Abs is Required for Maintenance of Insc Protein Levels, Basal Localization of Cell Fate Determinants, and Spindle Orientation in Mitotic Neuroblasts

Mitotic neuroblasts form an apical cortical protein complex containing Bazooka (the Drosophila homolog of nematode and mammalian Par-3), Par-6, atypical Protein Kinase C, Inscuteable (Insc), Partner-of-Inscuteable, and Gαi proteins [20–24]. These apical proteins have three functions: to promote basal cell fate determinant localization, to orient the mitotic spindle along the apical/basal axis, and to promote the formation of an asymmetric spindle leading to the generation of daughters of unequal size [25]. The basally localized determinants include Miranda (Mir) and Numb (Nb), which were used as markers in this study. Their basolocalization ensures their preferential segregation into the basal daughter cell, called ganglion mother cell (GMC), during neuroblast division and ensures proper GMC fate specification [6, 14–16, 26–28].

To assay abs function, we used a temperature-sensitive allele in combination with a small deficiency uncovering the abs locus (abs14B/Dr(3R)231-5, hereafter referred to as abs140 embryos) in which the maternally contributed Abs protein can be inactivated by a shift to the restrictive temperature [17]. Wild-type embryos at the restrictive temperature and abs140 embryos at the permissive temperature showed normal apical (Insc) and basal (Mir) cortical protein crescents in mitotic neuroblasts, as well as normal apicobasal orientation of the mitotic spindle (Figures 1A and 1B). In contrast, abs140 embryos that are shifted to the restrictive temperature display severe defects in neuroblast polarity: Mir frequently shows uniform cortical distribution or occasionally accumulates as mispositioned lateral crescents (Figures 1C and 1D). Furthermore, mitotic spindles occasionally fail to orient along the apical-basal axis (Figure 1C, insets). The similarity of these phenotypes and those that were previously reported for mutations affecting components of the Insc complex [28, 29] prompted us to assay Insc protein localization in the abs mutants. Interestingly, Insc protein is not detectable above background levels at the restrictive temperature in abs140 embryos.
The ABS Dead Box Protein Regulates Cell Asymmetry

Figure 1. abstrakt Regulates Insc Levels, Basal Protein Localization, and Spindle Orientation in Neuroblasts

Insc (top row) and Mir (middle row) protein localization in wild-type embryos (A) and abs14B embryos (B and C). α-Tubulin was used for identifying metaphase neuroblasts and assaying spindle orientation. Arrowheads indicate the apical side of neuroblasts; in all panels (including insets) apical is up and basal is down. Wild-type (wt) embryos at the restrictive temperature (A) and abs14B embryos at the permissive temperature (B) show normal apical (Insc) crescents and basal (Mir) cortical protein crescents in mitotic neuroblasts, as well as normal apicobasal orientation of the mitotic spindle. In contrast, abs14B embryos at the restrictive temperature (C) show no detectable Insc protein, misaligned spindles, and delocalized Mir. We noticed that Mir occasionally showed elevated levels in the cytoplasm in mitotic neuroblasts of both abs14B and wild-type control embryos at the restrictive temperature; this is not an abs-specific phenotype. Panel (D) shows the quantitation of the Mir localization phenotype in metaphase neuroblasts of embryos displayed in panels (A)–(C).

mutant neuroblasts (Figure 1C), although apical Insc localization is not affected in abs14B embryos at the permissive temperature or in wild-type embryos at the restrictive temperature (Figures 1A and 1B). We conclude that loss of abs function leads to the loss of detectable Insc protein in neuroblasts and generates the phenotype previously seen in insc mutants. The simplest interpretation is that Insc expression and/or Insc protein stability is impaired in abs mutants, which leads to the observed defects in neuroblast asymmetric cell division.

Abs Is Required during Asymmetric GMC4-2a Division and for Proper Resolution of Sibling Cell Fates in the CNS and Mesoderm

To elucidate the origin of the duplicated RP2 neurons, we used anti-Eve staining to follow the development of the GMC4-2a lineage in wild-type (Figure 2I) and abs14B (Figure 2J) embryos. Our results indicate that the extra RP2 neuron arises as the result of a transformation of the RPS2sib to the RP2 cell fate. Pon directly binds Nb protein and reflects the localization of Nb in all cells analyzed so far [34]. In control abs24:14B and in wild-type embryos shifted to the restrictive temperature (33°C), Pon localizes as a basal crescent in mitotic GMC4-2a cells (19/19; Figure 2I) and abs14B (Figure 2J) embryos. The absence of Pon crescents (data not shown).

