

Maternally expressed and partially redundant β -tubulins in *Caenorhabditis elegans* are autoregulated

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Summary

The mitotic spindle, which partitions replicated chromosomes to daughter cells during cell division, is composed of microtubule assemblies of α/β -tubulin heterodimers. Positioning of the mitotic spindle influences the size and location of daughter cells, and can be important for the proper partitioning of developmental determinants. We describe two semi-dominant mis-sense mutations in *tbb-2*, one of two *C. elegans* β -tubulin genes that are maternally expressed and together are required for microtubule-dependent processes in the early embryo. These mutations result in a posteriorly displaced and mis-oriented mitotic spindle during the first cell division. In contrast, a probable *tbb-2* null allele is recessive, and when homozygous results in less severe spindle positioning defects and only partially penetrant embryonic lethality.

Two of the *tbb-2* mutations result in reduced levels of TBB-2 protein, and increased levels of a second maternally expressed β -tubulin, TBB-1. However, levels of TBB-1 are not increased in a *tbb-2* mutant with an allele that does not result in reduced levels of TBB-2 protein. We conclude that feedback regulation influences maternal β -tubulin expression in *C. elegans*, but cannot fully restore normal microtubule function in the absence of one β -tubulin isoform.

Movies available online

Key words: β -tubulin, Meiosis, Microtubules, Mitotic spindle, Polarity, Asymmetric cell division

Introduction

Microtubules are dynamic polymers and are essential for cellular processes including motility, transport of intracellular components and cell division. A microtubule is composed of repeating α/β -tubulin heterodimer subunits assembled into linear protofilaments arranged to form a hollow tube. On average, the α and β subunits share 40% amino-acid identity (Kraus et al., 1981; Ponstingl et al., 1981), and their three dimensional structures are nearly identical (Nogales et al., 1998). Both subunits share domains required for dimerization and subsequent polymerization into functional microtubules, and both bind GTP. However, only β -tubulin contains an exchangeable site in which the bound GTP is hydrolyzed to GDP upon microtubule polymerization. The variable nucleotide state of β -tubulin, and the activities of MT-associated proteins (MAPs), account for the dynamic instability of microtubules, enabling cells to rapidly alter their cytoskeletal architecture in response to temporal and spatial factors (Valiron et al., 2001).

The bipolar mitotic spindle is composed of microtubules that emanate from the two centrosomes, or microtubule organizing centers (MTOCs), one at each pole (Karsenti and Vernos, 2001; Wittmann et al., 2001). It includes kinetochore microtubules, which attach to and aid in the separation of chromosomes during mitosis, and astral microtubules, which radiate in all directions from centrosomes and frequently contact the cell cortex. Astral microtubule contact with the cortex, possibly

aided by interactions with dynein and dynactin, can be important for orienting the mitotic spindle (Carminati and Stearns, 1997; Gönczy et al., 1999a; Shaw et al., 1997; Skop and White, 1998). Moreover, the position of the mitotic spindle determines the plane of cleavage during cytokinesis and thus dictates the position and relative size of daughter cells after mitosis (Lyczak et al., 2002; Satterwhite and Pollard, 1992; Schuyler and Pellman, 2001). Thus, the orientation of the mitotic spindle relative to axes of cell polarity can influence the partitioning of cell fate determinants to daughter cells during development.

We report that in *C. elegans*, two β -tubulins called TBB-1 and TBB-2 both assemble into spindle microtubules in early embryonic cells. We characterize two semi-dominant mis-sense mutations in *tbb-2* that are more deleterious in effect than a probable null mutation. We also show that the steady state levels of TBB-1 are substantially elevated in two mutants with reduced TBB-2 levels, but not in a *tbb-2* mutant with roughly wild-type levels of mutant tubulin isoform. These data provide genetic evidence confirming earlier studies suggesting that tubulin synthesis is autoregulated (reviewed by Cleveland, 1988). Nevertheless, complete loss of TBB-2 function results in unstable spindle positioning and, especially at higher temperatures, partially penetrant embryonic lethality. We conclude that while TBB-1 and TBB-2 are partially redundant, autoregulation is not fully sufficient to compensate for loss of one isoform.

Materials and Methods

C. elegans strains, alleles and genetic analysis

All strains were cultured by standard methods (Brenner, 1974). The Bristol Strain N2 was the standard wild-type strain used in this study. The following alleles were used: LGIII *unc-32(e189)*, *tbb-2(or362)*, *tbb-2(t1623)*, *tbb-2(gk129)*, *dpy-17(e164)*, *unc-93(e1500)*. LGIV *him-8(e1489)*, *him-3(e1147)*. Deficiencies and duplications: qC1 *dpy-19(e1259)* *glp-1(q339)* (III), sDf130(s2427) *unc-32(e189)* III; sDp3(III; f), nDf15/*unc-93(e1500)* *dpy-17(e164)* III, sDf121(s2098) *unc-32(e189)* III; sDp3 (III; f).

