DEVELOPMENTAL DYNAMICS 238:3248–3256, 2009

Asator, a Tau-Tubulin Kinase Homolog in *Drosophila* Localizes to the Mitotic Spindle

Hongying Qi, Changfu Yao, Weili Cai, Jack Girton, Kristen M. Johansen, and Jørgen Johansen*

We have used a yeast two-hybrid interaction assay to identify Asator, a tau-tubulin kinase homolog in *Drosophila* that interacts directly with the spindle matrix protein Megator. Using immunocytochemical labeling by an Asator-specific mAb as well as by transgenic expression of a GFP-labeled Asator construct, we show that Asator is localized to the cytoplasm during interphase but redistributes to the spindle region during mitosis. Determination of transcript levels using qRT-PCR suggested that Asator is expressed throughout development but at relatively low levels. By P-element excision, we generated a null or strong hypomorphic *Asator*^{exc} allele that resulted in complete adult lethality when homozygous, indicating that *Asator* is an essential gene. That the observed lethality was caused by impaired Asator function was further supported by the partial restoration of viability by transgenic expression of Asator-GFP in the *Asator*^{exc} homozygous mutant background. The finding that Asator localizes to the spindle region during mitosis and directly can interact with Megator suggests that its kinase activity may be involved in regulating microtubule dynamics and microtubule spindle function. *Developmental Dynamics 238:3248–3256, 2009*.

Key words: tau-tubulin kinase; microtubule spindle; spindle matrix; mitosis; *Drosophila*

Accepted 29 September 2009

INTRODUCTION

The coiled-coil protein, Megator, occupies the interchromosomal space surrounding the chromosomes at interphase (Zimowska et al., 1997; Qi et al., 2004) but redistributes during mitosis to form a molecular spindle matrix complex together with three other nuclear-derived proteins Skeletor, Chromator, and EAST (Walker et al., 2000; Rath et al., 2004; Qi et al., 2004, 2005). This complex forms a fusiform spindle structure that persists in the absence of polymerized tubulin and, based on theoretical considerations of the requirements for force production, has been proposed to help support the microtubule spindle apparatus during mitosis (reviewed in Johansen and Johansen, 2007). While Skeletor, Chromator, and EAST appear to have no obvious mammalian homologs, Megator is a 260-kD protein with a large NH_2-terminal coiled-coil domain and a shorter COOH-terminal acidic region that shows overall structural and sequence similarity to the mammalian nuclear pore complex Tpr protein (Zimowska et al., 1997). Recently, it has been demonstrated that Megator and Tpr both function as spatial regulators of the spindle assembly checkpoint (SAC) ensuring a timely and effective recruitment of Mad2 and Mps1 to unattached kinetochores as cells enter mitosis (Lince-Faria et al., 2009).

In searching for other components of the spindle matrix complex, we used a yeast two-hybrid screen to identify a protein directly interacting with the coiled-coil region of Megator that we have named Asator. Asator contains a kinase domain with 78% amino acid identity to that of the mammalian tau-tubulin kinase (TTBK) family members TTBK1 (Sato et al., 2006) and TTBK2 (Houlden et al., 2007) and belongs to the casein kinase 1 (CK1) superfamily (Manning et al., 2002; Sato et al., 2008). Mammalian

Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa
Grant sponsor: NSF; Grant number: MCB0817107.
***Correspondence to: Jørgen Johansen, Department of Biochemistry, Biophysics, and Molecular Biology, 3156 Molecular Biology Building, Iowa State University, Ames, IA 50011. E-mail: jorgen@iastate.edu**

DOI 10.1002/dvdy.22150
Published online 6 November 2009 in Wiley InterScience (www.interscience.wiley.com).

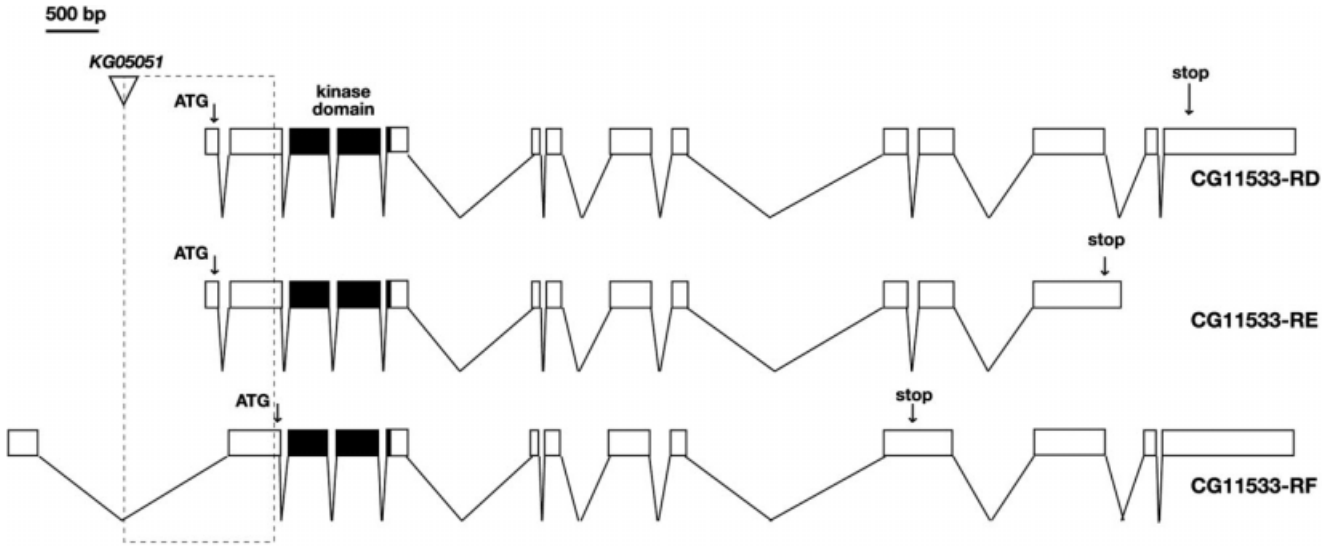

Fig. 1. The organization and protein coding potential of the *Asator* locus. The *Asator* locus gives rise to at least three different transcripts, CG11533-RD, CG11533-RE, and CG11533-RF, due to variant use of different 5′ exons, starting ATG sites, and stop codons. Each transcript, however, contains the same predicted kinase domain with mammalian tau-tubulin kinase homology (in black). The position of the P element *KG05051* within the locus is indicated by a triangle. The grey stippled box shows the sequence removed in the imprecise excision allele $Asator^{exc}$. The figure is modified from Flybase version FB2009_04.

