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Levels of the ubiquitin ligase substrate adaptor MEL-26 are inversely correlated with MEI-1/katanin microtubule-severing activity during both meiosis and mitosis

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Abstract

The MEI-1/MEI-2 microtubule-severing complex, katanin, is required for oocyte meiotic spindle formation and function in *C. elegans*, but the microtubule-severing activity must be quickly downregulated so that it does not interfere with formation of the first mitotic spindle. Post-meiotic MEI-1 inactivation is accomplished by two parallel protein degradation pathways, one of which requires MEL-26, the substrate-specific adaptor that recruits MEI-1 to a CUL-3 based ubiquitin ligase. Here we address the question of how MEL-26 mediated MEI-1 degradation is triggered only after the completion of MEI-1's meiotic function. We find that MEL-26 is present only at low levels until the completion of meiosis, after which protein levels increase substantially, likely increasing the post-meiotic degradation of MEI-1. During meiosis, MEL-26 levels are kept low by the action of another type of ubiquitin ligase, which contains CUL-2. However, we find that the low levels of meiotic MEL-26 have a subtle function, acting to moderate MEI-1 activity during meiosis. We also show that MEI-1 is the only essential target for MEL-26, and possibly for the E3 ubiquitin ligase CUL-3, but the upstream ubiquitin ligase activating enzyme RFL-1 has additional essential targets.

Keywords

Meiosis; Protein degradation; Spindle; *C. elegans*

Introduction

Fertilization triggers a cascade of effects on the oocyte, including maturation, cytoskeletal rearrangements and completion of meiosis (Harris et al., 2006; McCarter et al., 1999; McNally and McNally, 2005). A dramatic change occurs a short time later during the transition from meiosis to mitosis. Meiotic and mitotic spindles form sequentially in the same cytoplasm, but differ considerably in size, morphology, cellular location and the presence of centrosomes only in the mitotic spindle (Albertson, 1984; Schatten, 1994). This transition from one type of spindle to the other necessitates strict regulation of the gene activities unique to each type of spindle: meiotic-specific spindle components must be quickly inactivated prior to mitotic spindle formation and mitotic-specific components must remain inactive until after the meiotic

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divisions are completed. This problem is particularly acute in the newly fertilized *C. elegans* embryo where the mitotic spindle forms only about 15 min after the completion of the second meiotic division (Kemphues et al., 1986; Yang et al., 2003). Recently, it has become apparent that rapid turnover of many maternally-supplied proteins is critical during the oocyte to embryo transition [reviewed in Bowerman and Kurz (2006) and DeRenzo and Seydoux (2004)].

The MEI-1/MEI-2 katanin microtubule-severing complex is an example of a protein that is required strictly for meiotic spindle formation in *C. elegans*. While essential for meiosis, MEI-1/MEI-2 must be inactivated prior to mitosis (Clark-Maguire and Mains, 1994a). During meiosis, MEI-1/MEI-2 are required to generate microtubule fragments that seed microtubule nucleation and later contribute to meiotic spindle shortening (McNally et al., 2006; Srayko et al., 2006) and these proteins are required for translocation of the meiotic spindle to the oocyte cortex (Yang et al., 2003). MEI-1 and MEI-2 localize to the meiotic spindle but disappear prior to mitosis (Clark-Maguire and Mains, 1994a; Srayko et al., 2000). *mel-26* encodes an ubiquitin E3 ligase substrate-specific adaptor essential for post-meiotic MEI-1 degradation (Dow and Mains, 1998; Furukawa et al., 2003; Pintard et al., 2003b; Xu et al., 2003). If MEI-1 degradation is blocked, e.g., in the absence of MEL-26 or in the presence of a gain-of-function (gf) *mei-1* mutation that prevents MEI-1 binding to MEL-26, MEI-1 microtubule-severing activity is then ectopically expressed during mitosis, leading to small and mis-positioned spindles (Clark-Maguire and Mains, 1994a). The question of why MEL-26 mediated MEI-1 degradation does not begin until the completion of meiosis is the subject of this paper.

Synthesis and degradation of proteins requires precise regulation to control the concentrations of active molecules present in cells at critical times. In eukaryotes, the bulk of protein degradation is performed by the 26S proteasome. Ubiquitination marks proteins for degradation by the proteasome, and ubiquitin addition involves a multienzyme pathway (Glickman and Ciechanover, 2002; Kerscher et al., 2006). The ubiquitin-activating enzyme (E1) forms a high energy thioester bond with ubiquitin, which is then passed to the ubiquitin-conjugating enzyme (E2). Ubiquitin is then transferred to the substrate by an ubiquitin-protein ligase (E3). The Cullin (CUL) family of E3 ubiquitin ligases bind to ubiquitin bound-E2 using the common Rbx subunit while substrate-specificity is provided by variable subunits, which include F-box family members for CUL-1/SCF E3 ligases and BC-box family members for CUL-2/VHL E3 ligases (Kipreos, 2005; Petroski and Deshaies, 2005). CUL-3 based E3-ubiquitin ligases use BTB family members for substrate-specificity, of which MEL-26 was a founding member (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003b; Xu et al., 2003).

There are several levels of regulation for E3 ubiquitin ligase induced protein degradation. The subunits conferring substrate-specificity to CUL-based E3 ligases are themselves subject to autodegradation, and this has been demonstrated for MEL-26 (Luke-Glaser et al., 2005; Pintard et al., 2003b). The association of the F-box substrate adaptors of CUL-1-based E3 ubiquitin ligases with their target substrates stabilizes the F-box against autoubiquitination and degradation (Galan and Peter, 1999; Wirbelauer et al., 2000). In addition, CUL-based ubiquitin E3 ligases are themselves conjugated to the NEDD8 type of ubiquitin-like molecule, which activates ubiquitin ligase activity (Parry and Estelle, 2004; Petroski and Deshaies, 2005). Cyclical neddylation/denedylation is required for activity of *C. elegans* CUL-3/MEL-26 (Pintard et al., 2003a). Another level of control includes different types of ubiquitin E3 ligases regulating one another (Bashir et al., 2004; Vodermaier, 2004; Wei et al., 2004).