Identity and molecular cloning of *tbb-2*

or362 was identified in a screen for temperature sensitive embryonic lethal mutants (Encalada et al., 2000). The average brood size is 246 at 15°C and 55 at 25°C (five broods scored at each temperature). To test for paternal contribution, *or362* hermaphrodites were mated with wild-type males at room temperature, which is still completely restrictive for this allele. Embryos from three worms that laid at least 150 embryos each were analyzed; 0/841 embryos hatched. Hermaphrodites of strain *dpy-17(e164)* *or362/+* were shifted as L4s to the restrictive temperature of 25°C and allowed to lay embryos. 82% (300/364) of these embryos hatched. Of those that survived, 19% (56/300) were Dpy, suggesting that essential zygotic requirements during embryogenesis do not account for the roughly 20% embryonic lethality from *or362/+* hermaphrodites. The *t1623* allele failed to complement *or362* and was previously identified in a screen for embryonic lethal non-conditional alleles on LGIII (Gönczy et al., 1999b). Like *or362*, *t1623* shows partial dominance, as 75% of embryos from *unc-32(e189)* *tbb-2(t1623)/qC1* [*dpy-19(e1259)* *glp-1(q339)*] (III); *him-3(e1147)* (IV) heterozygous mothers hatch (176/232). Of the survivors, 54/176 (30%) were Unc, again suggesting that zygotic requirements during embryogenesis are not responsible for the observed levels of lethality. The lethality observed with *t1623* is not rescued paternally, as only 2/774 embryos hatched when wild-type males were crossed into homozygous *unc-32(e189)* *tbb-2(t1623)* mothers. *tbb-2(gk129)* was outcrossed twice and is homozygous viable at both 15°C and 25°C (see Results). The average brood size for *gk129* from five worms is 290 at 15°C, and 117 at 25°C. The *gk129* allele was provided by the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia. The *gk129* allele was sequenced at the *C. elegans* Reverse Genetics Core Facility at UBC and shown to contain a 766 bp deletion. The breakpoints of the deletion are at sites 29296 and 30063 of C36E8.5.

We mapped *or362* between -4.66 and -4.13 map units on chromosome 3, using the deficiency nDf15 and a single nucleotide polymorphism located at position 9408 on the cosmid C30D11, respectively. To confirm the identity of *or362* and *t1623*, we sequenced C36E8.5 (GenBank accession number CE00913) in homozygous mutants. DNA fragments of 700-800 bp, overlapping roughly 100 bp, were amplified from genomic DNA using PCR. Bands were excised from agarose gels and purified with GeneClean II (Bio 101) and cloned into pGEM-T vector (Promega). Sequencing was done at the University of Oregon DNA Sequencing Facility, using an ABI 377 Prism automated fluorescent sequencer. Clones from two independent PCR reactions for each allele were sequenced and compared with sequences from *lin-2(e1309)* animals for *or362*, and from *unc-32(e189)* for *t1623*, the parental strains used for mutagenesis. Both alleles were sequenced 1187 base pairs 5' to the start ATG, and 1163 base pairs 3' to the putative stop codon. Codon 141 of *tbb-2(or362)* is mutated from GGA to GAA resulting in a G to E substitution. Codon 313 of *tbb-2(t1623)* is mutated from GTG to ATG resulting in a V to M substitution.

Microscopy and immunofluorescence

For time-lapse digital microscopy, gravid hermaphrodites were dissected and their embryos placed on a 3% agarose pad and overlaid

with a glass coverslip. DIC images were captured every 5 seconds using a Dage MT1 VE1000 digital camera and Scion Image software, and displayed at 7 frames/second. Measurements of pronuclear meeting position, spindle angles and centrosome position were obtained using Object Image Software.

For immunofluorescence, embryos were placed onto a polylysine-coated slide. The eggshell was permeabilized by the freeze-crack method (for details, see Bowerman et al., 1993). For P granule staining, the slides were fixed in methanol for 15 minutes at -20°C. Microtubule staining was detected using a monoclonal anti- α -tubulin antibody (clone DM1 α ; Sigma), diluted 1:250. For DM1 α , anti-TBB-2 or anti-TBB-1 double staining, DM1 α was again used at 1:250, and mixed and incubated at the same time as rabbit anti-TBB-2 or rabbit anti-TBB-1, which was diluted 1:100. Anti-TBB-1 single staining was also used at a concentration of 1:100. Fluorescently tagged secondary antibodies were used at 1:200 dilutions: FITC-conjugated goat anti-mouse or anti-rabbit (Jackson ImmunoResearch Laboratories) and rhodamine-conjugated goat anti-mouse (Molecular Probes). DNA was labeled with 0.2 μ M TOTO3 (Molecular Probes).

