Abstrakt, a DEAD Box Protein, Regulates Insc Levels and Asymmetric Division of Neural and Mesodermal Progenitors

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Summary

Asymmetric cell division generates cell diversity in bacteria, yeast, and higher eukaryotes [1–3]. In *Drosophila*, both neural and muscle progenitors divide asymmetrically [4–16]. In these cells the Inscuteable (Insc) protein complex coordinates cell polarity and spindle orientation. Abstrakt (Abs) is a DEAD-box protein that regulates aspects of cell polarity in oocytes and embryos [17]. We use a conditional allele of *abs* to investigate its role in neural and muscle progenitor cell polarity. In neuroblasts we observe loss of apical Insc crescents, failure in basal protein targeting, and defects in spindle orientation. In the GMC4-2a cell we observe loss of apical Insc crescents, defects in basal protein targeting, and equalization of sibling neuron fates; muscle precursors show a similar equalization of sibling cell fates. These phenotypes resemble those of *insc* mutants; indeed, *abs* mutants show a striking loss of Insc protein levels but no change of *insc* RNA levels. Furthermore, we find that the Abs protein physically interacts with *insc* RNA. Our results demonstrate a novel role for Abs in the posttranscriptional regulation of *insc* expression, which is essential for proper cell polarity, spindle orientation, and the establishment of distinct sibling cell fates within embryonic neural and muscle progenitors.

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Results and Discussion

Abstrakt (Abs) is a member of a family of RNA-dependent ATPases called DEAD-box proteins; other family members regulate various aspects of RNA metabolism, including pre-mRNA splicing, RNA transport, and translation [18, 19]. Previous work has demonstrated that *abs* function is required for certain aspects of oocyte and epithelial polarity [17]; however, the mechanism of *abs*-dependent regulation of polarity in these systems remained elusive. Furthermore, it was unclear whether *abs* was also involved in regulating cell polarity in other developmental contexts. Here we investigate the function of *abs* in regulating Insc levels and asymmetric cell division in neural and muscle precursors.

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 basal localization 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 (*abs*[14B]/*Df(3R)231-5*, hereafter referred to as *abs*[14B] 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 *abs*[14B] 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, *abs*[14B] 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 *abs*[14B]
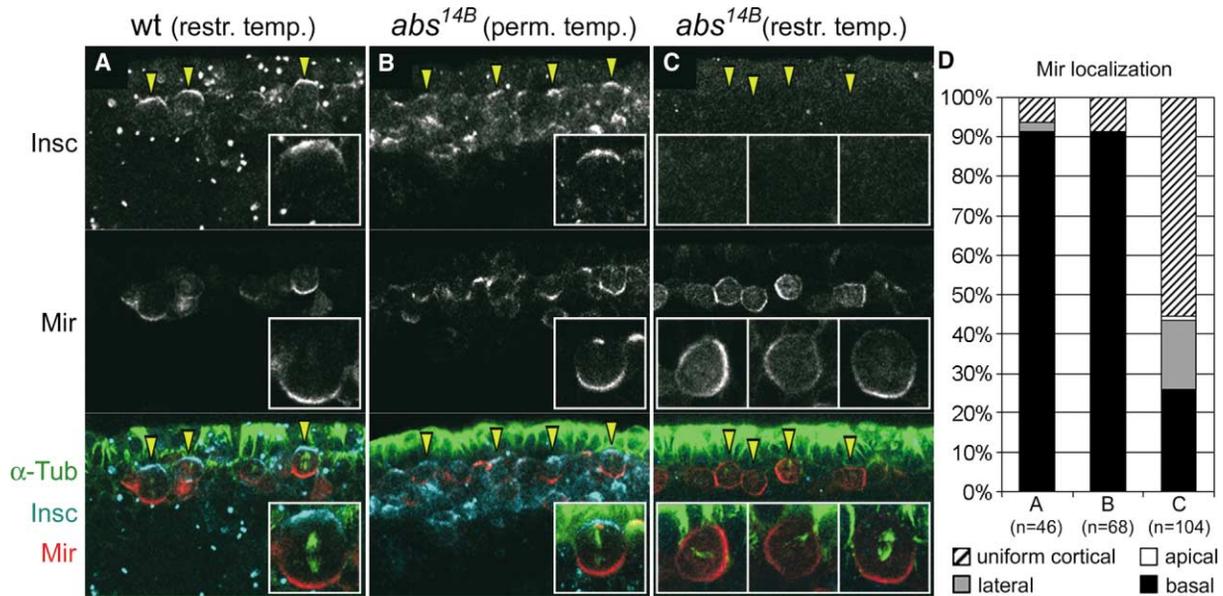
Figure 1. *abstrakt* Regulates Insc Levels, Basal Protein Localization, and Spindle Orientation in Neuroblasts