position in wild-type embryos (Figure 2A). However, in abs14B embryos shifted to the restrictive temperature prior to GMC4-2a division, approximately 32% of the hemisegments had a duplicated Eve- cell at the RP2 position (Figure 2B). This phenotype was rarely seen either in control embryos (from a stock homozygous for abs14B along with two copies of a functional abs14B+ transgene, henceforth referred to as abs24:14B, that rescues the abs lethality [17]) subjected to the same temperature-shift regime (Figure 2K) or in abs14B embryos at the permissive temperature (not shown). The duplicated Eve+ cells are likely to be duplicated RP2 neurons because they express two additional markers (22C10 [32] and Zfh1 [33]) for mature RP2 neurons (Figures 2C–2F).
Figure 2. abstract

Is Required for Establishing Distinct Sibling Cell Fates in the GMC4-2a Lineage

(A and B) In control abs^{24:14B} and wild-type embryos, there is only one Eve-positive RP2 neuron per hemisegment [A, arrows], whereas in abs^{14B} embryos at the nonpermissive temperature, about 32% of the total hemisegments contain two Eve-positive RP2 neurons [B, arrows]. Images are taken from stage 15 embryos. Anterior is left.

(C–F) Duplicated RP2 neurons arise as the result of an RP2sib-to-RP2 cell fate transformation. Images are of one hemisegment from stage 15 embryos. Embryos were double-labeled either with anti-Eve (red) plus anti-Zfh1 (green) (C and D) or with anti-Eve (red) plus mab22C10 (green) (E and F). The duplicated RP2 neurons in abs^{14B} embryos are Eve- (D and F), 22C10- (E and F), and Zfh1- (C and D) positive like wild-type RP2 neurons.

(G and H) The muscle DA1, which is Eve-positive, is duplicated in abs^{14B} embryos at the nonpermissive temperature (H, outlined with white lines) as compared to the wild-type and control embryos (G, outlined with white lines).

(I and J) A series of panels, each showing a single hemisegment, depicts the temporal profiles of Eve expression in control (abs^{24:14B}) (I) and in abs^{14B} (J) embryos exposed to a nonpermissive (33°C) temperature shift. (I) In control embryos, Eve-positive GMC4-2a divides and generates two Eve-positive daughter cells with distinct nuclear sizes; RP2 has the larger nucleus. The Eve expression in RP2sib rapidly disappears, and only RP2 maintains strong Eve expression in stage14 and later embryos. (J) In abs^{14B} embryos, GMC4-2a produces two Eve-positive cells with equal nuclear size as judged by Eve staining; both daughters retain Eve expression and become mature RP2 neurons. A schematic representation of the temporal profiles of Eve-expression is shown below the images. Anterior is up; the midline is toward the left.

(K) Quantitations of the phenotypes.

Hence, the symmetric segregation of Numb to both daughter cells in a proportion of the dividing GMC4-2a cells could account for the RP2 duplication phenotype seen in the abs^{14B} embryos.

Because the abs phenotype is similar to the insc phenotype in both neuroblasts and GMC4-2a, we also investigated Insc localization during the GMC4-2a cell division. In control embryos, Insc always forms an apical crescent (16/16) in metaphase GMC4-2a cells (Figure 3E). In contrast, at the restrictive temperature, the majority of the abs^{14B} mutant GMC4-2a cells (14/17) show no clear apical crescents of Insc (Figure 3F). Consistent with the finding that Insc localization is affected in abs^{14B}, the duplicated RP2 cells seen at the restrictive temperature (Figure 2) exhibit equal nuclear size, as is also seen in insc embryos but not in mutants that disrupt sibling cell fate choice at the postmitotic level [9].

We additionally investigated the role of abs during embryonic muscle progenitor divisions. The muscle progenitor P15 divides asymmetrically to produce two daughter cells with distinct identities [7, 8]. Nb is asymmetrically localized in the dividing P15 and preferentially segregates to the daughter cell that will become the founder for the single Eve-positive muscle DA1; the sibling cell is Eve-negative [35]. abs^{14B} embryos subjected to a 45 min pulse at the restrictive temperature showed duplications of the Eve-positive DA1 in 34% (23/68) of the hemisegments (Figure 2H). In the control abs^{24:14B} embryos, 135/136 of the hemisegments showed a clear apical crescents of Insc (Figure 3F). Consistent with the finding that Insc localization is affected in abs^{14B}, the duplicated RP2 cells seen at the restrictive temperature (Figure 2) exhibit equal nuclear size, as is also seen in insc embryos but not in mutants that disrupt sibling cell fate choice at the postmitotic level [9].

