(B) Schematic diagram depicting the roles played by *insc* and *stau* during the asymmetric NB cell division and their hierarchical relationship.

to the apical cortex; this apical localization may be a reflection of the inherent architecture of an interphase somatic cell and may act in conjunction with apical vectorial transport of *pros* RNA from the nucleus (see Francis-Lang et al., 1996). From this viewpoint, it is interesting to note that when ectopically expressed, localization sequences associated with three mRNAs that are actively transported in the oocyte, *K10*, *bicoid*, and *oskar* RNA, mediate microtubule-dependent, apical localization in somatic follicle cells (Karlin-McGinness et al., 1996). In addition, *bcd* transcripts localize apically in nurse cells where they are synthesized (St. Johnston et al., 1989; see Davis et al. [1993] for an interesting discussion), and *bcd* 3′ sequences required to localize maternal transcripts anteriorly during oogenesis can localize zygotic reporter transcripts apically at the blastoderm stage (Davis and Ish-Horowicz, 1991). We speculate that the apical cortex of interphase NBs (and perhaps other somatic cells) may serve as a distribution point for molecules that may ultimately be targeted to a different final destination.

How Does *prospero* RNA Get to the Basal Cortex during Mitosis?

We have shown that the basal localization of *pros* RNA during mitosis requires both *insc* and *stau*; in the absence of either *insc* or (zygotic) *stau*, *pros* RNA remains

apically localized during mitosis. We infer from these observations that Stau and Insc are both required for *pros* RNA to move to the basal cortex during the start of mitosis and that in the absence of either function, the *pros* RNA fails to move and stays associated with the apical cortex; the continued association of *pros* RNA with the apical cortex when either *insc* or zygotic *stau* is absent has three implications. First, since the RNA remains on the apical cortex during mitosis, it suggests that the *insc*/*stau*-independent mechanism that operates to effect localization to the apical cortex during interphase is normally overridden by the *insc*/*stau*-mediated process during mitosis. Second, since the RNA is apical in the absence of *insc*, cues distinct from those provided by *insc* must be used to read the apical basal axis of the NB during interphase. Third, since the interphase apical RNA localization is unchanged in *stau* mutant NB, one can also rule out models in which Stau translocates *pros* RNA along astral microtubules stabilized at the apical surface by interaction with Insc (or an Insc-nucleated complex).

Stau is required for the transport of *bicoid* and *oskar* RNA in the oocyte (see Introduction). There is strong evidence that suggests that specific signals localized in the 3′UTR of *bcd* can recruit Stau to form RNP particles that are subsequently transported in a process that requires intact microtubules (Ferrandon et al., 1994); the localization of *osk* mRNA to the posterior pole appears to additionally require actin filaments (Erdelyi et al., 1995). It is therefore appealing to suggest that Stau might play a similar role in the NB to transport *pros* RNA from the apical to basal cortex in the transition between interphase and mitosis. In support of this model, we have shown that Stau is capable of binding the *pros* 3′UTR. However, during the critical period between interphase and prophase, when the localization of *pros* RNA changes from the apical to the basal cortex, we see no obvious change in the distribution of Stau protein in wild-type NBs. This may just be a reflection of the fact that in NBs Stau protein is in excess and the relative amount of protein that escorts the RNA to the basal region is small. Nevertheless, it is possible that Stau is not directly involved in transporting *pros* RNA to the basal cortex but rather Insc and Stau might be required to bring *pros* RNA in contact with other factor(s) that are involved in its transport.

An Insc/Stau/*pros* RNA Complex on the NB Apical Cortex during Interphase?

We have shown that in NBs *pros* RNA is apically localized during late interphase when both Insc and Stau proteins are also enriched on the apical cortex. Insc and Stau proteins can interact via their respective C-terminal regions as judged by yeast two-hybrid or in vitro protein interaction assays; in addition, a region of the Stau protein that is distinct from its Insc interaction domain, that includes a previously defined RNA-binding motif (St. Johnston et al., 1992), can bind to RNA containing the *pros* 3′UTR. Since Stau can (in theory) bind both Insc protein and *pros* RNA simultaneously utilizing separate domains, these observations are consistent with the notion that an apical complex that includes Insc and Stau proteins as well as *pros* RNA may be formed at this time. Since Insc is required for several independent processes, we speculate that it might interact with a range of proteins, in addition to Stau, to effect not only RNA localization but also protein localization and mitotic spindle orientation. We therefore view Insc as a protein that nucleates the formation of an RNA/protein complex that plays a role in a variety of processes including that of providing a cortical centrosome attachment site (see Kraut et al., 1996). In this scenario, the loss of certain components from this complex may disrupt only a subset of the functions that require *insc*. For example, the absence of Stau from this postulated multifunctional complex affects only the localization of *pros* RNA; the basal cortical localization of Pros protein during mitosis and the apical/basal orientation of the mitotic spindle remain normal. With respect to *pros* RNA localization, the role of Insc may be to facilitate Stau protein/*pros* RNA interaction with perhaps other components necessary for the transport of the *pros* RNA.