TTBKs were originally identified as microtubule-associated proteins (MAPs) that directly can phosphorylate both tubulin and tau at multiple sites (Takahashi et al., 1995; Tomizawa et al., 2001). TTBK1 is a neuron-specific kinase that has been linked to tau phosphorylation and aggregation at Alzheimer's disease–related sites (Sato et al., 2006, 2008). In contrast, TTBK2 is ubiquitously expressed (Takahashi et al., 1995; Tomizawa et al., 2001) and mutations in the gene encoding TTBK2 have been identified as the cause of spinocerebellar ataxia type 11 (Houlden et al., 2007). Here we characterize the expression and localization of Asator, the *Drosophila* homolog of TTBK1 and TTBK2. We show that Asator is an essential protein that during interphase is localized to the cytoplasm but during mitosis localizes to the spindle region.

RESULTS

The Spindle Matrix Protein Megator Interacts With the TTBK Homolog Asator in *Drosophila*

In order to identify candidates for proteins interacting with the spindle matrix macromolecular complex in *Drosophila* (reviewed in Johansen and Johansen 2007), we conducted yeast two-hybrid interaction assays using a Megator bait construct from its coiled-coil region containing amino acids 173 through 360, which alone was unable to activate transcription of the reporter genes. An embryonic yeast two-hybrid library (0–21 hr) was screened and we identified one interacting clone comprised of partial *CG11533* coding sequence from a gene that we named *Asator*. Analysis of the isolated Asator yeast two-hybrid library clone suggests that the interaction region with Megator is COOH-terminally located. The *Asator* locus is located on the 4th chromosome and has at least three alternative transcripts due to variant use of different 5′ exons, starting methionine sites, and stop codons as depicted in Figure 1, giving rise to three predicted proteins $Asator^{RD}$, $Asator^{RE}$, and $Asator^{RF}$ of 1,349, 1,262, and 811 amino acids, respectively. Each transcript, however, contains the same predicted kinase domain with 78% amino acid identity to that of human TTBK family members (Fig. 2A). Outside of the kinase domain, Asator does not contain any previously described conserved motifs. To further determine the phylogenetic relationship of Asator within the casein kinase 1 superfamily (Manning et al., 2002), we constructed phylogenetic trees using maximum parsimony. The results show that Asator forms a monophyletic clade with other TTBKs with 100% bootstrap support that is distinct from the CK1 family members and that vertebrate TTBK1 and TTBK2 diverged after the origin of Asator (Fig. 2B).

To confirm the physical interaction of Asator with Megator, we performed in vitro pull-down experiments using a PinPoint vector construct that produces biotinylated Asator fusion protein and GST-Megator fusion protein produced in *Escherichia coli*. Whereas the biotinylation target peptide encoded by the PinPoint vector alone was not able to pull down Megator when purified using avidin beads, biotinylated Asator PinPoint fusion protein pulled down a band corresponding to the size of GST-Megator (Fig. 3A). In the converse experiment, GST-Megator fusion protein was able to pull down biotinylated Asator using GST-beads whereas GST protein alone was not (Fig. 3B). These results support the existence of a direct physical interaction between Megator and Asator.