Western blotting

Two independent rabbit polyclonal antisera were generated against a peptide of the sequence EPLDEFAGEG[C added], which corresponds to the unique 10 amino acid peptide within the C terminus of TBB-1, and subsequently affinity purified (Quality Controlled Biochemicals). To examine TBB-1 and TBB-2 levels in the non-conditional *tbb-2(t1623)* mutants, which are not homozygous viable, 75 gravid adult homozygous mutant or wild-type worms were snap frozen in 15 μ l M9 and then lysed by adding 15 μ l 2 \times sample buffer (125 mM Tris, pH 6.8, 6% SFS wt/vol, 10% β -mercaptoethanol vol/vol, 20% glycerol vol/vol) followed by incubation at 100°C for 10 minutes. The samples were then loaded and run out on a 14% SDS-PAGE acrylamide gel and subsequently transferred to a Hybond ECL Nitrocellulose membrane (Amersham Pharmacia Biotech). TBB-2 immunoblots were probed using rabbit anti-TBB-2 (kindly provided by C. Lu and P. Mains, U. Calgary) (1:3000) overnight at 4°C, and subsequently with mouse anti-actin (1:10,000; ICN clone C4) as a loading control for 3-4 hours at room temperature. TBB-1 immunoblots were probed using rabbit anti-TBB-1 (1:2500) overnight at 4°C, and subsequently with mouse anti-actin (1:5000; ICN clone C4) as a loading control for 3-4 hours at room temperature. Blots were detected using an HRP-conjugated secondary antibody (1:5000; Amersham Life Sciences). To examine TBB-1 and TBB-2 levels in the homozygous viable *tbb-2(or362)* and *tbb-2(gk129)* mutants, embryonic extracts were prepared. For each strain, 10,000 homozygous mutant or wild-type L1 larvae were plated onto twenty 15 cm egg-supplemented NGM agar plates with OP50 and incubated at 15°C until they reached the gravid adult stage. For the *or362* temperature shifted sample, L4 animals were shifted to 25°C prior to harvesting gravid adults. Worms were collected in M9 buffer and treated with hypochlorite solution to obtain embryos, which were stored at -80°C until lysates were made. Extracts were prepared by adding two volumes of breaking buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM EDTA). Complete protease inhibitor tablets (Roche) were included according to the manufacturer's instructions. One volume of 0.5 mm glass beads were added and the samples were homogenized for three cycles of 1 minute each (with ice incubation between cycles) in a Mini-Bead-Beater-8 (BioSpec Products, Inc.). Crude lysates were centrifuged in a microfuge at 2000 *g* for 10 minutes at 4°C to yield a low speed sup fraction. Bradford protein assays were performed and 20 μ g total protein was loaded per lane on an SDS-PAGE minigel. Western blotting was performed following standard procedures. TBST buffer and 6% dry milk was used to block the blot. The blot was probed multiple times after being stripped as follows: the membrane was incubated in 2% SDS, 100 mM β -mercaptoethanol, 62.5 mM Tris-HCl pH 6.8 for 30 minutes at 70°C.

Elevated levels of TBB-1 in *tbb-2* (*gk129*) and *tbb-2* (*or362*) were determined using Image J software (National Institutes of Health).

RNA interference

To make *tbb-1* dsRNA, PCR was used to amplify a genomic region corresponding to the unique 3' UTR of *tbb-1*. Primers used were as follows: f-gag aca tac gag tct gag c and r-tgc ttc aag tcc ata gct g. Bands were excised from agarose gels and purified with GeneClean II (Bio 101) and cloned into pGEM-T vector (Promega). PCR with T7 and SP6+ was then used to amplify the inserts. dsRNA was synthesized using T7 RNA polymerase (Promega), and purified by phenol/chloroform extraction and ethanol precipitation. Double-stranded RNA was microinjected into the syncytial gonad of young *rff-3* hermaphrodites by standard methods (Fire et al., 1998; Simmer et al., 2002). Embryos from injected animals were observed approximately 24 hours postinjection.

Online supplemental material

Videos 1-4 correspond to Fig. 2A, and show events associated with the first mitotic division in *C. elegans*. Each video begins shortly after fertilization, with anterior to the left, and posterior to the right. Video 1 is of a wild-type embryo, video 2 is of a *tbb-2*(*or362*) embryo, video 3 is of a *tbb-2*(*t1623*) embryo, and video 4 is of a *tbb-2*(*gk129*) embryo.

Results

tbb-2 encodes an embryonic isoform of the microtubule subunit β-tubulin

To identify genes required for mitotic spindle orientation in *C. elegans* embryos, we screened chemically mutagenized populations of nematodes for temperature-sensitive, embryonic-lethal mutants with abnormal spindle positioning in early embryonic cells. We identified a total of sixteen mutants with defects in positioning of the first mitotic spindle. Ten have mutations in genes previously identified (data not shown), including *dnc-1* (Skop and White, 1998), *mel-26* (Dow and Mains, 1998), *zyg-8* (Gönczy et al., 2001) and *zyg-9* (Matthews et al., 1998). One mutant, *or362*, did not map near any genes known to be required for spindle positioning. The *or362* mutation is partially conditional: at 15°C, 52% of the embryos from homozygous *or362* hermaphrodites hatched ($n=716$), whereas at 25°C none of the embryos hatched ($n=752$; see Table 1). Genetic map data placed *or362* on chromosome 3, which includes *t1623*, a previously described mutation in an unidentified locus that also causes defects in spindle positioning (Gönczy et al., 1999b). The *or362* and *t1623* mutations failed to complement each other in genetic crosses, suggesting they are two mutant alleles of the same gene (data not shown). While *or362* is partially conditional (see above), *t1623* is much less conditional: 9% of *t1623* mutant embryos produced at 15°C hatched ($n=492$), while none produced at 25°C hatched ($n=228$; see Table 1). Furthermore, *or362* and *t1623* are semi-dominant mutations, as heterozygous *or362*/+ hermaphrodites produced 82% ($n=364$) hatching embryos, and *t1623*/+ hermaphrodites produced 75% ($n=232$) hatching embryos at the restrictive temperature (Table 1; see Materials and Methods).

We mapped *or362* to an interval that includes the predicted gene C36E8.5 (see Materials and Methods), previously identified by sequence as a β-tubulin gene and named *tbb-2*

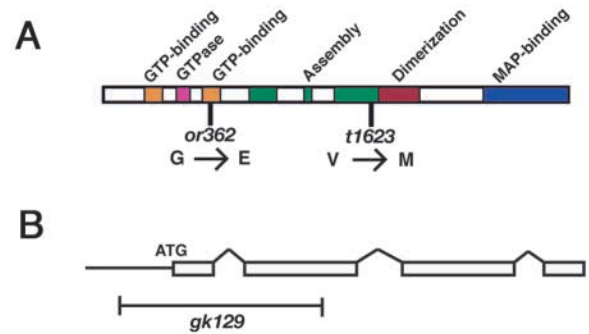


Fig. 1. Structure and homology of *C. elegans* β-tubulin. (A) β-tubulin domains indicated as described by Savage et al. (Savage et al., 1994). *or362* is a glycine to glutamic acid substitution in a GTP-binding domain, while *t1623* is a valine to methionine substitution in an assembly domain. (B) The exon/intron structure of *tbb-2* and approximate location of the deletion *gk129*.

