for our approach is found in tensile stress measurements of bulk materials, which are often made with the assumption of a constant cross-sectional area (*15*). We also ignored the possibility of any spatial gradients in π within the monolayer. Measurements of π were consistently taken from the center of the trough with the compression direction normal to the face of the Wilhelmy plate to ensure reproducibility.

25. Conventionally, the strain rate for the compression or expansion of a monolayer is given by $d(\ln A)/dt$, which is a true strain rate. When dealing with tensile stress-strain experiments conducted on bulk solids, however, the engineering strain $\varepsilon = \Delta L/L_0$ is customarily measured (ΔL, change in length; L_0, original length) (*15*). For such tensile deformation studies, it is necessary to maintain a constant geometry between experiments for the purposes of accurate comparison. Because we draw from the literature developed from the mechanical response of bulk materials to tensile deformation, we use an engineering strain and strain rate. To ensure the validity of comparison between experiments, we spread approximately equal amounts of amphiphilic material at the air-water interface for each experiment.
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Abnormal Spindle Protein, Asp, and the Integrity of Mitotic Centrosomal Microtubule Organizing Centers

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The product of the *abnormal spindle* (*asp*) gene was found to be an asymmetrically localized component of the centrosome during mitosis, required to focus the poles of the mitotic spindle in vivo. Removing Asp protein function from *Drosophila melanogaster* embryo extracts, either by mutation or immunodepletion, resulted in loss of their ability to restore microtubule-organizing center activity to salt-stripped centrosome preparations. This was corrected by addition of purified Asp protein. Thus, Asp appears to hold together the microtubule-nucleating γ-tubulin ring complexes that organize the mitotic centrosome.

The microtubule-nucleating capacity of the animal cell centrosome requires a ring-shaped complex of proteins associated with γ-tubulin present within the pericentriolar material (PCM) (*1–3*). The PCM contains lattice-like structures in which γ-tubulin has been found associated with pericentrin (*4, 5*). It is not known how the properties of the PCM might change during mitosis specifically to nucleate spindle microtubules.

Asp is a 220-kD microtubule-associated protein (MAP) found at the poles of mitotic spindles in the syncytial embryos of *Drosophila melanogaster* (*6*). It has consensus phosphorylation sites for $p34^{cdc2}$ and mitogen-activated protein kinases and putative binding domains for actin and calmodulin (*6*). Mutations in *asp* result in abnormal spindle morphology leading to mitotic arrest or to a high frequency of meiotic nondisjunction (*7, 8*). Because the mitotic defects of *asp* mutants are best studied in the larval central nervous system, we sought to examine its distribution more carefully in cells of whole-mount preparations of this tissue. We found that Asp became and remained associated with the centrosome throughout mitosis (Fig. 1, A through C). At telophase, it migrated to microtubules on the spindle side of both daughter nuclei (*9, 10*) and was not associated with the centrosome in interphase cells. At all mitotic stages from prophase to anaphase, the Asp protein was asymmetrically localized around the γ-tubulin in the PCM, where it appeared to

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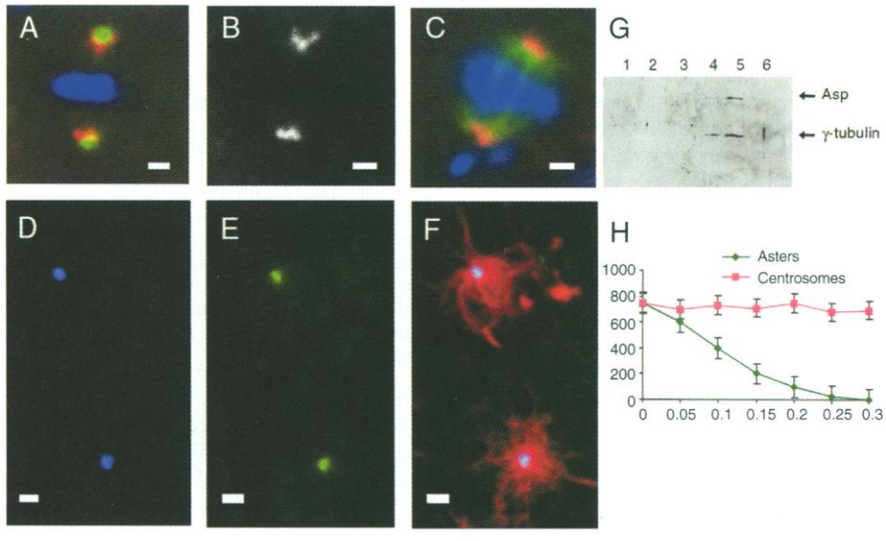