Expression and Localization of Asator

In order to study the expression and localization of Asator, we generated

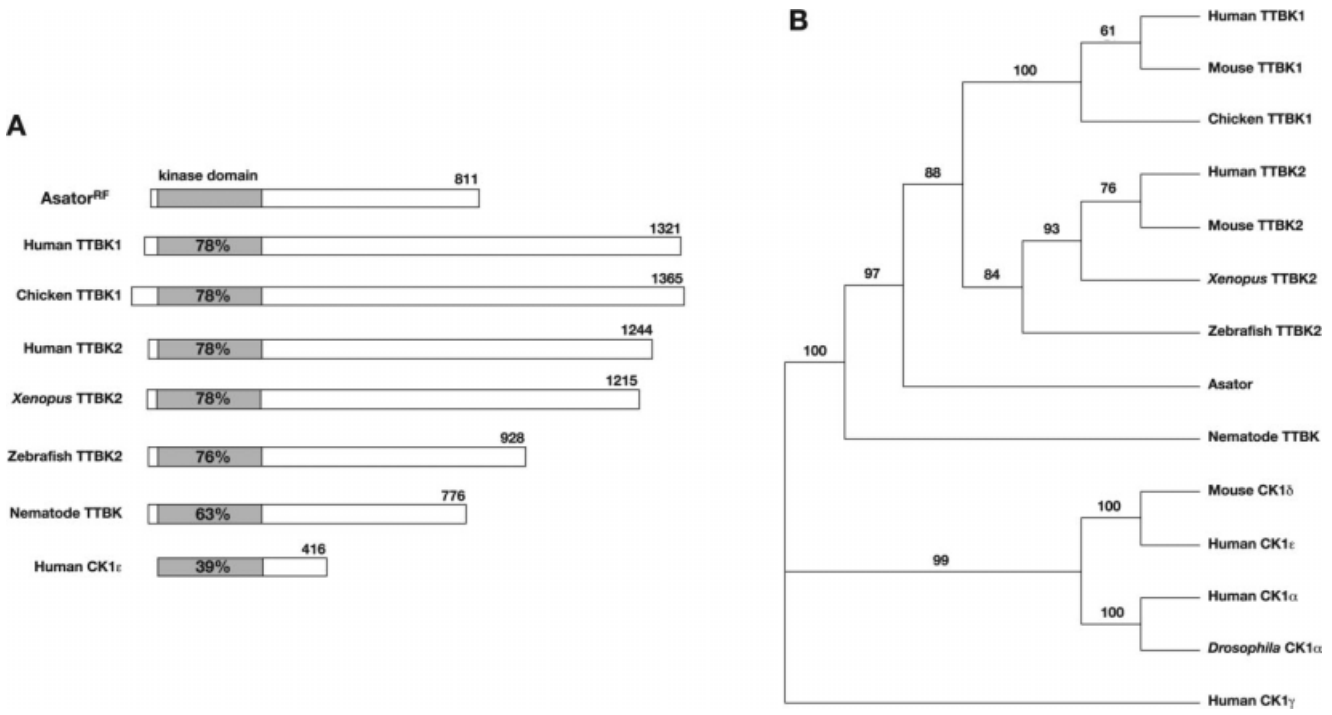

Fig. 2. **A**: Domain structure of Asator^{RF} compared to the most closely related TTBK family members from other organisms as well as to human CK1ε. The grey boxes indicate the location of the kinase domains and the level of amino acid identity with Asator's kinase domain is shown in percent. In addition, the number of residues of each protein is indicated. **B:** Phylogenetic relationship of Asator with other casein kinase 1 superfamily members. The consensus maximum parsimony tree was derived from an alignment of the conserved kinase domain. The tree was rooted using human CK1γ sequence and is depicted with the associated bootstrap support values from 1,000 iterations.

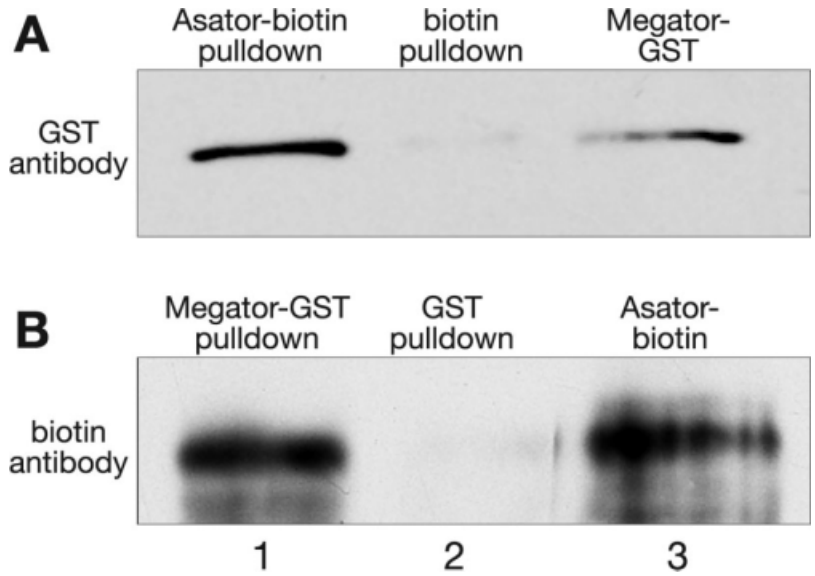

Fig. 3. Asator and Megator pull-down assays. **A:** An Asator-biotin construct pulls down Megator-GST as detected by GST antibody (lane 1). A biotin only pull-down control was negative (lane 2). Lane 3 shows the position of the Chromator-GST fusion protein. **B:** A Megator-GST construct pulls down biotinylated Asator as detected by anti-biotin antibody conjugated with HRP (Biotin antibody) (lane 1). A GST-only pull-down control was negative (lane 2). Lane 3 shows the position of the Asator-biotin fusion protein.

mAbs to GST-fusion proteins containing various regions of Asator. While most of these antibodies proved specific to Asator and could recognize biotinylated Asator as well as Asator-GFP (see below) on dot and immunoblots, we were not able to identify endogenous Asator on immunoblots of protein extracts from either embryos, third instar larvae, adult flies, or S2 cells. In addition, only one mAb, 3B8, raised to an amino acid sequence shared by all three Asator isoforms was able in a few cases to detect endogenous Asator protein above background levels in immunocytological stainings of fixed preparations. This is illustrated in Figure 4A–C where three examples of the faint labeling of the mitotic spindle region at metaphase by mAb 3B8 in dividing S2 cells is shown. A potential explanation for these results is that Asator is only transcribed and/or translated at very low levels. To test this possibility, we used qRT-PCR to measure *Asator* mRNA transcript levels in relation to that of the microtubule-associated motor protein Ncd (Endow et al., 1994). Primers were designed that would amplify all three transcripts from the *Asator* gene and primers specific to the gene encoding *ncd* (Ding et al., 2009) were used for normalization as previously described

(Cai et al., 2008). We performed several independent experiments in which total mRNA was isolated from 0–24-hr embryos, 3rd instar larvae, and adult flies, and in which qRT-PCR determination of transcript levels was performed in duplicate. As illustrated in Figure 4D, the results show that *Asator* is expressed throughout development but at much lower levels (3–20%) relative to *ncd*. In addition, we verified that Asator is expressed in the nervous system by determining *Asator* transcript levels in extracts of mRNA from dissected third instar larval brains (Fig. 4D).

The mAb 3B8 labeling in S2 cells suggested that Asator may be localized to the spindle region during mitosis. To further explore this possibility, we over-expressed a GFP-tagged pUAST Asator full-length construct corresponding to the *CG11533-RF* splice form transgenically in third instar larval brains using an *elav-GAL4* driver line. As illustrated in Figure 5A and C, Asator-GFP localized to the cytoplasm during interphase but redistributed to the mitotic spindle in dividing neuroblasts and GMCs, confirming the localization detected by Asator antibody. On immunoblots, the Asator-GFP transgene was detected as a 116-kD protein by both GFP pAb and Asator mAb 3B8 (Fig. 5B). In addition, we confirmed this expression pattern in S2 cells transiently transfected with a full-length Asator-V5 tagged construct. Figure 5D shows that Asator-V5 is present in the cytoplasm during interphase but is localized to the spindle during cell division as detected by single immunolabeling with V5-antibody.