Substrates are often phosphorylated prior to ubiquitination (Glickman and Ciechanover, 2002; Hunter, 2007; Petroski and Deshaies, 2005), and phosphorylation of MEI-1 by the DYRK minibrain kinase MBK-2 is required for timely degradation of MEI-1 (Ming Pang et al., 2004; Pellettieri et al., 2003; Quintin et al., 2003; Stitzel et al., 2006). MBK-2 has additional

targets in the oocyte, including the OMA-1/OMA-2 and MEX-5/MEX-6 proteins and components of the germ plasm (DeRenzo et al., 2003; Feng et al., 1999; Nishi and Lin, 2005; Shirayama et al., 2006; Stitzel et al., 2006). However, we recently found that MBK-2 activity is not necessary for MEL-26 induced MEI-1 degradation, and instead MBK-2 and MEL-26 act in parallel pathways that are both required for MEI-1 degradation (Lu and Mains, 2007). MBK-2 mediated degradation appears to be coupled with meiotic exit after which MEL-26 then completes the process of MEI-1 degradation.

The parallel MEL-26 and MBK-2 systems explain how MEI-1 microtubule-severing activity is degraded during mitosis, but this leads to the question of how MEI-1 degradation is delayed until the completion of meiosis. Stitzel et al. (2007) recently showed that the timing of the MBK-2 branch of the MEI-1 degradation pathway is regulated by the degradation of the cortical protein EGG-3 by the anaphase promoting complex (APC) as meiosis proceeds. EGG-3 degradation releases MBK-2 from the cortex, giving it access to cytoplasmic substrates. In this paper, we characterize the temporal regulation of MEL-26/CUL-3 mediated MEI-1 degradation. Immuno-localization indicates that MEL-26 protein does not accumulate to high levels until after meiosis has been completed and accumulation of high levels of MEL-26 is prevented during meiosis by another type of E3 ubiquitin ligase, one that includes CUL-2. However, the low levels of MEL-26 present during meiosis are active and function to moderate MEI-1 during that time, although ectopic high levels of MEL-26, like premature release of cortical MBK-2, are not sufficient to block MEI-1 function. Finally, we show that MEI-1 is the major target of MEL-26, and probably also of CUL-3, while RFL-1, an enzyme in the neddylation pathway that activates ubiquitin ligases, has additional essential targets.

Materials and methods

Strains and culture conditions

C. elegans strains were cultured at 15 °C as described by Brenner (1974). L4 stage animals were upshifted to the appropriate temperatures, and complete broods were scored for hatching as described in Mains et al. (1990b). Between 500 and 5000 embryos from at least four hermaphrodites are reported for each strain. The following alleles were used: LG I, *mei-1* (*ct46* and *ct46ct101*), *mei-2* (*sb39*, *ct98* and *ct102*), *mel-26* (*ct61* and *ct61sb4*), *unc-29* (*e1072*); LG II, *emb-27* (*g48*); LG III, *tbb-2* (*sb26*), *rft-1* (*or198*), *cul-2* (*ek1*), *pod-1* (*ye11* and *or548*), *lon-1* (*e185*), *dpy-18* (*e364*); LG IV, *mbk-2* (*dd5*), *dpy-20* (*e1280*). Mutations were often linked to morphological mutations listed above to ease strain construction, and the chromosomal translocation hT2(I;III) (Zetka and Rose, 1992) was used to balance *cul-2* and *mel-26*.

Antibody production

Fragments of *mel-26* cDNA were amplified by RT-PCR using Superscript II (Invitrogen) on gravid hermaphrodite poly(A)⁺ RNA isolated with the FasTrack mRNA Isolation System (Invitrogen). A 366 bp fragment was then PCR amplified with Herculase (Stratagene) and forward (gggatccccgctttgctcattgctgccgat) and reverse (ctgaatt-cattacagtcagatggagtg) primers. A 230 bp region was amplified by substituting gggatcctgagaaaacgagctgttacg for the former forward primer. Both fragments were cloned into pGEX-3X (Pharmacia) and transformed into BL21 CodonPlus (DE3)-RP to produce glutathione S-transferase (GST) fusion proteins. Recombinant protein was isolated using a glutathione Sepharose 4B column (Pharmacia Biotech) with EDTA-free protease inhibitor cocktail (Roche) added to the re-suspended bacterial pellet according to the GST Gene Fusion System (Pharmacia Biotech). The larger fusion protein was excised from an SDS-PAGE gel and injected into rats or rabbits (Southern Alberta Cancer Research Institute and the Animal Resource Centre, University of Calgary). The smaller MEL-26 protein was coupled to cyanogen bromide (CNBr)-activated

Sepharose (Pharmacia Biotech). Antiserum was affinity purified by passage through an immobilized *E. coli lysate* affinity column (Pierce) and then through the column containing the smaller MEL-26 fragment before use.

Microscopy

Temperature sensitive (*ts*) strains were upshifted 18–24 h prior to fixation, except for stains with *emb-27*, which were upshifted for 2 h to avoid degeneration of embryos. Embryos were fixed with methanol-acetone (Miller and Shakes, 1995) and blocked with either goat and/or donkey 20% normal serum. Rat anti-MEL-26 antibodies (1:50) and mouse anti- α -tubulin monoclonal antibody (Sigma clone M1A, 1:200) were applied for 1 h at 37 °C, indocarbocyanine (Cy3; Jackson ImmunoResearch) donkey anti-rat and fluorescein (FITC, TAGO Inc.) goat anti-mouse (both at 1:100) were incubated at room temperature for 1 h. DNA was then stained with 1 μ g/ml 4',6-diamidino-2'-phenylindole dichloride (DAPI, Roche) for 20–30 s, and specimens were mounted in Slowfade (Molecular Probes). Images were collected by a Hamamatsu Orca ER digital camera using Axiovision 3.0. Z-stacks (1 μ m per slice) were taken from a Zeiss Axioplan 2. Images were processed by digital deconvolution using the Axiovision 3.0 constrained iterative algorithm and processed with Adobe Photoshop. Where a uniform black background was desirable, the non-staining regions outside of the embryo have been digitally removed. Meiotic stages were judged based upon the number of DAPI stained polar bodies.