Experimental Procedures

Flies, Immunocytochemistry, and Confocal Microscopy

Embryos derived from wild type (Canton-S), null alleles of *insc* (*insc*^{P72} or *insc*^{P49}), and *stau* (*w; cn stau*^{D3} *sp*) were fixed and stained with anti-Insc and anti-Pros antibodies as previously described (Kraut et al., 1996). A "blue balancer," *CyO P*[w^+, *ftz-LacZ*], was used to facilitate identification of homozygous mutant embryos. Cy3-conjugated and FITC-conjugated antibodies (Jackson Immunoresearch) were used as secondary antibodies.

An anti-Stau rabbit serum made against a full-length Stau fusion protein (a gift from D. St. Johnston) was used at ~1:3000 dilution. A second anti-Stau antibody was generated by immunizing rabbits with a GST-Stau fusion protein containing the C-terminal portion (aa 769–1026) of Stau and used at 1:1000 dilution. The specificity of both serums was verified by lack of staining in *stau*^{D3} null embryos. Identical results were obtained with both serums.

RNA in situ localization experiments were performed essentially as described by Tautz and Pfeifle (1989), except that digoxygenin-labeled (Boehringer Mannheim) anti-sense and sense single-stranded RNA probes were generated using runoff-transcription reactions with T7 RNA polymerase. Hybridizations were carried out at 56°C overnight. Mouse anti-digoxygenin antibody (1:600 dilution, Boehringer Mannheim) was incubated with the hybridized embryos for 1 hr, and Cy3-conjugated anti-mouse antibody (1:300, Jackson Immunoresearch) was used to detect the signal. Stained embryos were mounted in DNA staining medium (Lundell and Hirsh, 1994), and images were collected and processed as previously described (Kraut et al., 1996).

Plasmid Construction

Vectors for constructing Insc baits and Stau preys were pEG202 and JG4-5, respectively (gifts from E. Golemis). pEG202 was modified by inserting oligonucleotide sequences encoding MYC epitope (5′-GGC CGCAGCTCGAGCAGAAGCTGATCAGCGA-GGAAGACCTG-3′ and 5′-TCGACAGGTCTTCCTCGCTGATCAGCTTCTGCT-CGAGCTGC-3′) into NotI–SalI sites to create pEG202 MYC. Corresponding domains of Insc were PCR amplified by specific oligonucleotides and subcloned into the BamHI–XhoI sites of pEG202MYC. The primers used to amplify YI-1 were inscup-2 (5′-GAAGATCTCGCCGTA-CAACAAT GAAACTGT-3′) and inscdown-3 (5′-ACGCGTCGACGAAACTCTCCT GACG-TGTTAA-3′); for YI-2, inscup2 and inscdown2 (5′-GAAGAT CTCGCCGTACAACAAT-GAAAACTGT-3′); for YI-3, inscup3 (5′-GAA GATCTTGGAATCCGCCGGCGAGAG-3′) and inscdown3; for YI-4, inscup3-1 (5′-GAGAAGATCTTTGATACCCTGGAG-CACCAG-3′) and inscdown3; for YI-5, inscup3-2 (5′-GAGAAGATCTCAGAGCTCCT GGAGCAGTGG-3′) and inscdown-3; for YI-6, inscup3 and inscdown3-1 (5′-ACCGCT-CGAGAATCAGCCGGAAGATGGC-3′). PCR-

amplified products for YI-3, YI-4, YI-5, and YI-6 were also subcloned into BamHI–XhoI sites of pGEX-5X-3 (Pharmacia) to generate In-1, In-3, In-4, and In-5 Insc-GST fusion constructs, respectively. PCR primers inscup-1 (5′-GAAGA-TCTCCTTTCAGCGCAGCTACAG-3′) and inscdown-1 (5′-CCGCTCGAGGGTGGCTCC-AAAAGTCACC-3′) were used to generate In-2.