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 determine if *abs* has a more general role in regulating Insc levels and asymmetric cell division, we examined asymmetric division of the ganglion mother cell GMC4-2a, which produces a pair of identified sibling neurons, RP2 and RP2sib [30]. During wild-type GMC4-2a divisions, the mitotic spindle is apicobasally oriented; Insc is localized to the apical cortex, whereas Nb is localized as a basal cortical crescent and segregates preferentially into the more basal daughter cell, where it acts to downregulate Notch (N) signaling and induce the RP2 cell fate. The RP2 sibling cell does not inherit Nb, cannot downregulate N signaling, and adopts the secondary RP2sib fate. GMC4-2a and RP2 express the Even-skipped (Eve) transcription factor, but RP2sib does not [30, 31]; thus, there is only one Eve+ cell at the RP2

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 *abs+* 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).

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 RP2sib 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 (19/19; Figure 3A). In *abs14B* embryos subjected to the same temperature shift regime, approximately 50% (18/34) of metaphase GMC4-2a cells show cortical distribution (Figure 3B), misplaced crescents (Figure 3C), or weak basal crescents (Figure 3D) of Pon, and approximately 25% (9/34) of the cells show no obvious Pon crescents (data not shown).
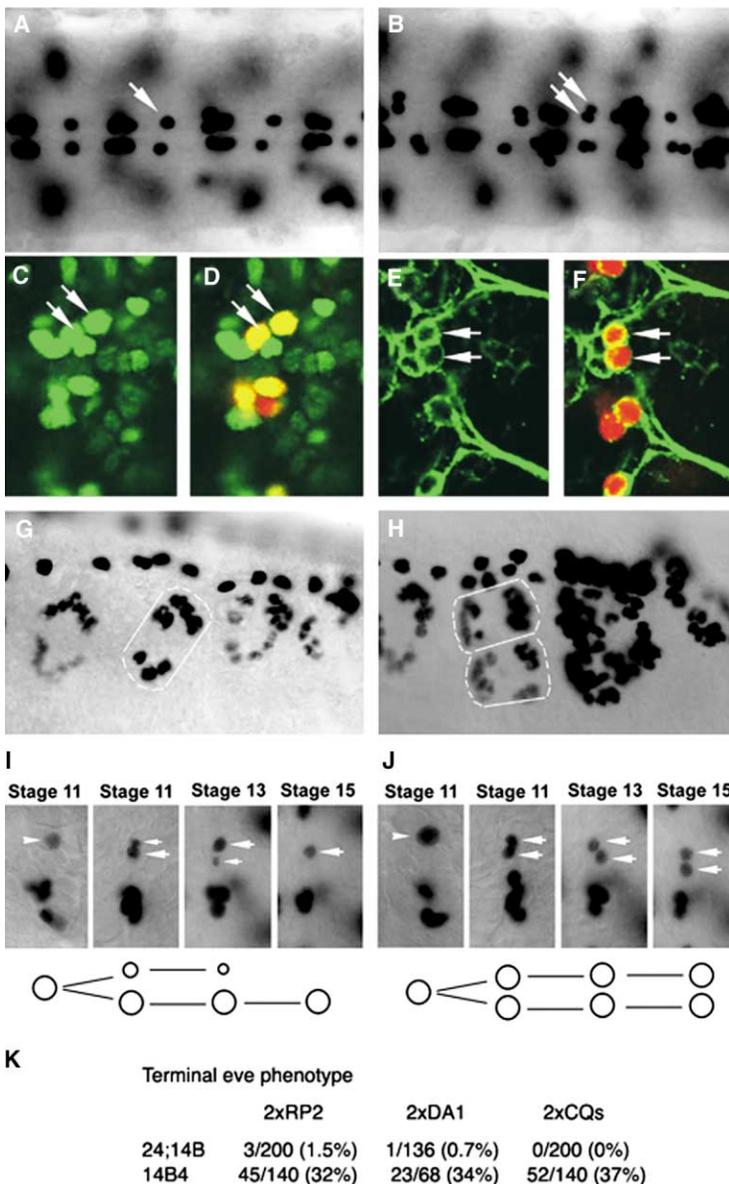
Figure 2. *abstrakt* Is Required for Establishing Distinct Sibling Cell Fates in the GMC4-2a Lineage

(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 GMC-4-2a 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 single Eve-positive DA1 (Figure 2G). Thus, *abs* is also required for the asymmetric division of some muscle progenitors.