Fig. 1. Antibodies to Asp decorate the centrosome and can block microtubule nucleating activity in vitro. [(A) through (C)] show the immunolocalization of Asp in wild-type neuroblasts from *Drosophila* third-instar larvae. (**A**) Immunolocalization of γ-tubulin (green), Asp (red), and DNA (blue) at metaphase. (**B**) The same cell is shown as in (A) but only with Asp staining. (**C**) An early anaphase cell showing α-tubulin (green), Asp (red), and DNA (blue). (D) through (F) show preparations of centrosomes from *Drosophila* embryos. Shown are (**D**) γ-tubulin (blue), (**E**) Asp (green), and (**F**) a merge of the two previous images also showing the asters of microtubules obtained after incubation with rhodamine-labeled tubulin. (**G**) An immunoblot of the fractions from the final sucrose gradient centrifugation in the centrosome purification procedure (*13*). The 70% sucrose cushion was discarded, and the indicated fractions are the first 6 of a total of 26 in the remaining gradient. Asp and γ-tubulin cosediment in fractions 4 and 5 as indicated. Fraction 5 was used in the experiments here. (**H**) shows antibody competition assays in which centrosomes were first incubated with 0 to 0.3 μg/ml affinity-purified anti-Asp before being used in microtubule nucleation assays and finally being immunostained to reveal γ-tubulin. Preparations were scored for the total number of asters of microtubules and total centrosomes as indicated by foci of γ-tubulin. Scale bars, 10 μm.

form a hemispherical cup contacting the spindle microtubules (Fig. 1B).

In several *asp* mutations, Asp did not immunostain at the spindle poles (*11*). The majority of mitotically arrested *asp* cells had bipolar spindles with broad unfocused poles of microtubules (Fig. 2, A and B). In a small proportion of cells, one pole could be sufficiently disorganized so that the spindle appeared monopolar (*12*). The γ-tubulin was not present within a well-organized centrosome in these cells, but was found in dispersed clumps at the spindle poles (Fig. 2, C and D). This suggested that Asp might be required to maintain the structure of the centrosomal microtubule-organizing center (MTOC) during mitosis.

To confirm that Asp was a centrosomal protein, centrosomes were partially purified from syncytial *Drosophila* embryos undergoing their rapid nuclear division cycles (*13*). Immunoblotting experiments (*14*) indicated that this preparation was enriched in both Asp and γ-tubulin (Fig. 1G). Moreover, these two proteins colocalized by immunofluorescence at in vitro organizing centers for rhodamine-labeled microtubules (Fig. 1, D through F). In contrast to our observations in vivo, Asp was found in all of the in vitro MTOCs and was distributed symmetrically. First, this suggests that the extract was in a mitotic-like state, probably due to the dominant effect of the active mitotic protein kinase $p34^{cdc2}$. Second, it implies that the asymmetric localization seen in intact cells requires that microtubules make contact with chromosomes to form a spindle. If Asp localizes to the "outside" of the centrosome, we wondered whether antibodies to Asp might interfere sterically with microtubule nucleation in the in vitro assay. The centrosome preparation was therefore incubated with either affinity-purified anti-Asp or control rabbit immunoglobulins before addition of rhodamine-labeled tubulin (*15*). The number of asters of microtubules formed decreased in proportion to the concentration of antibody (Fig. 1H). However, the number of γ-tubulin–positive bodies remained constant, suggesting that the antibody was blocking microtubule nucleation rather than disrupting the centrosomes.

Extraction of centrosomes with KI effectively destroyed their microtubule-nucleating activity, leaving a centrosomal scaffold (Fig. 3C) (*16, 17*). Ability to nucleate microtubules can be restored to such KI-extracted centrosomes by incubation with the soluble fraction of a *Drosophila* embryonic extract (Fig. 3, D and E) (*17*). Although γ-tubulin and its ring complex (γTuRC) are required to rescue the aster-forming ability of KI-extracted centrosomes, they are not sufficient and have to be supplemented by a high molecular weight microtubule-associated factor speculated to be pericentrin (*17*). To test whether Asp protein might be required to reconstitute MTOCs from KI-extracted centrosomes, we immunodepleted Asp from a soluble embryonic extract under conditions where several other centrosomally associated proteins, including γ-tubulin, CP190, KLP61F, and Polo, were not removed (Fig. 3G) (*18*). This immunodepleted extract was unable to restore the ability of KI-extracted centrosomes to nucleate microtubules into asters. However, many linear arrays of microtubules were seen (Fig. 3F), suggesting that the Asp-depleted extract provided microtubule nucleation ability, but that it was not organized into discrete centers.