(Gremke, 1987). We sequenced the *tbb-2* open reading frame in genomic DNA from *or362* and *t1623* mutants and found mis-sense lesions in a GTP-binding domain and in an assembly domain, respectively (Fig. 1A; see Materials and Methods). Further confirming the gene identity, TBB-2 protein levels are reduced in *or362* mutant embryos (see below).

Semi-dominant mutations in *tbb-2* disrupt meiotic and mitotic spindle function in the early *C. elegans* embryo

To characterize the defects in *tbb-2* mutant embryos, we compared the first embryonic cell division in mutant and wild-type embryos. Following fertilization and the completion of meiosis in a wild-type one-cell zygote, the maternal pronucleus migrates towards the posterior pole to meet the paternal pronucleus (Fig. 2A). Before and during pronuclear migration, the sperm pronucleus-associated centrosomes nucleate microtubules that assemble into a mitotic spindle. After the two pronuclei meet, they move in association with the two centrosomal asters towards the center of the embryo. During this process of centration, prior to nuclear envelope breakdown, the centrosome/pronuclear complex rotates approximately 90°. This rotation serves to align the mitotic spindle along the anterior-posterior axis of the embryo. During anaphase, an anterior-posterior asymmetry in forces that pull on astral MTs at the cell cortex displaces the spindle towards the posterior pole (Grill et al., 2001). Pronuclear migration, meiotic and mitotic spindle assembly and spindle positioning all require microtubules (Albertson, 1984; Hyman and White, 1987).

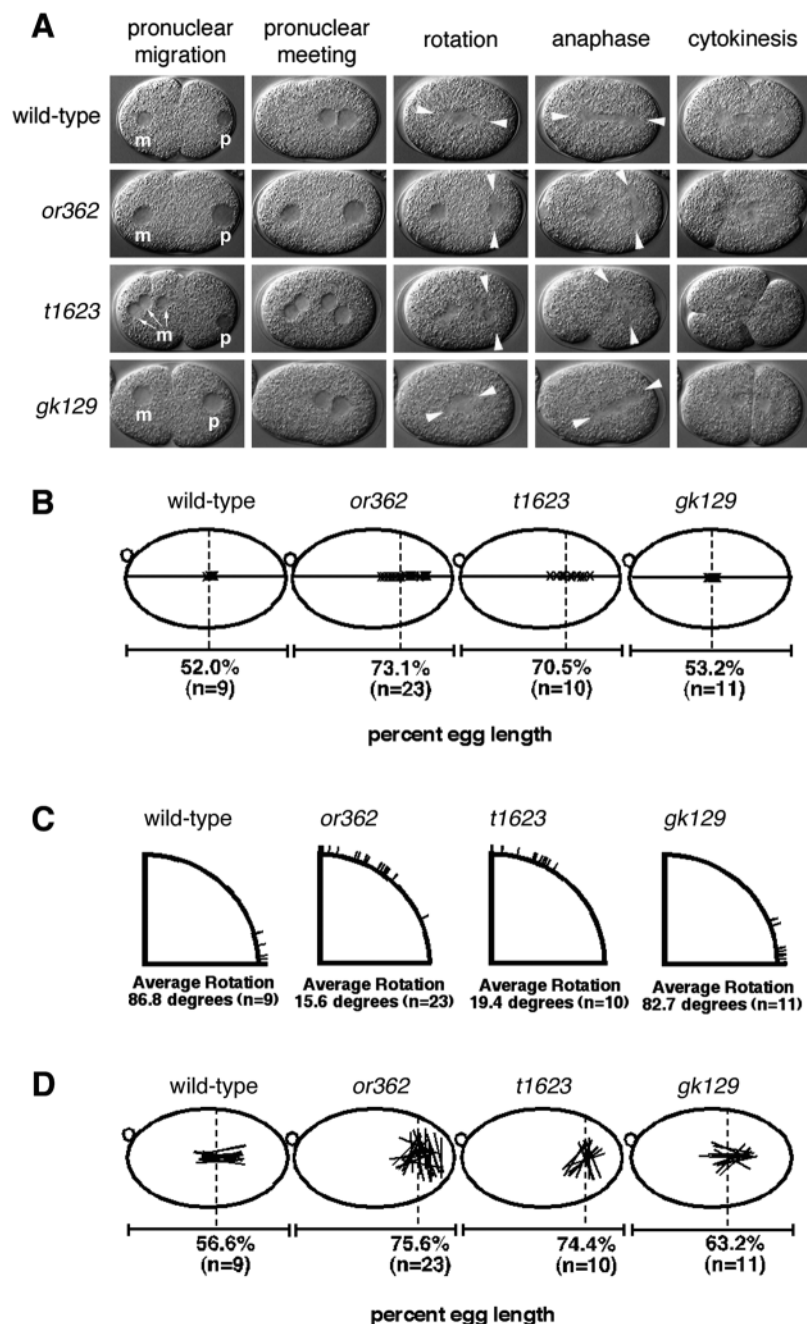
In embryos produced by homozygous *tbb-2* hermaphrodites, which were upshifted from the permissive temperature of 15°C to the restrictive temperature of 25°C as L4 larvae (hereafter referred to as *tbb-2* mutant embryos), we observed defects in meiosis, pronuclear migration and rotation of the centrosome/pronuclear complex (Fig. 2A,B; see also Movies 1-4, <http://jcs.biologists.org/supplemental/>). Pronuclei met prior to nuclear envelope breakdown (NEB) in 52% of *or362* mutant embryos ($n=23$), and in 90% of *t1623* mutant embryos ($n=10$). In contrast to the apparently weaker defect in pronuclear migration, *t1623* mutant embryos are more defective in meiosis, as inferred from the presence of multiple

maternal pronuclei in 7/9 embryos (Fig. 2A). We observed a meiosis defect in only 1/52 *or362* mutant embryos produced at the restrictive temperature.