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 Insc protein crescents in neuroblasts (Figure 1C),
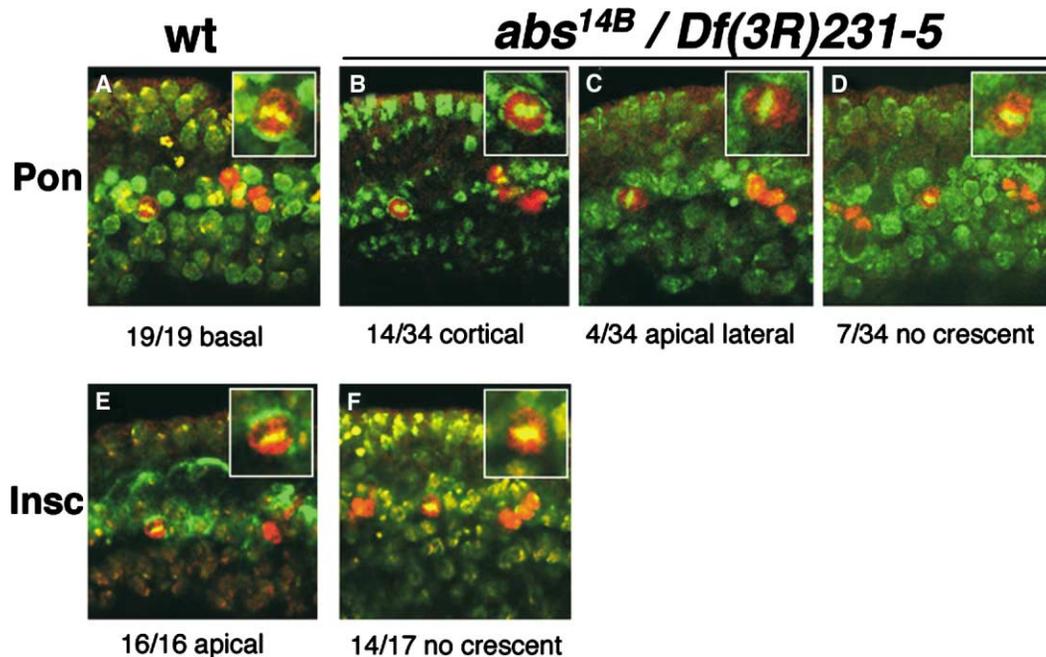
Figure 3. *abstrakt* Is Required for Asymmetric Protein Localization in GMC4-2a

Triple-labeled confocal images showing Insc (green) and Pon (green) localization in dividing GMC4-2a (stained for Eve in red and DNA in yellow). In $abs^{24:14B}$ and wild-type embryos, Pon forms a basal crescent in dividing GMC4-2a (A). In abs^{14B} embryos, Pon is often mislocalized (C), cortical (B), or not detectable (D) in dividing GMC4-2a. Insc is apically localized in GMC4-2a in control embryos (E), whereas in abs^{14B} embryos, the majority of mitotic GMC4-2a lack a clear Insc apical crescent (F). Anterior is toward the left; apical is toward the bottom. The inserts are enlarged images of GMC4-2a in the corresponding panels.

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 abs^{14B} embryos were shifted to the restrictive temperature and analyzed after 0, 30, and 60 min. The levels of Insc protein decreased progressively in abs^{14B} 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
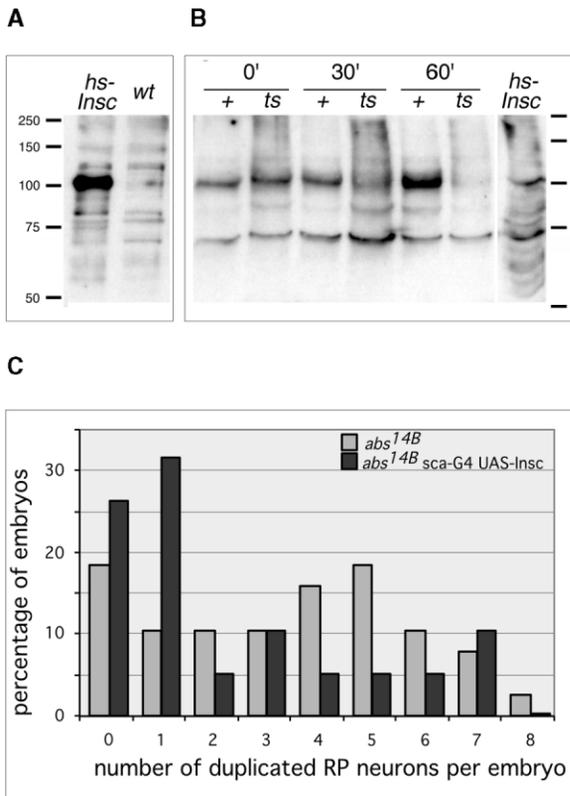
A