In contrast to the soluble extract of wild-type embryos, the equivalent soluble fraction prepared from *asp*-derived mutant embryos was unable to restore the ability of KI-extracted centrosomes to nucleate asters of microtubules (Fig. 4A). We then investigated whether addi-

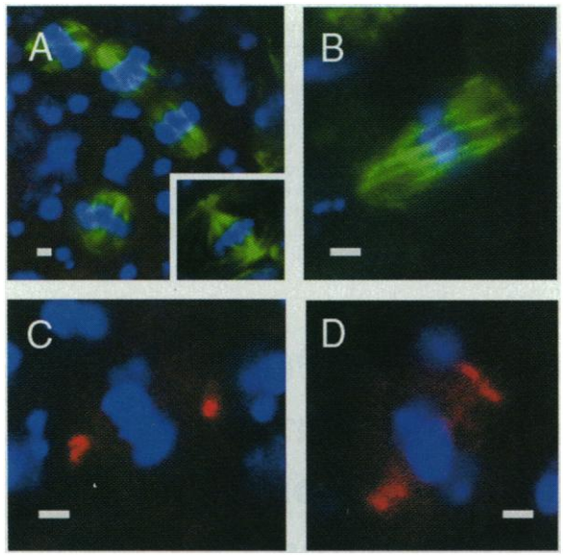
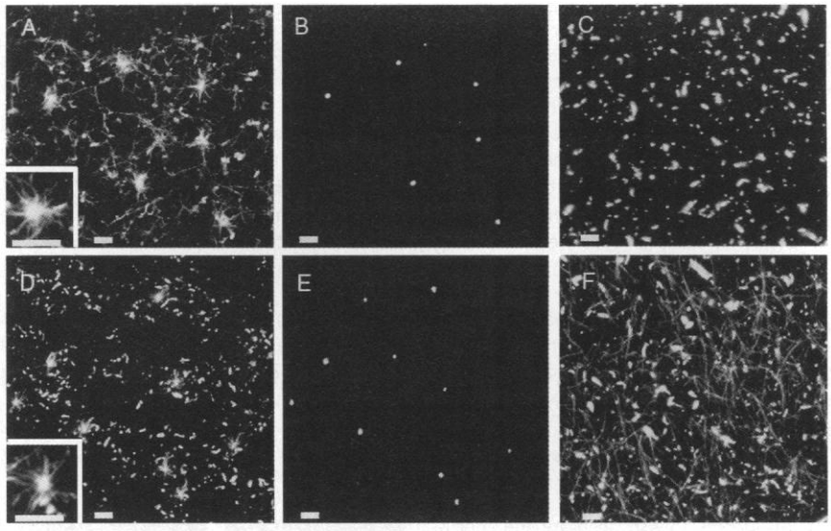


Fig. 2. Mitoses in *asp* mutants have unfocused spindle poles with disorganized γ-tubulin. (**A**) and (**B**) show metaphase-arrested cells in asp^{dd4} mutant (*23*) brains in which α-tubulin is stained green and DNA is stained blue. A wild-type spindle is shown in the inset of (A). (**C**) and (**D**) show γ-tubulin (red) and DNA (blue) in asp^{dd4} brains (compare with the wild-type cell in Fig. 1A). Scale bars, 10 μm.