Asator Is an Essential Gene

A *SUPor-P* (Roseman et al., 1995) element has been found to be inserted into the *CG11533* region (Fig. 1). We verified the P element insertion sites by PCR analysis using primers corresponding to genomic sequences flanking the region and by sequencing the PCR product. In *P{SUPor-P}Asator^{KG05051}* flies, the P element is inserted within the first intron of transcript *CG11533-RF* (Fig. 1). The P insertion line is homozygous viable; however, the position of the P insertion is not likely to affect all three transcripts. Thus, in order to generate an *Asator* null or strong hypomorphic allele, we mobilized the P element in *P{SUPor-P}Asator^{KG05051}* flies using the Δ2–3 transposase (Robertson et al., 1988) and screened for imprecise excision events indicated by a white eye color. From these excisions, we recovered one allele, *Asator^{exc}*, with complete adult lethality when homozygous. By PCR mapping, we determined that the excision event removed part of the P element as well as exonic sequence from all three transcripts including the start codons for *CG11533-RD* and *CG11533-RE* and with the 3′ excision site located 2 bp before the starting methionine of the *CG11533-RF* transcript (Fig. 1). Thus, this excision allele is likely to interrupt the transcription of all three isoforms.

To verify that the observed lethality was caused by impaired Asator function due to the *Asator^{exc}* allele, we used *Asator-GFP* as a rescue construct using a *tub-GAL4* driver line and the dominant *eyeless* allele *ey^{D}* (Lindsley and Zimm, 1992) as a marker for the fourth chromosome. Thus, in the following cross: *yw/y; UAST-Asator-GFP/UAST-Asator-GFP; Asator^{exc}/ey^{D} X yw/yw; tub-GAL4/TM3; Asator^{exc}/ey^{D}* rescue would be indicated by the presence of adult progeny that lack the *ey^{D}* phenotype of malformed eyes. Out of 117 adult flies examined from such a cross, we found 6 flies with wild type eye morphology whereas 17 would be expected in the case of full rescue. This indicates that the Asator-GFP construct can provide partial rescue function (35%) supporting that the *Asator^{exc}* is a null or strong hypomorphic *Asator* allele and that *Asator* is an essential gene.

We determined the stage of lethality of *Asator^{exc}* homozygous mutants by crossing *y^{1} w^{67c23}/y^{1} w^{67c23}; P{SUPorP}Asator^{KG05051}, y^{+}w^{+}/Asator^{exc}* females with *y^{1} w^{67c23}/Y; {SUP orP}Asator^{KG05051}, y^{+}w^{+}/Asator^{exc}* males and collecting and scoring the resulting larvae. In this cross, since the *Asator^{exc}* chromosome does not contain *y^{+}* sequences, all male and female *Asator^{exc}/Asator^{exc}* larvae would be identifiable by a yellow phenotype. This phenotype is readily detectable in first through third instar larval stages by examination of the larval mouthparts (Lindsley and Zimm, 1992). The expected Mendelian ratio of the *Asator^{exc}/Asator^{exc}* genotype in the progeny was 25%, and in a sample of 131 larvae from this cross no larvae were found with yellow mouthparts. This result indicates that *Asator^{exc}/Asator^{exc}* mutants do not survive past embryonic stages precluding analysis of possible mitotic phenotypes in third instar larval brains. In addition, it should be noted that in RNAi depletion experiments of Asator in S2 cells, no obvious phenotypes were observed and Megator localization was unaffected (H. Qi and C. Yao, unpublished results).

DISCUSSION

In this study, we provide evidence that the spindle matrix protein Megator in *Drosophila* interacts with the TTBK homolog, Asator. This interaction was first detected in a yeast two-hybrid screen and subsequently confirmed by pull-down assays. Using immunocytochemical labeling by an Asator-specific mAb as well as by transgenic over-expression of a GFP-labeled Asator construct, we show that Asator is localized to the cytoplasm during interphase but redistributes to the spindle region during mitosis. Furthermore, immunocytochemical and immunoblot analysis indicated that Asator, as is the case for many kinases and other enzymes, is present only at low expression levels. Direct determination of transcript levels using qRT-PCR determination suggested that Asator is expressed thoughout development including in the nervous system, but at levels only 3–20% that of the microtubule-associated motor protein Ncd. By P-element excision, we generated a null or strong hypomorphic *Asator* allele that resulted in complete adult lethality when homozygous indicating that *Asator* is an essential gene. That the observed lethality was caused by impaired Asator function was further supported by the partial restoration of viability by transgenic expression of Asator-GFP in the *Asator^{exc}* homozygous mutant background. That complete rescue was not obtained could be due to differences in expression levels of the transgene or that one of the other Asator isoforms has

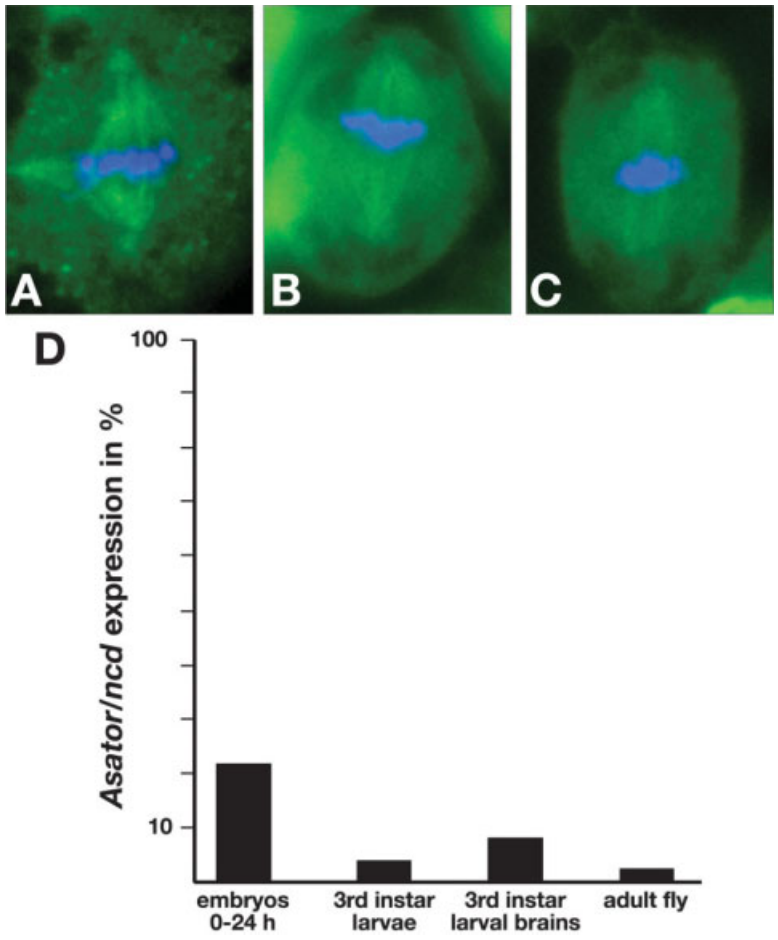

Fig. 4.

function(s) not fully covered by the Asator^{RF} isoform.