In utero spindle imaging

Wild-type and *mei-1(ct46)* worms expressing GFP-tubulin were anesthetised, mounted and subjected to time-lapse fluorescence microscopy as described previously (McNally et al., 2006). Images were captured at 15 second intervals with a Photometrics Quantix CCD controlled by IP Lab software using shuttered, attenuated Hg-arc illumination and a Nikon Microphot SA wide field microscope with a 60X Plan Apo 1.4 objective. Spindle length was determined from individual time-lapse images using the Measure Length function of IP Lab Spectrum.

RNAi

Feeding RNAi was done using the library described by Kamath et al. (2003). L4 animals were grown on bacteria induced to produce the appropriate double stranded RNA for 24 h, and were transferred to fresh plates spread with RNA producing bacteria. Broods from the second set of plates were reported.

Results

MEL-26 does not accumulate to high levels until the completion of meiosis

mel-26 encodes a maternally required protein whose mRNA is present predominantly in the gonad of hermaphrodites and in fertilized embryos (Dow and Mains, 1998). MEL-26 acts as the substrate-specific adaptor for the CUL-3 based E3 ubiquitin ligase to down-regulate MEI-1 microtubule-severing activity when meiosis is completed (Clark-Maguire and Mains, 1994a; Furukawa et al., 2003; Pintard et al., 2003b; Xu et al., 2003). However, MEL-26 mediated degradation of MEI-1 must not occur until MEI-1 fulfills its meiotic functions. To address the timing of MEL-26 function, we generated a polyclonal antibody against the C-terminal region of MEL-26 to determine when and where it is present in the newly fertilized embryo.

MEL-26 was detected in a punctate cytoplasmic pattern in the newly fertilized embryo. Levels are low during meiosis I and II (Figs. 1A–D), consistent with the requirement for MEL-26 for viability only following completion of meiosis (Mains et al., 1990b). MEL-26 levels

immediately increase when the pronuclei form after meiosis (Figs. 1E, F and 2A), and high levels of MEL-26 were present throughout the early mitotic divisions (Figs. 1G–N). As shown in Fig. 2G, among embryos in meiosis I or meiosis II, we found that a minority of embryos (5/17 and 3/17, respectively), had levels of MEL-26 noticeably higher than that shown in Figs. 1A–D, and none had levels comparable to post-meiotic embryos. In contrast, after completion of meiosis, 28/30 embryos had high levels of MEL-26 staining (Fig. 2G). In multicellular embryos, MEL-26 is enriched at the membranes between cells (Figs. 1K–N and Luke-Glaser et al., 2005). MEL-26 expression remained strong at the 16-cell stage, but decreased to low levels by the 64-cell stage (data not shown).

To demonstrate antibody-specificity, we used the genetic null allele *mel-26(ct61sb4)* (Lu and Mains, 2007). This mutation encodes a truncated protein missing the terminal 75 amino acids (Dow and Mains, 1998), which contains the region used to affinity purify the antisera (Materials and Methods). As shown in Figs. 10, P, no MEL-26 staining was apparent in embryos with this null mutation. This demonstrates that the low meiotic levels of MEL-26 seen in Figs. 1A–D and 2A represent a true signal. We conclude that the timing of the post-meiotic disappearance of MEI-1 corresponds to the period when MEL-26, as measured by immunostaining, accumulates to higher levels (*C. elegans* meiotic stage embryos cannot be isolated for Western analysis to independently quantify MEL-26 levels).

Timing of MEL-26 accumulation is not regulated by autodegradation

We next sought to identify the factors that regulate the accumulation of MEL-26 during meiosis. Many ubiquitin substrate targeting subunits are themselves regulated by autoubiquitination and degradation, and this has been demonstrated by accumulation of higher levels of MEL-26 in *cul-3(RNAi)* embryos (Luke-Glaser et al., 2005; Pintard et al., 2003b). While we also observed higher levels of MEL-26 in post-meiotic *cul-3(RNAi)* embryos consistent with previous reports, we did not observe higher levels of MEL-26 during meiosis (data not shown). Thus autodegradation affects the levels, but not the timing, of post-meiotic MEL-26 accumulation. Likewise, the presence of the substrate can stabilize ubiquitin ligase substrate adaptors (Galan and Peter, 1999; Wirbelauer et al., 2000); however, we found MEL-26 accumulation was not altered in the background of the null allele *mei-1(ct46ct101)* (data not shown).

The CUL-2 ubiquitin ligase is required to regulate meiotic levels of MEL-26

cul-2 encodes another E3 ligase implicated in many processes in the newly fertilized *C. elegans* embryo, including meiotic progression (Feng et al., 1999; Liu et al., 2004; Sonnevile and Gonczy, 2004; Vasudevan et al., 2007; Zhong et al., 2003). While a *cul-2* null allele had no obvious effect on post-meiotic levels of MEL-26, we found that this mutation resulted in higher MEL-26 levels in 16/24 embryos in meiosis I (Fig. 2B). As *cul-2* embryos proceed from meiosis I to meiosis II, there was an increase in the proportion of embryos with high levels of MEL-26 staining compared to wild type (Fig. 2G).

rfl-1 is part of the heterodimeric E1 activating enzyme in the ubiquitin-like neddylation pathway that activates ubiquitin E3 ligases. During mitosis, RFL-1 activates the E3 ligase containing CUL-3 and MEL-26 to degrade MEI-1 (Kurz et al., 2002; Pintard et al., 2003a). *rfl-1* mutants, like *cul-2* mutants, had increased accumulation of MEL-26 during meiosis (Fig. 2C). The increase in MEL-26 accumulation seen in both *rfl-1* and *cul-2* mutant embryos suggests that during meiosis *rfl-1* might regulate CUL-2 based E3 ligases.

Two other genes previously implicated in MEL-26 function were also examined. MBK-2 has been shown to act in parallel with MEL-26 for MEI-1 degradation (Lu and Mains, 2007) and is also required for the coordinated regulation of several maternally-provided proteins (Ming

Pang et al., 2004; Nishi and Lin, 2005; Pellettieri et al., 2003; Quintin et al., 2003). *mbk-2* mutant embryos had no effect on the timing of MEL-26 expression (data not shown), consistent with our previous observation that *mbk-2* and *mel-26* act independently of one another (Lu and Mains, 2007). MEL-26 membrane localization requires POD-1 (Luke-Glaser et al., 2005), an actin-binding protein (Rappleye et al., 1999). However, *pod-1* mutants did not affect the temporal pattern of MEL-26 expression (data not shown).