Domains of Stau were PCR amplified and subcloned into EcoRI and XhoI sites of JG4-5 vector. The primers used to amplify YS-1 were stauup1 (5′-GGCCGAATTCCAGCACA-ACGTTCATGCC-3′) and staudown1 (5′-GGCCCTCGAGTCAATGCTTGTACA-TGGTTTC-3′); for YS-2, stauup2S (5′-GGCCGTCGACCCGCCGCCGAAGATTCGC-3′) and staudown2S (5′-GGCCGTCGACTCATGCCAATTTCTTGCTGTT-3′); for YS-4, Bco-1 (5′-CCAGCCTCTTGCTGAGTGGAGATG-3′) and stauup3′-1 (5′-TCCGCTCGAGTCACTCTTTGGATTCCACGT-3′); for YS-5, BcoI and stauup3′-2 (5′-TCCGCTCGAGTCACTCCGTGTTGTTTTTACT-3′); for YS-6, Bco2 (5′-GACAAGCCGACAACCTTGATTGGA-3′) and St3′-5 (GATCGAATTCAGTAGCAG-CAACAGTACGAGT-3′). PCR-amplified products for YS-1, YS-2, and YS-3 were also used to generate S-1, S-2, and S-8. Primers used to amplify S-3 were stauup2S and stR3-3 (5′-GGAAGTCGACACTCACTTGGTGGGCGTAAGGGG-3′); for S-4, stR3-2 (5′-GGAAGAATTCCA-GGTTCACGAGATCGGA-3′) and stR3-4 (5′-GGAAGTCGACACTCACATCTTTTCCGCGGCACG-3′); for S-5, stR3-1 (5′-GGAAGAATTCTCTGCCTCTGAGGAGGCT-3′) and stR3-4; for S-6, stR3-2 and stR-–3; for S-7, stR3-1 and stR3-3. All PCR-amplified fragments were subcloned into EcoRI and XhoI sites of the pGEX 4T-1 (Pharmacia) vector, except for YS-2, which was subcloned into the XhoI site of the vector.

Yeast Two-Hybrid Screening

Primary Drosophila embryonic activation–tagged cDNA library, LacZ reporter pSH18-34, yeast strain EGY48, and repression assay vector JK101 were generous gifts from Russ Finley and Roger Brent (Massachusetts General Hospital, Harvard Medical Center, Boston, MA). mRNA for the library was extracted from collection of 0–4, 4–8, and 8–12 hr embryos. The procedures for library amplification, yeast culture, yeast transformation, positive clone identification, plasmid rescue, and β-galactosidase assay were described by Finley and Brent (1994).

In Vitro Insc/Stau Protein Interaction Assay

GST protein production and purification were conducted according to Ausubel et al. (1987–1996). Beads containing about 4 μg of GST-Insc fusion proteins were used in each binding assay. ^{35}S-labeled full-length Stau was generated by using TNT in vitro transcription and translation kit (Promega) and T7 polymerase from a *stau* "full-length" cDNA clone E10 (from D. St. Johnston). Lysate (5 μl) containing ^{35}S-labeled Stau was used to incubate with beads containing GST-Insc fusion proteins in PBS for one hour and washed five times with PBS+1% Triton. Proteins bound to beads were eluted in 2× SDS buffer, separated on SDS–PAGE gels, and autoradiographed.

RNA–Protein Interaction

The in vitro RNA–protein binding assay was performed essentially as described by St. Johnston et al. (1992). GST-Stau fusion proteins were generated as described above. ^{32}P-labeled *pros* cDNA 3′UTR was generated by using [^{32}P]UTP and in vitro transcription kit from Promega. The amount of probe used in each binding reaction was 1×10^6 cpm/ml.

Western Blot Analysis

Yeast strains that contain the corresponding fusion proteins for either Insc or Stau were cultured to $OD_{600} = 1$, and cells were lysed by freezing in liquid N_2 for 5 min in SDS loading buffer. Proteins were separated on SDS–PAGE gel, transferred to nitrocellulose filter, and blocked with 3% BSA in PBS+1% Triton overnight. Monoclonal antibodies against MYC and HA were used, respectively, and positive signals were detected by ECL (Amersham).

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