The direct physical interaction between Asator and Megator suggests that Asator may be involved in spindle matrix function. The spindle matrix is hypothesized to provide a stationary or elastic molecular matrix that can provide a substrate for motor molecules to interact with during microtubule sliding and that can stabilize the spindle during force production (Pickett-Heaps et al., 1997; Forer et al., 2008). During mitosis, the Megator-defined spindle matrix forms a fusiform spindle-like structure that is co-aligned with the microtubule-based spindle apparatus and that persists in the absence of microtubules (reviewed in Johansen and Johansen, 2007).

Fig. 4. Asator expression and localization. **A–C:** Double labelings of mitotic S2 cells with the Asator mAb 3B8 (in green) and of DNA with Hoechst (in blue). **D:** Transcript levels of *Asator* mRNA in 0–24-hr embryos, third instar larvae, third instar larval brains, and adult flies. *Asator* transcript levels from all three isoforms were determined by qRT-PCR and normalized to the mRNA levels of the microtubule associated motor protein Ncd. Each determination was performed in duplicate.

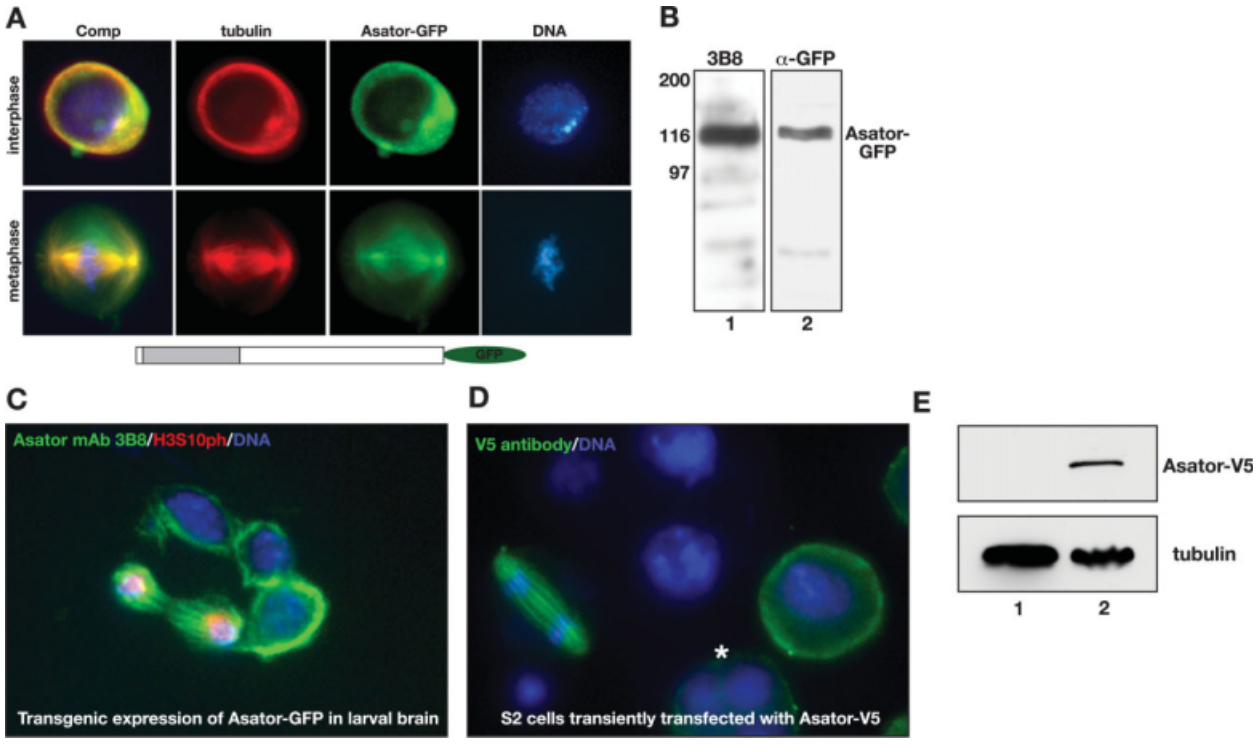

Fig. 5.

Furthermore, molecules forming a spindle matrix complex would be expected to exhibit several characteristics including that one or more members of the complex should interact with microtubules or microtubule-associated molecules. Mammalian TTBKs have been demonstrated to have at least dual substrate specificity and be able to phosphorylate both tubulin and tau proteins (Takahashi et al., 1995; Tomizawa et al., 2001). Considering the high percentage of amino acid identity between the kinase domains of mammalian TTBKs and Asator, it is likely that Asator may have similar properties. Thus, the finding that Asator localizes to the spindle region during mitosis and can interact directly with Megator suggests that its kinase activity may be involved in regulating microtubule dynamics and microtubule spindle function. Such regulation of microtubule dynamics during mitosis by tubulin phosphorylation has been previously reported for the cyclin-dependent kinase Cdk1 (Fourest-Lieuvin et al., 2006). While mammalian TTBKs were first purified as microtubule-associated proteins, a significant fraction was also detected in the MAP-free supernatant indicating that not all TTBK is necessarily associated with microtubules (Takahashi et al., 1995). It is, therefore, possible that Asator in *Drosophila* may have binding affinity for both Megator and microtubules and potentially could represent a link between the spindle matrix and the microtubule-based spindle apparatus. Alternatively, Megator and the spindle matrix may serve as a spatial and temporal regulator of Asator function during mitosis in a way similar to its recently described role in sequestering the SAC proteins Mad2 and the Mps1 kinase (Lince-Faria et al., 2009). Thus, it will be of interest in future studies to elucidate the functional role of Asator in mitosis.