In summary, MEL-26 levels are kept low during meiosis through the action of a CUL-2 based ubiquitin ligase, which in turn may be regulated by RFL-1 mediated neddylation.

CUL-2 regulates the accumulation of MEL-26 during meiosis

We next asked if the increased levels of MEL-26 seen in *rfl-1* and *cul-2* mutants requires progression through meiosis, or if fertilization *per se* triggers increased MEL-26 accumulation. Embryos mutant for *emb-27*, which encodes the APC subunit APC-6, arrest at metaphase of meiosis I (Golden et al., 2000). These embryos expressed low levels of MEL-26 (Fig. 2D), indicating that induction of higher MEL-26 levels requires at least passage through the first meiotic division.

Mutations in *cul-2* result in a prolonged meiosis II (Liu et al., 2004; Sonnevile and Gonczy, 2004), and so it is possible that the increased levels of meiotic MEL-26 in *cul-2* mutants are misleading. Many mutant *cul-2* meiotic embryos could simply be chronologically older than the corresponding wild-type meiotic embryos. Similarly, we have found that mutant *rfl-1* embryos also appear to have an extended meiosis (J. Johnson and E. Raharjo, unpublished), which might explain the ectopic meiotic MEL-26 observed in *rfl-1* mutants. To test whether increased chronological age leads to the premature meiotic increase in MEL-26 accumulation, we examined *zyg-11* mutants. The *zyg-11* gene encodes the substrate adaptor that acts with CUL-2 to control meiotic progression (Vasudevan et al., 2007). Although *zyg-11* mutants also display a prolonged meiosis II (Liu et al., 2004; Sonnevile and Gonczy, 2004), these mutant embryos did not show increased meiotic MEL-26 levels (Fig. 2E). This result argues that CUL-2 and RFL-1 may play a direct role in MEL-26 regulation, and additionally that ZYG-11 is not the substrate adaptor for MEL-26.

Another way to test the possibility that CUL-2 is involved in regulating MEL-26 other than by altering meiotic timing is to examine *emb-27*; *cul-2* double mutants. If CUL-2 is actively involved in MEL-26 regulation prior to passage through meiosis II, then the high levels of meiotic MEL-26 in *cul-2* mutants would be epistatic to the low levels seen in *emb-27* APC mutants blocked at meiosis I. As shown in Figs. 2B, D, F, G, we found that *cul-2(+)* function is required to prevent MEL-26 accumulation in *emb-27* mutant embryos. Thus CUL-2 is responsible for keeping MEL-26 levels low during meiosis, through a mechanism independent of its role in meiotic progression.

The premature increase in meiotic MEL-26 in *cul-2* mutants could lead to a decrease in MEI-1 levels during meiosis. By scoring levels of cytoplasmic anti-MEI-1 staining in meiotic embryos we determined that meiotic MEI-1 staining in *cul-2* mutant embryos did not decrease. Compared to later mitotic stage embryos, *cul-2* meiotic embryos (9/11) showed higher levels of MEI-1 (Fig. 3B). This was similar to wild type, where 21/26 meiotic embryos displayed higher cytoplasmic MEI-1 staining compared to later-stage embryos (Fig. 3A). Interestingly, both meiotic and mitotic MEI-1 levels appeared higher in *cul-2* mutants than corresponding wild-type embryos, consistent with the previous report that *cul-2(+)* is required for efficient MEI-1 degradation (Stitzel et al., 2006). Thus any increase in MEI-1 degradation in *cul-2* mutants due to ectopic MEL-26 may be offset by loss of CUL-2 dependent MEI-1 degradation. In this scenario, CUL-2 rather than MEL-26 is limiting in *cul-2* mutants.

Other factors also might limit meiotic MEI-1 degradation when MEL-26 is ectopically expressed in *cul-2* mutants. One candidate is MBK-2, a component of the pathway that degrades MEI-1 in parallel to MEL-26. Ectopic MBK-2 expression in *egg-3* mutants leads to decreased levels of cytoplasmic MEI-1:GFP during meiosis, resulting in meiotic embryos with the same level of cytoplasmic MEI-1 as later-stage mitotic embryos (Stitzel et al., 2007). As was observed with MEI-1:GFP, we found that cytoplasmic anti-MEI-1 staining was reduced in *egg-3(RNAi)* in 14/20 meiotic embryos (Fig. 3C). However, it should be noted that meiotic spindles still formed in *egg-3(RNAi)* embryos indicating that ectopic MBK-2 alone is not sufficient to block MEI-1 function [Fig. 3C and Maruyama et al. (2007) and Stitzel et al. (2007)]. To test the possibility that both the MBK-2 and MEL-26 pathways are required to prevent MEI-1 dependent meiotic spindle formation, we examined *egg-3(RNAi); cul-2*. These embryos resembled both of the *egg-3(RNAi)* and *cul-2* single mutants. 7/10 meiotic embryos showed decreased levels of cytoplasmic MEI-1 during meiosis similar to those seen in mitosis, like *egg-3* alone, indicating premature loss of cytoplasmic MEI-1. MEI-1 levels in *egg-3(RNAi); cul-2* embryos were increased during both meiotic and mitotic divisions, as seen in *cul-2* single mutants. However, MEI-1 levels were still sufficient for meiotic spindle formation (Fig. 3D). Therefore factors, which may include CUL-2, appear to be required in addition to MBK-2 and MEL-26 to eliminate sufficient MEI-1 to prevent meiotic spindle formation.