Abs Associates with Insc RNA and Is Required for Maintenance of Insc Protein Levels but not Insc RNA Levels

The abs and insc mutant phenotypes in asymmetrically dividing cells are very similar, and abs mutants show a loss of Ins protein crescents in neuroblasts (Figure 1C),
in GMCs (Figure 3F), and throughout the embryo (Figure 1C). Thus, the abs phenotype can be most simply modeled as a defect in establishing or maintaining normal levels of apical Insc protein in all of these cell types. The loss of Insc crescents could be caused either by an overall decrease in the levels of Insc or by a failure to localize Insc correctly in these cells. In situ hybridization experiments revealed no reduction in insc RNA expression, so abs does not appear to regulate insc at the transcriptional level (not shown). We used Western blots to test whether the total amount of Insc protein was affected in abs mutant embryos. The Insc protein migrates as an approximately 100 kDa band (Figure 4A; [15]). Wild-type and abs14B embryos were shifted to the restrictive temperature and analyzed after 0, 30, and 60 min. The levels of Insc protein decreased progressively in abs14B embryos until they were nearly undetectable at 60 min, whereas they remained constant or even increased (depending on the age distribution of embryos at the beginning of the experiment) in wild-type embryos (Figure 4B). Other proteins remain constant, and several proteins can be translated de novo at the restrictive temperature, indicating that abs function is not generally required for protein synthesis [17]. Together, these data indicate that the most upstream defect associated with a reduction in abs function is a reduction in the levels of the Insc protein.

If Abs indeed acts on asymmetric cell divisions by maintaining high levels of Insc, it should be possible to circumvent the requirement for Abs at least in part by raising Insc levels experimentally. To test this, we used the GAL4-UAS system to express high levels of insc within neuroblasts in embryos lacking functional Abs protein. This led to a marked rescue of the RP2 phenotype (fewer duplications; Figure 4C).

Because Abs is a DEAD-box protein, it seemed conceivable that it might exert its effect on InsC protein levels by a direct interaction with insc RNA. We used a yeast-three hybrid assay to test this. The assay is based on the interaction of the HIV-1 RNA binding protein Rev with RNA molecules containing a Rev responsive element (RRE) [36]. Rev is fused to the GAL4 DNA binding domain, whereas the putative RNA binding protein, in this case Abs, is fused to the activation domain. The two fusion proteins are then bridged by a hybrid RNA consisting of an RRE-containing sequence fused to the RNA to be tested, in this case insc RNA. We find that insc RNA is clearly able to interact with Abs in this system (Figure 5). Both the full-length RNA and a construct lacking the 5’ third of the RNA show an interaction. However, we found that no single fragment of the 3’ part of the RNA was able to interact with Abs. Control RNAs (e.g., bicoid, crb, and sog) did not interact with Abs in this assay (Figure 5 and U.I., unpublished data).

**Conclusions**

DEAD-box proteins have been implicated in many aspects of RNA metabolism, including ribosome biogenesis, pre-mRNA splicing, nuclear export, and translational regulation [18, 19]. Here we demonstrate that the DEAD-box protein Abs directly binds Insc mRNA in vitro, that loss of Abs leads to lowered Insc protein levels but not...
Western Blots

Flies were heat shocked at 37°C for 1 hr, left to recover at room temperature for 30 min, and lysed in SDS sample buffer (20 flies per 0.2 ml). Embryos from staged collections (3–10 hr after egg laying) were transferred to test tubes in groups of 100, shifted to the restrictive temperature (32°C) for the indicated time, and lysed in 0.2 ml SDS sample buffer. The lysates were boiled for 5 min, and the proteins were separated on an SDS PAGE (equivalent of two flies or 20 embryos per lane). The gels were blotted onto nitrocellulose membranes, which were then stained with anti-Inscutableable and HRP-labeled goat anti-rabbit and developed with the ECL kit (Amer-sham) for detection.

Yeast Three-Hybrid Assay

For the detection of protein-RNA interactions, a three-hybrid system based on the interaction of HIV-1 Rev protein with RNA molecules containing a Rev responsive element (RRE) was used [36]. For the creation of hybrid RNAs with an RRE followed by different parts of the insc transcript, full-length insc and several smaller fragments (see Figure 5) were cloned into the Smal site of pRevRX [37] via BamHI (blunted), SphI (blunted), or both Dral and Clal (blunted). pRevRX is a modified version of pDBRevM10 that allows the expression of hybrid RNA and a fusion protein between the DNA binding domain of GAL4 and RevM10, a mutated version of the HIV-1 Rev protein, with specific primers to incorporate an EcoRI and a SalI site and subsequent cloning of the fragment into...
pGAD-C2 [38]. Both plasmids were transformed into the yeast strain PJ69-4a, and growing the transformants on plates lacking tryptophane, leucine, histidine, and adenine tested the interaction.

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