B

C

Figure 4. Maintenance of Inscuteable Protein in abs^{14B} Embryos

(A and B) Western blots of lysates of embryos and adult flies stained with antibodies against Insc.

(A) Specificity of the Insc antibody. Extracts of heat-shocked wild-type flies and flies carrying an *insc* transgene under heat-shock control. The hs-Insc flies show a strong band of the expected size in addition to the background bands seen in both wild-type and hs-Insc flies.

(B) Extracts of wild-type and abs^{14B} embryos that had been shifted to the restrictive temperature for the times indicated above the lanes. The embryos were between 3 and 10 hr old at the beginning of the experiment. The Insc band disappears in abs^{14B} but not in wild-type embryos after 60 min at the restrictive temperature. Background bands and other proteins tested were not affected (not shown). Extract of adult heat-shocked hs-Insc flies was run on the same gel as a control. The heat-shock induction of Insc was weaker in this case than that shown in (B). Markers at the left and right correspond to 250, 150, 100, 75, and 50 kDa.

(C) Suppression of the abs^{14B} phenotype by overexpression of Insc. The frequency of RP2 neuron duplications was compared between heat-shocked embryos derived from $abs^{14B}/Df(3R)231-5$ parents and those derived from *sca-GAL4/+, abs^{14B}UAS-Insc/Df(3R)231-5* parents. Both groups of embryos lack functional Abs protein. In the second case, 9/16 of the embryos carry both the sca-GAL4 driver construct and the UAS-Insc construct and thus express high levels of Insc. The number of hemisegments with duplicated RP2 neurons was counted for embryos of each cross (abs^{14B}: 38 embryos. abs^{14B} sca-GAL4, UAS-Insc: 19 embryos), and the percentages of embryos with the indicated numbers of duplications were plotted. The frequency of duplications in abs^{14B} embryos is drastically reduced by the expression of high levels of Insc. The group of embryos from the abs^{14B} sca-GAL4, UAS-Insc cross that show three or more duplications (42% of all embryos) most likely include mainly those embryos that do not carry both transgenes (expected 44%).

lowered mRNA levels, and that loss of Abs leads to a failure to properly localized cell fate determinants in at least three asymmetrically dividing cell types: neuro-

blasts, GMCs and muscle progenitors. We conclude that Abs has a role in controlling cell polarity and asymmetric cell division in multiple cell types, in part through the posttranscriptional regulation of Insc levels.

Experimental Procedures

Processing of Embryos and Immunocytochemistry

Embryos were fixed and processed for immuno-histochemical or -fluorescent labeling according to previously described methods [30], except that embryos labeled for α-Tubulin were fixed in 9% formaldehyde, 100 mM PIPES (pH 6.9), 1 mM EGTA, and 2 mM MgCl$_2$ for 20 min. Staged embryos were labeled with the following primary antibodies: rabbit anti-Inscuteable (1:1000), rat anti-Miranda (raised against a peptide encompassing the N-terminal 21 amino acids MSFSKAKLKRFNDVDVAICGS, 1:1000), rabbit anti-Pon (1:1000) [34], rabbit anti-Even-skipped (1:2000, from Manfred Frasch), mouse anti-Even-skipped (mab2B8, 1:30, from Kai Zinn), anti-Zfh-1 (1:1000) [33], mouse monoclonal anti-α-Tubulin (Sigma T-9026, 1:5000), and mouse mab22C10 (1:5) [32]. For confocal imaging, appropriate fluorescently conjugated secondary antibodies (1:400; LRSC, FITC, Cy3 and Cy5 conjugates, Jackson Labs; Alexa 488 conjugates, Molecular Probes) were used. Anti-Eve staining was used for detection of RP2 and DA1.