MEL-26 has a meiotic function

The low levels of MEL-26 present at meiosis are clearly above the background seen in a *mel-26* null mutant (Fig. 1, A–D compared to O–P). Therefore, we asked if MEL-26 has a meiotic function, perhaps in moderating MEI-1 activity. Any meiotic function of MEL-26 examined by loss of function mutations would be obscured by the subsequent mitotic lethality caused by ectopic MEI-1 expression, therefore we examined double mutants of a null *mel-26* allele with *tbb-2(sb26)*. This mutant β -tubulin rescues lethality by partially inhibiting ectopic MEI-1 severing of mitotic microtubules (Lu et al., 2004). Among the survivors of this strain, the appearance of males is a measure of their meiotic abnormalities: while autosomal meiotic nondisjunction leads to lethality, nondisjunction of the X chromosome instead results in viable males. We have previously used the high incidence of males (Him) phenotype as a sensitive metric of *mei-1* function (Clandinin and Mains, 1993; Clark-Maguire and Mains, 1994a; Lu and Mains, 2005). Among self progeny of wild-type hermaphrodites (Hodgkin et al., 1979) and *tbb-2(sb26)* (Lu et al., 2004), males are rare, ~0.2% of the progeny. However, we found approximately a 6-fold increase to 1.3% among the surviving *mel-26(null); tbb-2(sb26)* progeny. Similar results (1.4% male) were seen for *mei-1(ct46); tbb-2(sb26)*. *ct46* is an allele that encodes a protein resistant to MEL-26 degradation (Clark-Maguire and Mains, 1994a; Pintard et al., 2003b; Xu et al., 2003), suggesting that MEI-1 is the relevant MEL-26 target leading to the high incidence of males.

To determine if the meiotic abnormalities seen in the absence of *mel-26* resulted in increased MEI-1 activity during meiosis, we measured the length of meiotic spindles in live *mei-1(ct46)* embryos that have the form of MEI-1 that appears completely resistant to MEL-26 action (Lu and Mains, 2007). The genetic behavior of *mei-1(ct46)* is identical to loss of *mel-26* (Dow and Mains, 1998; Mains et al., 1990a). Since partial loss of MEI-1 activity results in metaphase meiotic spindles that are longer than wild type (McNally et al., 2006), excess MEI-1 activity due to loss of MEL-26 inhibition in *mei-1(ct46)* should result in meiotic spindles that are shorter than wild type. Indeed, time-lapse imaging of GFP-tubulin in *mei-1(ct46)* embryos revealed meiotic metaphase spindles that were significantly shorter ($p < 0.005$) than wild type (Fig. 4). Following completion of metaphase, *C. elegans* meiotic spindles undergo a period of shortening and rotation, and by this time mutant and wild-type spindles were of comparable sizes.

As an independent test of MEL-26 regulation of meiotic MEI-1, we examined genetic interactions between these genes. If low levels of MEL-26 do indeed antagonize MEI-1 function in meiotic spindles, then decreasing MEL-26 activity should compensate for mutations with sub-optimal levels of microtubule-severing activity. To investigate this possibility, we made use of partial loss-of-function alleles of *mei-2* (Mains et al., 1990a; Srayko et al., 2000). Since MEI-1 and MEI-2 are co-dependent on one another for localization (Clark-Maguire and Mains, 1994a; Srayko et al., 2000) and MEI-1 requires MEI-2 for microtubule-severing activity (McNally et al., 2006), we reasoned that decreasing the level of MEI-1 degradation with a *mel-26* mutant might lead to increased levels of MEI-2, thereby rescuing the meiotic lethality caused by *mei-2* partial loss-of-function mutations. Indeed, when we partially lowered the level of *mel-26* using a strain heterozygous for the dominant-negative (*dn*) allele *mel-26(ct61)* (Mains et al., 1990b), we increased the hatching of the temperature-sensitive (*ts*) allele *mei-2(sb39)* at all temperatures tested (Table 1, lines 1–3). We further lowered microtubule-severing activity using a strong *mei-2* allele in combination with *mei-2(sb39)* and again observed rescue with the addition of *mel-26(dn)/+* (lines 4–5). Finally, to guard against possible background effects in the strains, we substituted a less *ts* *mei-2* allele, *ct98, for sb39* and the *mei-1(ct46)* allele that is refractory to MEL-26 regulation in place of *mel-26(dn)*. We again saw a rescue of the meiotic lethality (lines 6–8). We conclude that the low levels of MEL-26 in meiosis appear to antagonize MEI-1/MEI-2 microtubule-severing activity in the meiotic spindle. [As previously observed (Mains et al., 1990a), the genetic interaction was reciprocal, decreasing the level of *mei-2* suppressed the mitotic lethality caused by *mel-26* and *mei-1(gf)* (Table 1)].

Thus, based on increased levels of nondisjunction, altered spindle morphology and genetic interactions, we conclude that MEL-26 moderates MEI-1 activity during meiosis.

We next asked whether the MBK-2 pathway of MEI-1 degradation is likewise active during meiosis. Using a similar strategy to that described above, we asked whether a *ts* allele of *mbk-2* can rescue *mei-2* hypomorphs. At 24 °C, where *ts* alleles of both genes were strongly compromised, the hatching rate of the double mutant increased from 2.6% to 17% (Table 1, lines 9–10) clearly demonstrating rescue of *mbk-2* lethality. This is expected because *mbk-2* lethality results in part due to ectopic mitotic MEI-1, and by lowering *mei-2* hatching increases (Quintin et al., 2003). Here we are concerned with the reciprocal genetic interaction, asking if lowering *mbk-2* in turn increases the viability of *mei-2* hypomorphs. The levels of hatching for *mei-2(ts)* (Table 1, lines 2 and 10) and *mei-2(ts)/mei-2(0)* (lines 6 and 11) were similar with or without *mbk-2(ts)* at all temperatures tested. Unless one assumes that *mei-2* rescued 100% of the lethality due to *mbk-2* (which seems unlikely, especially given MBK-2's many other targets), then these values indicate that the reciprocal suppression of *mei-2* meiotic lethality by *mbk-2* was occurring, preventing increased lethality in the double mutant. For example, if decreased *mei-2* suppressed half of the *mbk-2* lethality (so ~50% hatching is then the theoretic maximum for hatching due to lethality caused by other MBK-2 targets), the observed hatching frequency of *mei-2; mbk-2* indicates an approximately two-fold suppression of the lethality seen in the *mei-2(ts)* single mutant. However, because we do not know the extent of suppression of *mbk-2* by *mei-2*, we cannot come to a firm conclusion as to whether MBK-2, like MEL-26, also moderates MEI-1/MEI-2 activity during meiosis.