Regardless of whether the pronuclei met in *tbb-2(or362)* and *tbb-2(t1623)* mutant embryos, the centrosomes associated with the sperm pronucleus always assembled a bipolar mitotic spindle (Fig. 2A). The centrosome/pronuclear complex failed to centrize in these *tbb-2* mutants, resulting in nuclear envelope breakdown occurring on average 73.1% egg length for *or362* embryos, and 70.5% egg length in *t1623*, as compared to 52% in wild-type embryos (Fig. 2B). Additionally, rotation of the centrosome/pronuclear complex was defective. On average, the centrosome/pronuclear complex rotated 15.6° ($n=23$) in *or362*, and 19.4° ($n=10$) in *t1623* mutant embryos, compared to 86.8°

($n=9$) in wild-type embryos (Fig. 2C). Owing to these defects in both centration and rotation in *tbb-2* mutant embryos, the midpoint between the two transversely oriented mitotic spindle poles was positioned on average at approximately 75% egg length, compared to about 57% egg length in wild type (Fig. 2D).

If the first mitotic spindle remained transversely oriented in *tbb-2* mutant embryos, an ectopic cleavage furrow bisected the spindle from the posterior pole, and a second circumferential furrow appeared near the anterior pole. These abnormal furrows usually resolved to produce two daughter cells, in 14/17 of *or362* and 5/6 *t1623* mutant embryos. In 3/17 *or362* and 1/6 *t1623* mutant embryos a third, anucleate cytoplasm was produced.



A deletion mutation is less deleterious than two semi-dominant mis-sense mutations in *tbb-2*

Although the *tbb-2* mis-sense mutations *or362* and *t1623* implicate TBB-2 in MT-dependent processes, these semi-dominant mutations probably do not eliminate TBB-2 function and may result in abnormal function. To further address the requirements for TBB-2, we obtained two deletion alleles, *tbb-2(gk129)* and *tbb-2(gk130)*, from the International *C. elegans* Gene Knockout Consortium (<http://elegans.bcgsc.bc.ca/knockout.shtml>). The *gk129* deletion removes 766 base pairs of genomic sequence that begins 328 base pairs 5' to the presumed *tbb-2* translational start site and removes approximately 37% of the coding sequences, whereas the *gk130* deletion removes 453 base pairs of genomic sequence and

Fig. 2. Microtubule-dependent processes are defective in *tbb-2* mutant embryos. (A) Nomarski differential interference contrast (DIC) micrographs of wild-type embryos beginning after the completion of meiosis II and ending during the first cytokinesis. In all panels in this and other figures, anterior is to the left and posterior to the right. m, maternal pronucleus; p, paternal pronucleus. Arrowheads mark positioning of the centrosomes during centrosome/pronuclear rotation and during anaphase (Movies 1–4, <http://jcs.biologists.org/supplemental>). (B) Centration in wild-type and *tbb-2* mutant embryos. Each X represents the position along the long axis of the midpoint between the two centrosomes of the centrosome/pronuclear complex at nuclear envelope breakdown. If the pronuclei did not meet, the measurement was taken during nuclear envelope breakdown of the centrosome/paternal pronuclear complex. (C) Extent of centrosome/pronuclear complex rotation in wild type and in *tbb-2* mutants, showing the angle of the mitotic spindle relative to the long axis just after nuclear envelope breakdown. (D) Anaphase spindle position in wild-type and in *tbb-2* mutant embryos. Each bar represents the spindle position roughly 2 minutes after nuclear envelope breakdown in one embryo. Percentage of embryo length indicates the average spindle position relative to the anterior pole of the embryo.

Table 1. Thermal sensitivity of *tbb-2* alleles

Maternal genotype	Percent hatching	
	15°C	25°C
<i>tbb-2(or362)</i>	52 (716)	0 (752)
<i>tbb-2(t1623)</i>	9 (491)	0 (228)
<i>tbb-2(gk129)</i>	76 (457)	21 (528)
<i>tbb-2(or362)/+</i>	–	82 (364)
<i>tbb-2(t1623)/+</i>	–	75 (232)
<i>tbb-2(gk129)/+</i>	–	100 (175)

10% of the coding sequences. (Fig. 1B; see Materials and Methods). Because *gk129* is a larger deletion, we chose this allele for further analysis.

In contrast to the fully penetrant embryonic lethality observed when homozygous *or362* and homozygous *t1623* hermaphrodites were raised at the restrictive temperature of 25°C following upshift from the permissive temperature, we found that homozygous *tbb-2(gk129)* and *tbb-2(gk130)* mutant strains are homozygous viable. While not absolutely essential, only 76% ($n=457$) of *gk129* mutant embryos produced at 15°C, and 21% ($n=528$) produced at 25°C hatched. Thus *tbb-2* is required for survival of most embryos produced at high temperatures. Furthermore, the *gk129* mutation is fully recessive, as 100% ($n=175$) of the embryos produced by *gk129/+* hermaphrodites raised at 25°C hatched (Table 1).