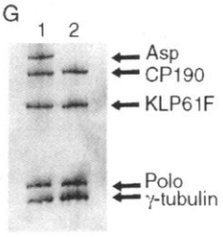


Fig. 3. Restoration of MTOC activity to salt-stripped centrosomes by soluble embryo extracts is prevented by immunodepletion of Asp. (**A**) Nucleation of rhodamine-labeled microtubules from partially purified centrosomes. The inset shows a single centrosome at higher magnification. (**B**) Same field as (A) immunostained to reveal γ-tubulin. Routinely, 80% of γ-tubulin–staining bodies were seen to nucleate asters of microtubules. (**C**) Microtubule nucleation after extraction of the centrosomes with 1 M KI. (**D**) Potassium iodide–extracted centrosomes incubated with soluble extract from wild-type *Drosophila* embryos before the microtubule nucleation assay. The inset shows a single centrosome at higher magnification. (**E**) Same field as (D) immunostained to reveal γ-tubulin. (**F**) Same field as (D), but using an extract immunodepleted of Asp. Scale bars, 10 μm. (**G**) Immunoblot of soluble embryo extract before (lane 1) and after (lane 2) immunodepletion of Asp. The blot was probed with antibodies to Asp, CP190, KLP61F, polo, and γ-tubulin as indicated.

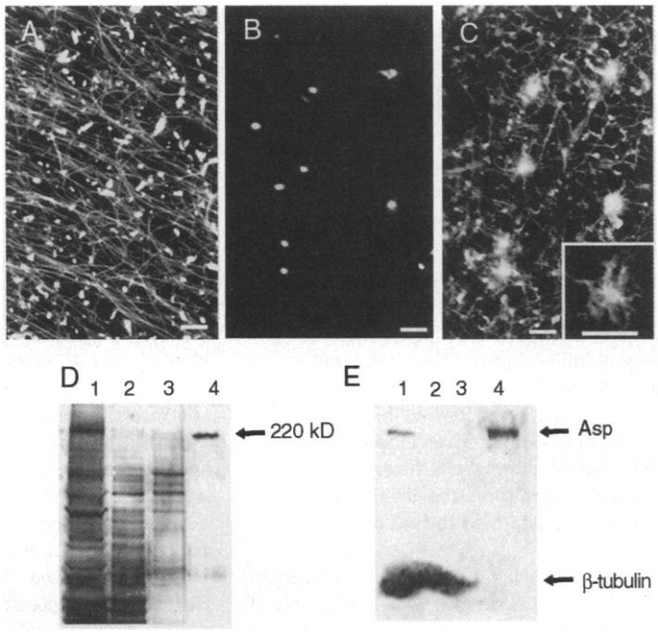


Fig. 4. A soluble extract from *asp*-derived embryos is unable to restore MTOC activity to salt-stripped centrosomes, but this ability can be rescued by purified Asp protein. (A) and (C) show the nucleation of rhodamine-labeled microtubules by KI-extracted centrosomes incubated with a soluble extract from a 3-hour collection of asp^{dd1}-derived (*23*) embryos in the absence (**A**) or presence (**C**) of purified Asp protein (estimated concentration of 5×10^{-4} pmol/μl). The inset in (C) shows an MTOC at higher magnification. (**B**) is the same field as (C) immunostained to reveal γ-tubulin. In this experiment, 70% of γ-tubulin–staining bodies were seen to nucleate asters of microtubules. Scale bars, 10 μm. [(D) and (E)] Purification of Asp. (**D**) Silver-stained SDS-PAGE gel and (**E**) the corresponding immunoblot probed with antibodies to Asp and β-tubulin. Lane 1, total extract; lanes 2 through 4, soluble fractions following sequential washes of GTP-taxol–pelleted mictotubules with 500 mM NaCl, 750 mM NaCl, and 1 M KI.

tion of purified Asp protein could restore this ability to the mutant embryo extract. Asp copurifies with microtubules from *Drosophila* embryos but is not released by concentrations of NaCl known to remove most MAPs (*8*). Thus, it seemed that if KI extracts Asp from the centrosomes, it probably would do so from such purified microtubules. We extracted NaCl-washed microtubule preparations with 2 M KI, and a 220-kD protein recognized by antibodies to Asp was found to be the main component of the resulting supernatant fraction (Fig. 4, D and E) (*19*). This purified Asp fraction was added to the soluble extract prepared from *asp*-derived embryos and found to restore the ability of KI-extracted centrosomes to nucleate asters of microtubules (Fig. 4, B and C).