MEI-1 is the major target of MEL-26 and CUL-3, but RFL-1 has additional targets

MEL-26 has an essential role in regulating MEI-1 (Clark-Maguire and Mains, 1994a) but MEL-26 also interacts with the actin-binding protein POD-1 and the katanin-related protein FIGL-1 (Luke-Glaser et al., 2005; Luke-Glaser et al., 2007). However, only the interaction between MEI-1 and MEL-26 appears to be essential, leading to complete lethality when this interaction is lost. Data in Table 2 (lines 1 and 2) shows that *mel-26* lethality is substantially

rescued by *tbb-2 (sb26)*, a tubulin mutation that partly inhibits MEI-1 microtubule severing (Lu et al., 2004). *mei-1(ct46)*, which encodes a product that appears to be completely refractory to MEL-26 regulation (Lu and Mains, 2007), is suppressed by *tbb-2(sb26)* to a greater extent than the *mel-26* null (lines 3 and 4), indicating that misregulation of other MEL-26 targets may contribute some incompletely penetrant lethality. However, we previously reported that a series of *mei-2* and *mei-1(dn)* alleles that suppress *mei-1(ct46)* also suppress two different *mel-26* mutations to a similar extent (Dow and Mains, 1998; Mains et al., 1990a). Thus, MEL-26 regulation of targets other than MEI-1 is not essential for viability.

Loss of *cul-3*, *rfl-1* and *mel-26* all leads to similar mitotic defects due to ectopic MEI-1 expression during mitosis (Clark-Maguire and Mains, 1994a; Kurz et al., 2002). We asked whether *cul-3* and *rfl-1* have essential targets in addition to MEI-1. As shown in Table 2 (lines 5 and 6), *rfl-1* lethality was not rescued by *tbb-2(sb26)*, the suppressor of ectopic MEI-1 activity. Similarly, the *mei-2* hypomorph *ct98*, which efficiently suppresses *mel-26(dn)* (Mains et al., 1990a), also failed to rescue *rfl-1* (line 7). Thus RFL-1 must have essential targets in addition to MEI-1. A mutation is not available for *cul-3*, but a substantial level of *cul-3 (RNAi)* lethality was rescued by *tbb-2(sb26)* (lines 8 and 9), demonstrating that MEI-1 is indeed the major target of CUL-3. However, because *cul-3(RNAi)* did not lead to complete lethality, there could be other essential targets that would only be revealed in situations where *cul-3* is null. We conclude that MEL-26, and likely CUL-3, have MEI-1 as their only essential target while RFL-1 must regulate essential pathways in addition to MEL-26/MEI-1.

Discussion

Upon fertilization, the embryo shifts from one developmental program, oogenesis, to another, zygotic development. One of the first events initiated is the completion of meiosis. In the *C. elegans* embryo, meiosis resumes from its prophase arrest, and the two divisions are rapidly completed (Albertson and Thomson, 1993; Kempthues et al., 1986; Yang et al., 2003). The zygote then transitions to another form of cell division, mitosis. Because the meiosis to mitosis transition is rapid in *C. elegans*, lasting only about 15 min, the regulation of meiotic-specific components such as the katanin microtubule-severing complex MEI-1/MEI-2 is especially critical. Post-meiotic MEI-1 degradation requires two independent pathways (Lu and Mains, 2007), one involving the MEL-26 substrate adaptor for the CUL-3 based ubiquitin ligase and another that includes the MBK-2 DYRK kinase. While MBK-2 has many other targets (DeRenzo et al., 2003; Feng et al., 1999; Nishi and Lin, 2005; Shirayama et al., 2006; Stitzel et al., 2006), MEL-26 (and likely CUL-3) have MEI-1 as their only essential target in the early embryo (Table 2).

Downregulation of MEI-1 by proteolysis presents the embryo with the problem of maintaining sufficient MEI-1 levels until the completion of meiosis. Recently Stitzel et al. (2007) demonstrated that, at fertilization, MBK-2 is sequestered to the cortex by EGG-3, a protein required for several other aspects of embryo activation (Maruyama et al., 2007). Meiotic APC activity then degrades EGG-3, releasing MBK-2 and giving it access to MEI-1 in the cytoplasm. Here we have described the regulation of the parallel, MEL-26 dependent pathway for MEI-1 degradation (Fig. 5). We find that MEL-26 levels are kept low in meiosis by the activity of another type of ubiquitin ligase that includes CUL-2 as well as by RFL-1, a protein that mediates ubiquitin ligase neddylation and activation. We also show that MEL-26 has a previously undescribed role during meiosis in moderating MEI-1 activity.

The level of MEL-26, as assessed by antibody staining, is low (but non-zero) during meiosis but then increases dramatically (Figs. 1–3). This dramatic increase likely is required because during mitosis MEL-26 does not have just a subtle role in moderating MEI-1 activity as it did during meiosis, but rather MEL-26 takes part in the total elimination of MEI-1 activity

following meiosis. MBK-2 activation occurs during meiosis (Stitzel et al., 2007; Stitzel et al., 2006) while the increase in MEL-26 levels appears to be delayed until meiosis is complete. This is consistent with our previous hypothesis that MBK-2 mediated MEI-1 degradation may link meiotic progression with a first wave of MEI-1 degradation, after which the MEL-26 pathway is required to complete the process (Lu and Mains, 2007).

While substrate adaptors, including MEL-26, are frequently targets of autodegradation, we found that a block to autodegradation alters the level (Luke-Glaser et al., 2005) but not the timing of MEL-26 expression. Instead, maintenance of low MEL-26 levels requires a CUL-2 based ubiquitin ligase: in *cul-2* mutants, MEL-26 accumulates to high levels at meiosis (Fig. 2). Since *cul-2* mutant embryos have a prolonged meiosis II (Liu et al., 2004; Sonnevile and Gonczy, 2004), it was possible that the higher levels of MEL-26 in this background resulted indirectly because embryos still in meiosis were simply chronologically older and so had more time to accumulate MEL-26. We determined that this was not the case because a mutation in *zyg-11*, the gene that functions with *cul-2* to regulate meiotic progression (Vasudevan et al., 2007), did not show premature MEL-26 accumulation. In addition, embryos blocked at meiosis I by the APC mutant *emb-27*, which by themselves have low MEL-26 levels, require CUL-2 activity to maintain those low levels (Figs. 2 and 3). While the relationship between CUL-2 and MEL-26 could be indirect, the simplest model is that prior to the end of meiosis, CUL-2 directly targets MEL-26 for degradation (Fig. 5). Progression through meiosis results in the inactivation of CUL-2 regulation of MEL-26, allowing accumulation of higher levels of MEL-26 in the embryo. Since CUL-2 is active against many other substrates in the developing embryo (Bowerman and Kurz, 2006; DeRenzo and Seydoux, 2004; Kipreos, 2005), the inhibition of CUL-2 induced degradation of MEL-26 may occur by the inactivation of a CUL-2 substrate-specific adaptor that targets MEL-26 rather than an inactivation of CUL-2 itself. Regulation of one ubiquitin ligase by another, as we see here for CUL-2 and CUL-3, has been described in other systems, such as CUL-1 by the APC ubiquitin ligase (Bashir et al., 2004; Vodermaier, 2004; Wei et al., 2004). During *C. elegans* sex determination, the FEM proteins provide substrate-specificity for CUL-2 mediated degradation of the terminal sex determination factor for TRA-1 (Starostina et al., 2007), and FEM proteins are themselves the target of a CUL-1 based ubiquitin ligase (Jager et al., 2004).