EXPERIMENTAL PROCEDURES

Drosophila melanogaster Stocks and Transgenes

Fly stocks were maintained according to standard protocols (Roberts, 1998). Canton S was used for wild-type preparations. The *P{SUPor-P}Asator*^{KG05051} and *ey*^{D} fly lines were obtained from the Bloomington Stock Center. For Asator-GFP, *Asator* full-length cDNA sequence corresponding to residues 1–811 of *CG11533-RF* was inserted into the pUAST vector (Brand and Perrimon, 1993) with a C-terminal GFP tag and transgenic lines were generated by standard P-element transformation (BestGene, Inc.) The expression of the transgenes was driven using the nervous system–specific *GAL4* driver *P{w[+mW.hs]=GawB}elav[C155]* or the *tub-GAL4 (P[tub>CD2>GAL4]* driver (Bloomington Stock Center) introduced by standard genetic crosses. Expression levels of the Asator-GFP construct were monitored by immunoblot analysis as described below. The fidelity of the construct was verified by sequencing at the Iowa State University DNA Facility. Viability assays were performed as in Zhang et al. (2003). Balancer chromosomes and markers are described in Lindsley and Zimm (1992).

P Element Excision

The *Asator* allele *Asator*^{exc} was isolated by mobilizing the P element in *P{SUPor-P}Asator*^{KG05051} flies using the Δ2–3 transposase chromosome (Robertson et al., 1988) and screening for imprecise excision events as previously described in Wang et al. (2001). Imprecise excisions were identified by a white eye color and mapped by (PCR) analysis using primers corresponding to genomic sequences flanking the insertion region and to sequences within the P-element. The *y w; Δ2-3 Sb/TM2 Ubx e* stock was the generous gift of Dr. Linda Ambrosio, Iowa State University.

Identification and Sequence Analysis of *Asator*

The Megator cDNA sequence encoding residues 173–360 in the NH_2-terminal coiled-coil domain was subcloned in-frame into the yeast two-hybrid bait vector pGBKT7 (Clontech) using standard methods (Sambrook and Russell, 2001) and verified by sequencing (Iowa State University Sequencing Facility). This Megator bait was used to screen 10^6 cDNA clones from a Clontech Matchmaker *Drosophila* 0–21-hr embryonic yeast two-hybrid library according to the manufacturer's instructions and as previously described (Bao et al., 2005; Rath et al., 2004). One positive cDNA clone was isolated, retransformed into yeast cells containing the Megator bait to verify the interaction, and sequenced. Homology searches identified the interacting clone as comprised of partial coding sequences from the *CG11533* (*Asator*) locus. The Asator sequence was compared with known and predicted sequences using Flybase and the National Center for Biotechnology Information BLAST server. The sequence was further analyzed using *SMART* (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/) to predict the domain organization of the protein. Alignments used to produce maximum parsimony trees were generated with the Clustalw version 1.7 program and encompassed the conserved kinase domain. Trees were constructed by maximum parsimony

Fig. 5. Transgenic expression of Asator in third instar larval brains and S2 cells. **A:** Neuroblasts triple-labeled with tubulin antibody (in red), Asator mAb 3B8 (in green), and Hoechst (in blue). Top: Labeling at interphase. Bottom: Cell at metaphase. The expressed Asator-GFP construct is diagrammed beneath the micrographs. **B:** The Asator-GFP construct was detected as a 116-kD protein on immunoblots labeled with both Asator mAb 3B8 (lane 1) and GFP antibody (lane 2). The relative migration of molecular size markers is indicated to the left in kD. **C:** Asator-GFP expressed in larval brain cells and triple-labeled with Asator mAb 3B8 (in green), H3S10ph antibody for identification of dividing cells (in red), and Hoechst (in blue). Three interphase cells and a dividing neuroblast are shown. **D:** S2 cells transiently transfected with an Asator-V5 tagged construct and double-labeled with V5 antibody (in green) and with Hoechst (in blue). Transfected S2 cells expressing the Asator-V5 construct at anaphase and interphase are shown together with a cell not expressing the Asator-V5 construct (asterisk). **E:** Immunoblot of protein extracts from untransfected (lane 1) and Asator-V5 transiently transfected (lane 2) S2 cells labeled with V5 antibody (top). Labeling with tubulin antibody was used as a loading control (bottom).

using the PAUP computer program version 4.0b (Swofford, 1993). All trees were generated by heuristic searches, and bootstrap values in percent of 1,000 replications are indicated on the bootstrap majority rule consensus tree.

Biochemical Analysis

Immunoblot analysis.

Protein extracts were prepared from embryos or third instar larvae (or in some experiments from dissected larval brains) homogenized in a buffer containing: 20 mM Tris-HCl pH8.0, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, 0.2% NP-40, 2 mM Na_3VO_4, 1 mM PMSF, 1.5 μg/ml aprotinin. Proteins were separated by SDS-PAGE according to standard procedures (Sambrook and Russell, 2001). Electroblot transfer was performed as in Towbin et al. (1979) with transfer buffer containing 20% methanol and in most cases including 0.04% SDS. For these experiments, we used the Bio-Rad Mini PROTEAN II system, electroblotting to 0.2 μm nitrocellulose, and using anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA) (1:3,000) for visualization of primary antibody. Antibody labeling was visualized using chemiluminescent detection methods (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL). The immunoblots were digitized using a flatbed scanner (Epson Expression 1680).

Pull-down experiments.

For in vitro pull-down assays, an Asator fragment consisting of the COOH-terminal 468 aa of Asator^{RF} (bio-Asator) was subcloned in-frame into the Pinpoint Xa-2 vector (Promega, Madison, WI) and the Megator bait sequence of residue 173–360 (GST-Megator-bait) was subcloned into the pGEX4T-1 vector. The biotinylated Asator protein and the GST-Megator-bait protein were expressed in XL-1 Blue cells (Stratagene, La Jolla, CA). For GST pull-down assays, approximately 3 μg of GST-Megator-bait or GST protein alone were coupled to glutathione agarose beads (Sigma, St. Louis, MO) and incubated with 0.5 ml of cell extract expressing bio-Asator protein in immunoprecipitation (ip) buffer (20 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.1% Triton X-100, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1.5 μg aprotinin) overnight at 4°C. The protein complex coupled beads were washed three times with 1 ml of ip buffer and analyzed by SDS-PAGE and immunoblotting using biotin antibody conjugated with HRP (Cell Signaling). Similarly, for avidin pull-down assays bio-Asator or the biotinylation tag alone was bound to immobilized Streptavidin beads (Pierce, Thermo Fischer Scientific, Rockford, IL) and incubated with 3 μg of Megator-GST-bait in 500 μl of immunoprecipitation buffer. The resulting complexes were then analyzed by SDS PAGE and immunoblotting using the GST mAb 8C7 (Rath et al., 2004).