Consistent with the partial viability of *tbb-2(gk129)* mutants, we also observed less severe defects in microtubule-dependent processes during early embryogenesis at 25°C, compared to those observed in *tbb-2(or362)* and *tbb-2(t1623)* mutant embryos. Meiosis appeared normal in all *gk129* embryos examined ($n=17$). Furthermore, rotation and centration of the centrosome/pronuclear complex occurred normally in 16/17 embryos, while remaining transversely oriented in only 1 of 17 (Fig. 2A,B; see QuickTime movies in Supplemental Data).

Although early steps in spindle assembly and positioning appeared roughly normal in one-cell stage *gk129* mutant embryos, the first mitotic spindle was displaced laterally late in mitosis in 15/17 embryos (Fig. 2A,C; see also Movies 1–4, <http://jcs.biologists.org/supplemental/>). The lateral displacements we observed were highly dynamic, with the spindle moving back and forth multiple times while remaining roughly aligned with the anterior-posterior (AP) axis (Fig. 2A). Moreover, the spindle frequently moved further than normal towards the posterior pole (Fig. 2C). Thus, a deletion that may fully eliminate *tbb-2* function results in a partially penetrant and conditional embryonic lethality, with defects at the one-cell stage substantially less severe than those caused by the semi-dominant *or362* and *t1623* mis-sense mutations.

TTB-2 protein levels are reduced in *tbb-2(or362)* but not in *tbb-2(t1623)* mutants

To further investigate *tbb-2*, we obtained polyclonal antibodies that specifically recognize a C-terminal TBB-2 peptide that is unique among *C. elegans* β-tubulins (a gift from C. Lu and P. Mains; see Materials and Methods) (Lu et al., 2003). On western blots, these antibodies detected a single protein of the size predicted for TBB-2 in wild-type extracts (Fig. 3A,B; see Materials and Methods). TBB-2 levels appeared normal in extracts from *tbb-2(t1623)* worms (Fig. 3B), but was not detectable in *tbb-2(or362)* embryonic extracts made from worms grown at either 15°C or 25°C, or in embryonic extracts made from *tbb-2(gk129)* mutants (Fig. 3A; see Materials and Methods).

We next investigated to what degree mutant TBB-2 proteins can assemble into spindle microtubules by double staining fixed *tbb-2* mutant embryos with rabbit antibodies that recognize the C terminus of TBB-2 (Lu et al., 2003), and a mouse antibody that recognizes α-tubulin (Fig. 3C, see Materials and Methods). During mitosis in wild-type one-cell stage embryos, astral microtubules radiated out from both centrosomes, contacting the cell cortex at many points, with the different antibodies exhibiting full overlap in their staining of spindle microtubules. In *tbb-2(or362)* and *tbb-2(t1623)* mutant embryos stained with antibodies that recognize α-tubulin, most astral microtubules were short and did not appear to contact the cortex (Fig. 3C). These defects presumably account for the abnormalities observed in pronuclear migration and mitotic spindle positioning. Astral microtubules appeared more normal in one-cell stage *tbb-2(gk129)* mutant embryos (Fig. 3C), consistent with the much less severe spindle positioning defects we observed in time lapse Nomarski movies (see above). TBB-2 was

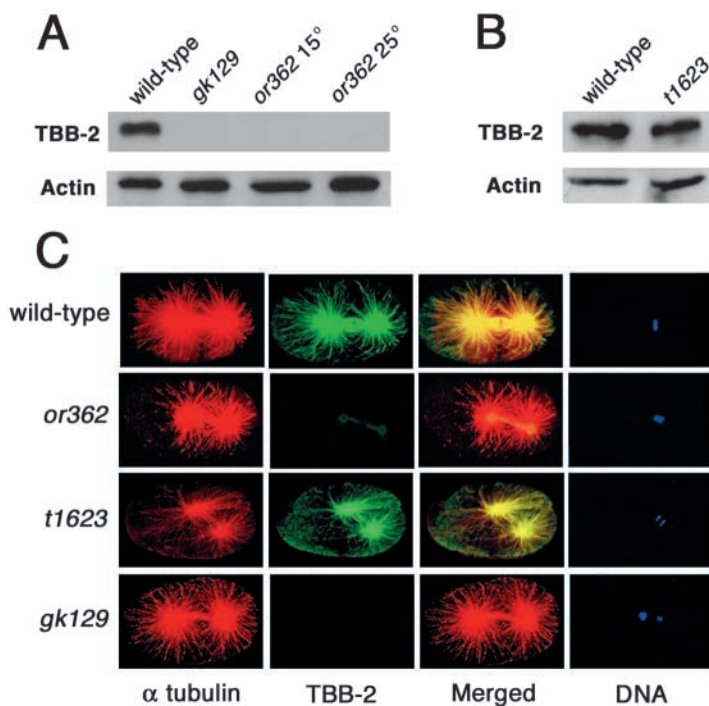


Fig. 3. Spindle microtubules and TBB-2 protein in wild-type and *tbb-2* mutant embryos and extracts. (A) Western blot showing TBB-2 levels relative to an actin loading control, in embryo extracts from wild-type, *gk129* and *or362* animals (see Materials and Methods). *or362* embryo extracts were prepared from worms matured at permissive or restrictive temperatures. (B) Western blot showing TBB-2 levels relative to an actin loading control in whole worm extracts prepared from wild-type and *t1623* animals. (C) Indirect immunofluorescence images of wild-type and *tbb-2* mutant embryos stained with antibodies that recognize α-tubulin (red) and TBB-2 (green); DNA was stained with TOTO (blue).