Meiotic spindle abnormalities might be expected in *cul-2* mutants due to increased MEL-26 accumulation, resulting in increased MEI-1 degradation. However, Liu et al. (2004) reported normal meiotic spindles in *cul-2* mutants, implying that MEL-26 is not rate limiting at that time, and we did not observe premature MEI-1 degradation (Fig. 3). However, in addition to increasing meiotic MEL-26, loss of *cul-2* also delays MEI-1 degradation [Stitzel et al. (2006) and B. Gavinolla and P. Mains, unpublished], and so these opposing effects of *cul-2* loss may cancel one another. Alternatively, although *mel-26* mutants demonstrate that the protein is necessary for meiotic MEI-1 regulation, an increase of MEL-26 alone might not be sufficient. The same appears to be true for MBK-2, whose ectopic expression during meiosis (alone or in combination with ectopic MEL-26) is also not sufficient to block MEI-1 dependent meiotic spindle formation. Thus other factors (e.g., CUL-2) might be limiting. Finally, MEI-1 might be resistant to MEL-26/MBK-2 degradation when assembled into a spindle and so efficient elimination of MEI-1 may only occur upon spindle disassembly once meiosis is completed.

Neddylation was the only other pathway that we found to regulate MEL-26. RFL-1 is a component of the Nedd-8 E1 complex that leads to ubiquitin ligase activation by coupling the Nedd8 ubiquitin-like molecule to the cullins (Parry and Estelle, 2004; Petroski and Deshaies, 2005). Previously described roles for RFL-1 in *C. elegans* included CUL-3/MEL-26 activation and early embryo interphase progression (Kurz et al., 2002; Pintard et al., 2003a). Here we find that RFL-1 has an earlier role, likely in activating CUL-2 mediated degradation of

MEL-26. Similar to *cul-2* mutants, *rfl-1* mutants resulted in premature MEL-26 accumulation (Fig. 2), and so the neddylation pathway likely acts on both CUL-2 and CUL-3.

While post-meiotic MEL-26 levels dramatically increase, lower amounts of the protein are clearly present during meiosis (Figs. 1 and 2). We have presented three independent lines of evidence that MEL-26 functions to moderate meiotic MEI-1 activity prior to its degradation of MEI-1 during mitosis. First, partial loss of *mel-26* rescues *mei-2* hypomorphic mutations, indicating that MEL-26 normally antagonizes meiotic MEI-2 (and by implication, MEI-1) activity (Table 1). Second, the absence of MEL-26 activity towards MEI-1 leads to hyperactive MEI-1 microtubule severing as evidenced by shortened metaphase spindles at both meiosis I and II (Fig. 4). Third, these shortened spindles are not fully functional since they result in higher levels of nondisjunction. MBK-2 may also moderate meiotic MEI-1 activity during meiosis, although the results were less definitive (Table 1). The opposite situation, hypoactive MEI-1, also leads to chromosome segregation defects (Clandinin and Mains, 1993; Clark-Maguire and Mains, 1994b), indicating that the levels of meiotic MEI-1 must be maintained within a precise range.

Why might MEL-26 (and perhaps MBK-2) be necessary to regulate MEI-1/MEI-2 during meiosis and why is ectopic expression of both MEL-26 and MBK-2 not sufficient for large-scale MEI-1 degradation? MEI-1/MEI-2 has multiple functions during meiosis, initially to increase the number of microtubule ends to aid in nucleating the meiotic spindle in the absence of centrosomal-based microtubule organizing centers (McNally et al., 2006; Srayko et al., 2006). Moments later, MEI-1/MEI-2 is required for a different event, spindle shortening and also for translocation of the spindle to the cortex (McNally et al., 2006; Yang et al., 2003). Meiotic spindle shortening is itself a biphasic event (McNally et al., 2006), with only the second stage requiring katanin activity. Perhaps these differing katanin functions require precise and dynamic regulation of microtubule-severing. While MEL-26 regulation of MEI-1/MEI-2 is not essential for completion of meiosis (the level of nondisjunction is relatively low in *mel-26* mutants), this regulation would be important in nature as it clearly increases the fidelity of meiotic chromosome segregation.