For the analysis of GMCs, RP2, and DA1, embryos from various stocks were collected at 1 hr intervals and aged at 25°C for a defined period (6.5 hr for 24;14B and *yw* flies and 7.5 hr for abs^{14B} flies because the growth rate of the latter was slower). The aged embryos were shifted to 33°C for 45 min and incubated at 18°C in a moist chamber to allow further development. Embryos were processed when they reached stage14–15 to score for RP2 and DA1. For analysis of Insc and Pon localization in GMC4-2a, embryos were allowed to develop at 25°C for 1 hr after the nonpermissive temperature shift before fixation. Higher temperatures could not be used for these experiments because they disrupted the morphology of late embryos and prevented scoring of RP2 and DA1.

For the analysis of neuroblasts, staged collections (3–7 hr after egg laying) of embryos derived from $abs^{14B}/Df(3R)231-5$ flies were maintained at the permissive temperature (25°C) or were shifted to the restrictive temperature (here 37°C) for 2 hr prior to fixation and preparation for immunofluorescence. *yw* embryos were treated similarly and used as wild-type controls. Fluorescently labeled embryos were analyzed on a Biorad Radiance confocal microscope.

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-Inscuteable and HRP-labeled goat-anti-rabbit and developed with the ECL kit (Amersham) 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 SmaI site of pRevRX [37] via BamHI (blunted), SphI (blunted), or both DraI and ClaI (blunted). pRevRX is a modified version of pDBRevM10 that allows the expression of the hybrid RNA and a fusion protein between the DNA binding domain of GAL4 and RevM10, a mutated version of the HIV-1 Rev protein that is not exported from the nucleus. The other fusion protein with the GAL4 activation domain was obtained by PCR amplification of the *abs*-ORF with specific primers to incorporate an EcoRI and a SalI site and subsequent cloning of the fragment into
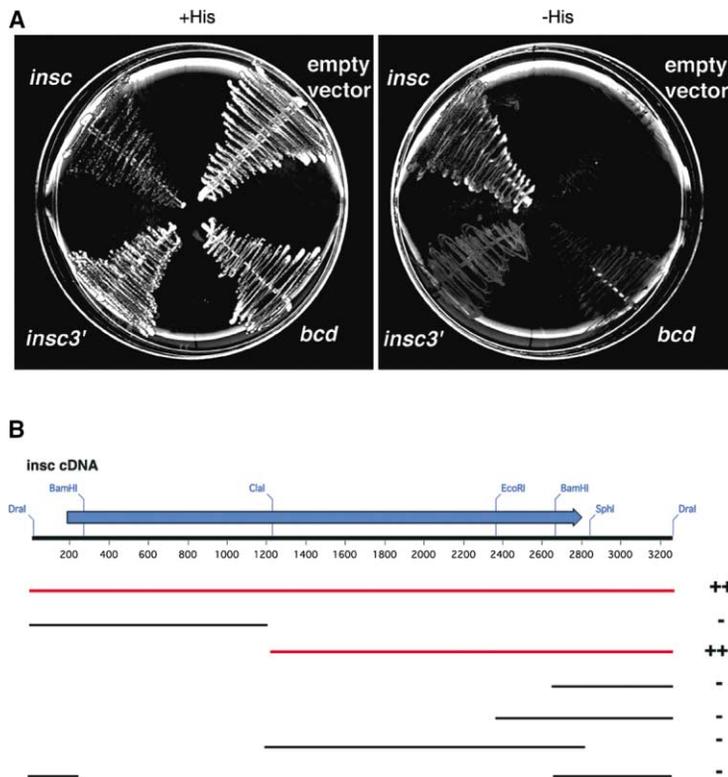
Figure 5. Abstrakt Interacts with Inscuteable RNA

(A) Yeast cells transformed with plasmids coding for the components of the three-hybrid system were plated on medium selecting only for the presence of the plasmids (left; medium minus tryptophane, and leucine) or for medium also selecting for an interaction between the encoded products (right; medium also lacking histidine). The RNA fusion constructs being tested contained full-length Insc RNA, the 3′ half of the Insc RNA, or Bicoid RNA fused to a Rev response element (RRE). As a negative control, the vector containing only the RRE (empty vector) was used. (B) Fragments of the Insc RNA that were tested in the three-hybrid assay for interaction with Abstrakt and results of the assay. Only the full-length RNA and the large fragment containing the 3′ two-thirds of the molecule were able to interact. Further subdivision of this fragment resulted only in fragments that were unable to bind to Abs.

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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