detected at greatly reduced levels in fixed *or362* mutant embryos, was undetectable in *tbb-2(gk129)* embryos, but appeared normal in *t1623* embryos, (Fig. 3C). Thus the mutant TBB-2 proteins are incorporated into spindle microtubules in both *or362* and *t1623* embryos. Presumably TBB-2 was not detectable in *or362* embryonic extracts on western blots (Fig. 3A) because only a small fraction of the embryonic cells are in mitosis when extracts are prepared (see Materials and Methods). It is not clear why the mis-sense mutation in *tbb-2(or362)* results in reduced protein levels, but no additional changes were detected in *tbb-2(or362)* genomic DNA after sequencing 1.19 kb 5' of the translational start site and 1.16 kb 3' of the predicted stop codon (see Materials and Methods), suggesting that the mis-sense mutation is responsible for either decreased production or stability of the encoded protein. Nevertheless, immunostaining revealed that a very small amount of TBB-2 mutant protein is produced and incorporated into microtubules in *or362* one-cell stage embryos. We conclude that the incorporation of abnormal β -tubulin subunits contributes to the microtubule defects in one-cell stage *tbb-2(or362)* and *tbb-2(t1623)* mutant embryos. However, the increased penetrance in embryonic lethality observed for *or362* and for *gk129* mutants at 25°C does not correlate with changes in TBB-2 levels (Fig. 3A), suggesting that other factors also contribute to the defects in microtubule-dependent processes.

TBB-1 protein levels are elevated in mutants with reduced levels of TBB-2

We next examined levels of TBB-1, the other *C. elegans* β -tubulin expressed in early *C. elegans* embryos (Baugh et al., 2003). We examined steady state TBB-1 levels in wild-type and mutant worm extracts after generating polyclonal antibodies, using the unique C-terminal TBB-1 peptide as an antigen (see Materials and Methods). To document the specificity of our TBB-1 antibodies, we double stained fixed embryos with antibodies that recognize both α -tubulin and TBB-1, after using 3'UTR-specific RNA interference to silence TBB-1 germline expression (see Materials and Methods). In these embryos, TBB-1 was no longer detectable (Fig. 4D). In *tbb-2(gk129)* and *tbb-2(or362)* worm extracts, TBB-1 levels were increased approximately threefold in comparison to wild-type extracts (Fig. 4A), although this increase is not temperature dependent (data not shown). In *t1623* extracts, where TBB-2 levels are wild type (Fig. 3B), TBB-1 levels are not elevated (Fig. 4B). We also stained *tbb-2(or362)* and *tbb-2(gk129)* with the TBB-1 antibodies and found that TBB-1 was still incorporated into spindle microtubules in *or362* and *gk129* mutant embryos (Fig. 4C).

Discussion

We have identified two semi-dominant mis-sense mutations in a maternally expressed *C. elegans* β -tubulin gene called *tbb-2*. Hermaphrodites homozygous for either mutation produce inviable mutant embryos with defects in microtubule-dependent processes at the one-cell stage. Defects are observed during meiosis, pronuclear migration, and mitotic spindle positioning, suggesting that *tbb-2* is involved in these processes. Although the incorporation of abnormal subunits affects multiple microtubule-dependent processes, polarity, as

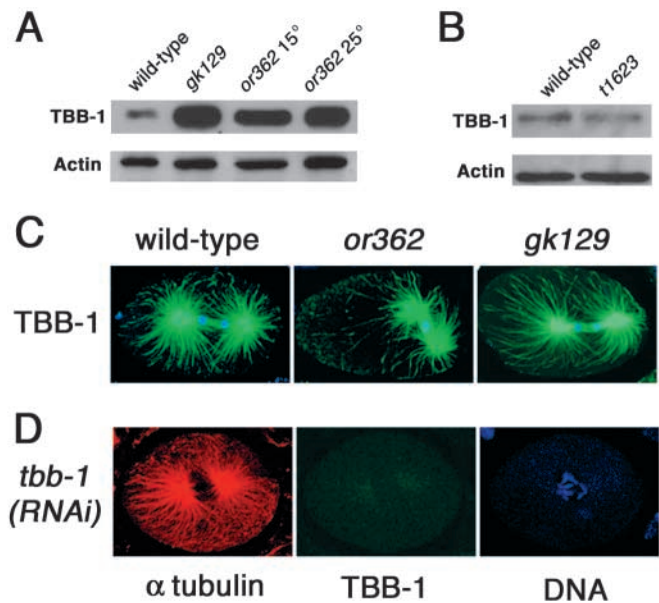


Fig. 4. TBB-1 is upregulated in the absence of TBB-2. (A) Western blots showing TBB-1 levels in wild-type and *tbb-2* embryo extracts that have reduced levels of TBB-2. Actin was used as an internal loading control. (B) Western blots showing TBB-1 levels in wild-type and *tbb-2 (t1623)* whole worm extracts, which have wild-type levels of TBB-2. (C) Fixed wild-type and mutant embryos stained with antibodies that recognize TBB-1 (green); DNA was stained with TOTO (blue). (D) Indirect immunofluorescence image of a fixed *tbb-1(3' UTR RNAi)* embryo stained with antibodies that recognize α -tubulin (red) and TBB-1 (green); DNA was stained with TOTO (blue).

measured by P-granule and PAR-2 localization, does not appear to be disrupted (data not shown). In contrast, a deletion that probably eliminates all *tbb-2* function results in a much less severe phenotype. Intriguingly, the mis-sense mutation *or362* and the deletion *gk129* result in substantially reduced levels of TBB-2, and in elevated levels of a second maternally expressed β -tubulin called TBB-1. However, neither TBB-1 nor TBB-2 levels are affected by the mis-sense mutation *tbb-2(t1623)*, suggesting that TBB-1 levels are upregulated in response to decreased levels of TBB-2 in *or362* and *gk129* mutants.