Asator Antibody

Various regions of the predicted Asator protein were subcloned using standard techniques (Sambrook and Russell, 2001) into the pGEX-4T-1 vector (Amersham Pharmacia Biotech) to generate GST-fusion proteins. The correct orientation and reading frame of the inserts were verified by sequencing. The GST-fusion proteins were expressed in XL1-Blue cells (Stratagene) and purified over a glutathione agarose column (Sigma-Aldrich), according to the pGEX manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). The mAb 3B8 was generated by injection of 50 μg of GST-fusion protein containing amino acids 103–210 of Asator^{RF} into BALB/c mice at 21-day intervals. After the third boost, mouse spleen cells were fused with Sp2 myeloma cells and monospecific hybridoma lines were established using standard procedures (Harlow and Lane, 1988). All procedures for mAb production were performed by the Iowa State University Hybridoma Facility.

Immunocytochemistry

Larval brain squashes were performed according to the protocol of Bonaccorsi et al., (2000) with minor modifications as described in Ding et al. (2009). Antibody labelings of 0–3-hr embryos were performed as described in Johansen and Johansen (2003) and S2 cell immunocytochemistry was performed as described in Qi et al. (2004). Primary antibodies used include the Asator-specific mAb 3B8 (this study), anti-α-tubulin mAb (Sigma-Aldrich), anti-H3S10ph pAb (Cell Signaling, Danvers, MA), anti-V5 mAb (Invitrogen, Carlsbad, CA), and anti-GFP pAb (Invitrogen). DNA was visualized by staining with Hoechst 33258 (Molecular Probes, Eugene, OR) in PBS. The appropriate species- and isotype-specific Texas Red-, TRITC-, and FITC-conjugated secondary antibodies (Cappel/ICN, Southern Biotech, Birmingham, AL) were used (1:200 dilution) to visualize primary antibody labeling. The final preparations were mounted in 90% glycerol containing 0.5% *n*-propyl gallate. The preparations were examined using epifluorescence optics on a Zeiss Axioskop microscope and images were captured and digitized using a high-resolution Spot CCD camera. Images were imported into Photoshop where they were pseudocolored, image processed, and merged. In some images, non-linear adjustments were made to the channel with Hoechst labeling for optimal visualization of chromosomes.

Expression of Asator-V5 in Transfected S2 Cells

A full-length Asator^{RF} (811 aa) construct was cloned into the pMT/V5-HisB vector (Invitrogen) with an in-frame V5 tag at the COOH-terminus using standard methods (Sambrook and Russell, 2001). *Drosophila* Schneider 2 (S2) cells were cultured in Shields and Sang M3 insect medium (Sigma) supplemented with 10% fetal or newborn bovine serum, antibiotic/antimycotic solution, and L-Glutamine (Gibco/BRL/Life Technologies, Gaithersburg, MD) at 25°C. The S2 cells were transfected with Asator-V5 using a calcium phosphate transfection kit (Invitrogen) and expression was induced by 0.5 mM $CuSO_4$. Cells expressing the Asator-V5 construct were harvested 12–24 hr after

induction and affixed onto poly-L-lysine coated coverslips for immunostaining and Hoechst labeling.

Analysis of Gene Expression by qRT-PCR

Total RNA was extracted from 0–24-hr embryo collections, whole third instar larvae, dissected third instar larval brains, and adult flies, respectively, using the MicroPoly(A)Purist Small-Scale mRNA Purification Kit (Ambion, Austin, TX) following the manufacturer's instructions. cDNA derived from this RNA using SuperScript II Reverse Transcriptase (Invitrogen) was used as template for quantitative real-time (qRT) PCR performed with the Stratagene Mx4000 real-time cycler. In addition, the PCR mixture contained Brilliant II SYBR Green QPCR Master Mix (Stratagene) as well as the corresponding primers: *Asator*, 5′-TCAGAAGTCAA TCGGTCAACGG-3′ and 5′-CGTAGTA TCCTCGGAATCATCAAAC-3′; *ncd*, 5′-GCCAAGAACAACAAGAACGACATC TACG-3′ and 5′-AAACTGCCGCTGTT GTTGCTCTGTGTG-3′. Cycling parameters were 10 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 60 sec at 60°C, and 30 sec at 72°C. Fluorescence intensities were plotted against the number of cycles using an algorithm provided by Stratagene. mRNA levels were quantified using a calibration curve based on dilution of concentrated cDNA. mRNA values for *Asator* transcripts were normalized to those for *ncd*.

ACKNOWLEDGMENTS

We thank members of the laboratory for discussion, advice, and critical reading of the manuscript. We also acknowledge Ms. V. Lephart for maintenance of fly stocks and Mr. Laurence Woodruff for technical assistance. We especially thank Dr. L. Ambrosio for providing the *y w; Δ2-3 Sb/TM2 Ubx e* stock. Work in the laboratory of J.J. and K.M.J. is supported by NSF grant MCB0817107.

REFERENCES

Bao X, Zhang W, Krencik R, Deng H, Wang Y, Girton J, Johansen J, Johansen KM. 2005. The JIL-1 kinase interacts with lamin Dm_0 and regulates nuclear lamina morphology of *Drosophila* nurse cells. J. Cell Sci 118:5079–5087.

Bonaccorsi S, Giansanti MG, Gatti M. 2000. Spindle assembly in *Drosophila* neuroblasts and ganglion mother cells. Nat Cell Biol 2:54–56.

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

Cai W, Bao X, Deng H, Jin Y, Girton J, Johansen J, Johansen KM. 2008. RNA polymerase II-mediated transcription at active loci does not require histone H3S10 phosphorylation in *Drosophila*. Development 135:2917–2925.