Thus, it appears that both Asp and γTuRC are required to restore microtubule nucleating activity to centrosome scaffolds. Our data do not rule out the possibility that in addition to Asp, other high molecular weight proteins are required for this function, as previously suggested (*17*). Because Asp neither copurifies with the γTuRC nor coimmunoprecipitates with γ-tubulin (*20*), it is unlikely to have a direct role in the nucleation process. Rather, the consequences of loss of Asp function upon spindle poles in vivo and upon MTOCs in vitro suggest that it is required to organize the γTuRC within the PCM to form a nucleating center for microtubules at mitosis. In *asp* mutants, a spindle can still form, most likely reflecting the known ability of mitotic chromatin and motor proteins to organize a bipolar spindle in the absence of centrosomes (*21*, *22*). However, one consequence of the disorganized centrosomes and spindle poles is that the cells arrest in a metaphase-like state, suggesting that the spindle integrity checkpoint has been activated. Asp protein function is likely to be modified later in the mitotic cycle, since it was observed to associate with the microtubules of the telophase spindle, a property consistent with its purification as a MAP. However, the lack of any obvious association with microtubules during interphase suggests that its properties have to be modulated, possibly by phosphorylation, during entry into mitosis in order to activate its essential role in maintaining the coherence of the centrosome at the spindle poles.

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9. Immunostaining of *Drosophila* brains was carried out as previously described (*12*). DNA was stained with TOTO3 (Molecular Probes) according to the suppliers instructions. Mitotic spindles were visualized by incubation with anti–α-tubulin monoclonal antibody (clone YL1/2 from Harlan Sera Labs) diluted 1:10. We detected γ-tubulin, using the monoclonal antibody from clone GTU88 (Sigma) at a 1:10 dilution. Anti-Asp was polyclonal rabbit serum Rb3133 (*6*) diluted 1:50. Secondary antibodies were purchased from Jackson Immunochemicals and used according to the supplier's instructions. Preparations were visualized in a Bio-Rad 1024 confocal scanning head in conjunction with a Nikon Optiphot microscope.
10. The immunolocalization of Asp on the telophase spindle in cells of the larval brain is not shown. Asp is found in the region between the nuclei and the central spindle.
11. We have found Asp immunostaining to be lost at spindle poles in all mutant alleles that we examined. These are asp^{1}, asp^{dd1}, asp^{dd4}, asp^{dd7}, and asp^{dd8} (*23*).
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13. Centrosomes were purified from *Drosophila* embryos according to published protocols for the preparation of centrosomes from Chinese hamster ovary cells (*24*). The final centrifugation was through a 20 to 62.5% (w/w) sucrose gradient over a 70% sucrose cushion in a SW27 (Beckman) rotor for 90 min at 65,000*g* at 4°C. Microtubule nucleation assays, extraction with 1 M KI, and complementation assays were carried out as previously described (*17*). Tubulin and rhodamine-labeled tubulin used in the centrosome nucleation assays were purchased from Molecular Probes.
14. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels were blotted to polyvinylidene difluoride membranes (Millipore). The following antibodies were used: anti-Asp, Rb3133 (*6*); anti-KLP61F (*25*); anti-Polo, MA294 (*26*); anti–γ-tubulin, GTU88 (Sigma); and anti–β-tubulin, BX69 (*26*). All primary antibodies were diluted 1:500, with the exception of MA294 and BX69, which were diluted 1:4. Peroxidase-conjugated secondary antibodies were purchased from Jackson Immunochemicals and used according to the suppliers instructions. Bound antibodies were detected by chemiluminescence using chemicals from Amersham (ECL) or Pierce (Supersignal).
15. A number of control antibodies have been used, including one against the centrosomal component CP190. None of these would prevent microtubule nucleation by the centrosome preparation.
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18. Asp was immunodepleted from 100 μl of *Drosophila* embryo extract by incubation with affinity-purified anti-Asp (20 to 50 μg) coupled to protein G–Sepharose beads (Sigma).
19. Microtubules were polymerized from *Drosophila* embryo extracts by addition of guanosine triphosphate (GTP) and taxol (*6*). The microtubule pellet was sequentially extracted with 500 mM NaCl, 750 mM NaCl, and 1 M KI. Soluble fractions were concentrated by ultrafiltration using Millipore Ultrafree systems.
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27. We are grateful to P. Ripoll for stimulating our interest in *asp* through the original observations of his laboratory and for his continued encouragement. We thank A. Desai, M. Moritz, and B. Lange for their valuable advice about protocols for centrosome purification and microtubule nucleation. We are grateful to L. Goldstein for the antibody to KLP61F. M.C.A. held an European Molecular Biology Organization fellowship during the initial stages of this work, which was supported by grants from the International Association of Cancer Research and Cancer Research Campaign of Great Britain.

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