Two partially redundant β -tubulin isoforms are incorporated into microtubules in early embryonic cells in *C. elegans*

Eukaryotic organisms have multiple genes encoding isoforms of both the α - and β -tubulin microtubule subunits (Gu et al., 1988; Lewis et al., 1987; Lopata and Cleveland, 1987). While genetic studies have shown that some tubulin isotypes appear to have specialized functions, others appear to be functionally equivalent (Ludueña, 1998). The *C. elegans* genome includes nine predicted α -tubulin and six predicted β -tubulin genes. While the requirements for most have not been determined, at least some appear to be specialized in function. For example, while most microtubules in *C. elegans* contain 11-protofilaments, the *mec-7* gene encodes a non-essential β -tubulin required for the assembly of 15-protofilament

microtubules involved in touch sensitivity later in development (Chalfie and Thomson, 1982; Savage et al., 1989). In contrast, during embryogenesis, expression studies have shown that two α -tubulins (*tba-1* and *tba-2*) and two β -tubulins (*tbb-1* and *tbb-2*) are maternally expressed (Baugh et al., 2003). Our analysis shows that *tbb-2* is involved in microtubule-dependent processes of the early *C. elegans* embryo.

Because *tbb-2(or362)* and *tbb-2(t1623)* are semi-dominant and the mutant proteins they encode assemble into microtubules, while the deletion allele *gk129* is recessive and less deleterious in effect compared to *or362* and *t1623*, we conclude that the cell division defects caused by the semi-dominant mis-sense mutations are, at least partly, the result of mutant subunit incorporation altering microtubule dynamics or stability. Destabilization of MTs by nocodazole treatment (Hyman and White, 1987) and by mutations in genes that influence MT stability (Mains et al., 1990; Matthews et al., 1998; Srayko et al., 2000), have been shown to result in a similar mutant phenotype in early *C. elegans* embryos. In *tbb-2(t1623)* mutant embryos, the mutant TBB-2 is present at roughly wild-type levels, more of the mutant protein is incorporated into microtubules compared to *tbb-2(or362)* mutants, and *t1623* is slightly more dominant than *or362*. Moreover, greater incorporation of abnormal β -tubulin subunits may account for the higher penetrance of meiotic defects in homozygous *t1623* embryos. However, pronuclear migration defects are more penetrant in *or362* embryos, suggesting that the lesion in *or362* may be more deleterious in effect than the lesion in *t1623*, given that much less of the *or362* mutant protein appears to be present and incorporated into microtubules. Because the increased penetrance in embryonic lethality observed in *tbb-2(or362)* and *tbb-2(gk129)* mutants shifted to 25°C does not appear to result from changes in TBB-2 levels, microtubules may be more sensitive at higher temperatures to the incorporation of abnormal subunits or loss of an isoform.

Autoregulation of tubulin synthesis is not fully sufficient to restore normal microtubule function when one of two embryonic isoforms is absent

Studies in mammalian cell culture have shown that tubulin translation responds to altered levels of un-polymerized tubulin subunits (Ben-Ze'ev et al., 1979; Cleveland et al., 1981; Pachter et al., 1987). This autoregulation involves changes in mRNA stability that occur during tubulin translation (Cleveland, 1988), and our data confirm in vivo that tubulin isoforms are subject to feedback regulation. Although TBB-1 and TBB-2 appear to be partially redundant, and TBB-1 is upregulated in both *tbb-2(or362)* and *tbb-2(gk129)* mutants, this autoregulation does not appear to fully restore microtubule function. The upregulation does not restore normal function as effectively in *tbb-2(or362)* mutants, apparently because of the incorporation of the abnormal *or362* mutant subunits into microtubules. The *gk129* allele is probably null, with TBB-1 upregulation largely but not entirely compensating for the loss of TBB-2 protein. This insufficiency of feedback regulation to fully restore normal microtubule function could be due to feedback regulation resulting in the production of either too little or too much TBB-1 in *tbb-2(gk129)* mutants. For example, overexpression of β -tubulin can be lethal in budding

yeast (Burke et al., 1989; Weinstein and Solomon, 1990). Alternatively, TBB-1 upregulation in *tbb-2(gk129)* embryos might produce appropriate levels of β -tubulin, with defects resulting from specialized requirements for TBB-2 (Lu et al., 2003). Whatever the explanation, microtubule-dependent processes are substantially more sensitive to the loss or disruption of TBB-2 at higher temperatures.

In conclusion, the *C. elegans* β -tubulin genes *tbb-1* and *tbb-2* are partially and conditionally redundant in early embryonic cells. Both TBB-1 and TBB-2 are incorporated into microtubules, and TBB-1 protein is upregulated in response to decreased TBB-2. However, this feedback regulation is not sufficient to fully restore microtubule function when one β -tubulin isoform is absent. We suggest that such feedback regulation is important for less drastic alterations in the levels of tubulin isoforms.

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