Ding Y, Yao C, Lince-Faria M, Rath U, Cai W, Maiato H, Girton J, Johansen KM, Johansen J. 2009. Chromator is required for proper microtubule spindle formation and mitosis in *Drosophila*. Dev Biol 334:253–263.

Endow SA, Chandra R, Komma DJ, Yamamoto AH, Salmon ED. 1994. Mutants of the *Drosophila* ncd microtubule motor protein cause centrosomal and spindle pole defects in mitosis. J Cell Sci 107:859–867.

Forer A, Pickett-Heaps JD, Spurk T. 2008. What generates flux of tubulin in kinetochore microtubules? Protoplasma 232:137–141.

Fourest-Lieuvin A, Peris L, Gache V, Garcia-Saez I, Juillan-Binard C, Lantez V. Job D. 2006. Microtubule regulation in mitosis: tubulin phosphorylation by the cyclin-dependent kinase Cdk1. Mol Biol Cell 17:1041–1050.

Harlow E, Lane E. 1988. Antibodies: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 726 pp.

Houlden H, Johnson J, Gardner-Thorpe C, Lashley T, Hernandez D, Worth P, Singleton AB, Davis MB, Giunti P, Wood NW2007. Mutations in *TTBK2*, encoding a kinase implicated in tau phosphorylation, segregate with spinocerebellar ataxia type 11. Nat Genet 39: 1434–1436.

Johansen KM, Johansen J. 2003. Studying nuclear organization in embryos using antibody tools. In: Henderson DS, editor.Drosophila cytogenetics protocols. Totowa, NJ: Humana Press. p 215–234.

Johansen KM, Johansen J. 2007. Cell and molecular biology of the spindle matrix. Int Rev Cytol 263:155–206.

Lindsley DL, Zimm GG. 1992. The genome of Drosophila melanogaster. New York: Academic Press. 1133 pp.

Lince-Faria M, Maffini S, Orr B, Ding Y, Florindo C, Sunkel CE, Tavares A, Johansen J, Johansen KM, Maiato H. 2009. Spatiotemporal control of mitosis by the conserved spindle matrix protein Megator. J Cell Biol 184:647–657.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. 2002. The protein kinase complement of the human genome. Science 298:1912–1934.

Pickett-Heaps JD, Forer A, Spurck T. 1997. Traction fibre: toward a "tensegral" model of the spindle. Cell Motil Cytoskeleton 37:1–6.

Qi H, Rath U, Wang D, Xu Y-Z, Ding Y, Zhang W, Blacketer M, Paddy M, Girton J, Johansen J, Johansen KM. 2004. Megator, an essential coiled-coil protein localizes to the putative spindle matrix during mitosis. Mol Biol Cell 15:4854–4865.

Qi H, Rath U, Ding Y, Ji Y, Blacketer MJ, Girton J, Johansen J, Johansen KM. 2005. EAST interacts with Megator and localizes to the putative spindle matrix during mitosis in *Drosophila*. J Cell Biochem 95:1284–1291.

Rath U, Wang D, Ding Y, Xu Y-Z, Qi H, Blacketer MJ, Girton J, Johansen J, Johansen KM. 2004. Chromator, a novel and essential chromodomain protein interacts directly with the putative spindle matrix protein Skeletor. J Cell Biochem 93:1033–1047.

Roberts DB. 1998. In *Drosophila*: a practical approach. Oxford, UK: IRL Press. 389 pp.

Robertson HM, Preston CR, Phillis RW, Johnson-Schlitz DM, Benz WK, Engels WR. 1988. A stable genomic source of P element transposase in *Drosophila melanogaster*. Genetics 118:461–470.

Roseman RR, Johnson EA, Rodesch CK, Bjerke M, Nagoshi RN, Geyer PK. 1995. A P element containing suppressor of hairy-wing binding regions has novel properties for mutagenesis in *Drosophila melanogaster*. Genetics 141: 1061–1074.

Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sato S, Cemy RL, Buescher JL, Ikezu T. 2006. Tau-tubulin kinase 1 (TTBK1), a neuron-specific tau kinase candidate, is involved in tau phosphorylation and aggregation. J Neurochem 98:1573–1584.

Sato S, Xu J, Okuyama S, Martinez LB, Walsh SM, Jacobsen MT, Swan RJ, Schlautman JD, Ciborowski P, Ikezu T. 2008. Spatial learning impairment, enhanced CDK5/p35 activity, and downregulation of NMDA receptor expression in transgenic mice expressing tau-tubulin kinase 1. J Neurosci 28: 14511–14521.

Swofford DL. 1993. Phylogenetic analysis using parsimony. Illinois Natural History Survey, Champaign, IL. 132 p.

Takahashi M, Tomizawa K, Sato K, Ohtake A, Omori A. 1995. A novel tau-tubulin kinase from bovine brain. FEBS Lett 372:59–64.

Tomizawa K, Omori A, Ohtake A, Sato K, Takahashi M. 2001. Tau-tubulin kinase phosphorylates tau at Ser-208 and Ser-210, sites found in paired helical filament-tau. FEBS Lett 492:221–227.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose

sheets: Procedure and some applications. Proc Natl Acad Sci USA 9:4350–4354.
Walker DL, Wang D, Jin Y, Rath U, Wang Y, Johansen J, Johansen KM. 2000. Skeletor, a novel chromosomal protein that redistributes during mitosis provides evidence for the formation of a spindle matrix. J Cell Biol 151: 1401–1411.
Wang Y, Zhang W, Jin Y, Johansen J, Johansen KM. 2001. The JIL-1 tandem kinase mediates histone H3 phosphorylation and is required for maintenance of chromatin structure in Drosophila. Cell 105:433–443.
Zhang W, Jin Y, Ji Y, Girton J, Johansen J, Johansen KM. 2003. Genetic and phenotypic analysis of alleles of the Drosophila chromosomal JIL-1 kinase reveals a functional requirement at multiple developmental stages. Genetics 165:1341–1354.
Zimowska G, Aris JP, Paddy MR. 1997. A *Drosophila* TPR protein homolog is localized both in the extrachromosomal channel network and to nuclear pore complexes. J Cell Sci 110:927–944.

Developmental Dynamics