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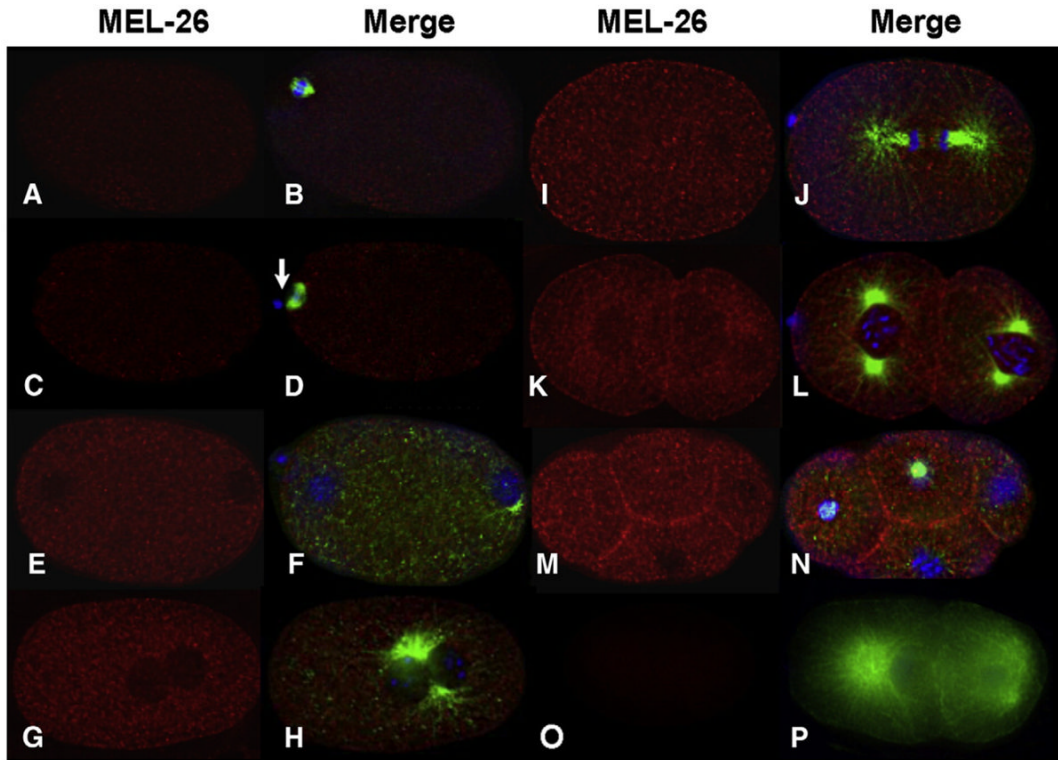


Fig. 1.

Time course of MEL-26 accumulation in wild-type embryos. The left column shows deconvolved indirect immunofluorescence images of embryos stained with anti-MEL-26 (red) while the right column shows merged images of the same embryos stained with anti-MEL-26 (red), anti-tubulin (green) and DAPI (blue). (A, B) meiosis I. (C, D) Meiosis II, note the first polar body (arrow). (E, F) Pronuclear formation, levels of MEL-26 have increased. (G, H) Pronuclear fusion. (I, J) Anaphase of the first mitotic division. (K, L) Late two cell stage, and (M, N) four cell stage. Note that MEL-26 is enriched at the membrane between cells of multicellular embryos. (O, P) Two cell *mel-26(ct61sb4); tbb-2(sb26)* shows no MEL-26 staining. The mutation encoded by the allele *ct61sb4* results in a truncation N-terminal to the region used to raise the antisera. *tbb-2(sb26)* suppresses *ct61sb4* lethality, restoring normal embryo morphology. Note that the low levels of MEL-26 present at meiosis I and II (A–D) are above the background seen in (O, P).

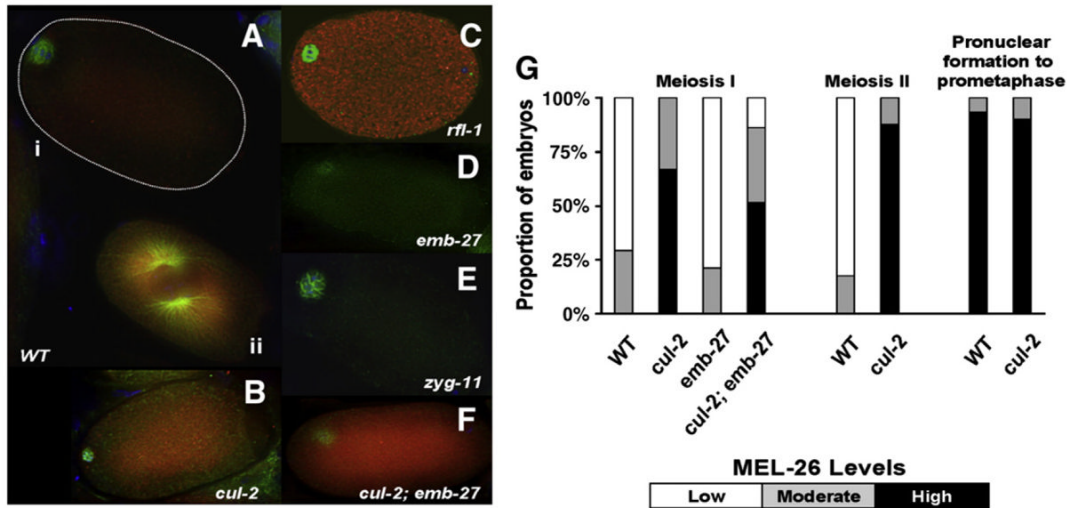


Fig. 2. Mutations affecting MEL-26 temporal expression. Merged images are of MEL-26 (red), tubulin (green) and DAPI (blue). (A) Low levels of MEL-26 are apparent in the meiotic stage wild-type embryo (i) compared to the pronuclear fusion stage wild-type embryo (ii) in the same field. *cul-2* (B) and *rfl-1* (C) embryos show increased MEL-26 staining at meiosis compared to wild type. (D) *emb-27* embryo arrested at meiosis I shows low levels of MEL-26. (E) *zyg-11* embryos have a prolonged meiosis (as do *cul-2* embryos), but levels of meiotic MEL-26 do not increase as they do in *cul-2*. Prolonged meiosis may result in very low levels of MEL-26 in (D) and (E). (F) The increased levels of meiotic MEL-26 characteristic of *cul-2* are epistatic to the low levels seen in *emb-27* in the *cul-2; emb-27* double mutant. (G) CUL-2 prevents premature accumulation of MEL-26. The histogram shows the percent of embryos showing very low (white), moderate (grey) and high (black) levels of MEL-26 at the stages indicated for each genotype. Wild-type embryos show an abrupt increase in MEL-26 after meiosis. *cul-2* embryos are strongly positive at meiosis I and increase slightly thereafter. An APC subunit (*emb-27*) mutant arrests at meiosis I and is similar to wild type at that stage, but *cul-2* is epistatic to *emb-27* as doubly mutant embryos are similar to *cul-2*. The numbers of embryos scored are as follows. Meiosis I: wild type (17), *cul-2* (29), *emb-27* (19), *cul-2; emb-27* (25). Meiosis II: wild type (17), *cul-2* (16). Prometaphase: wild type (30), *cul-2* (20).

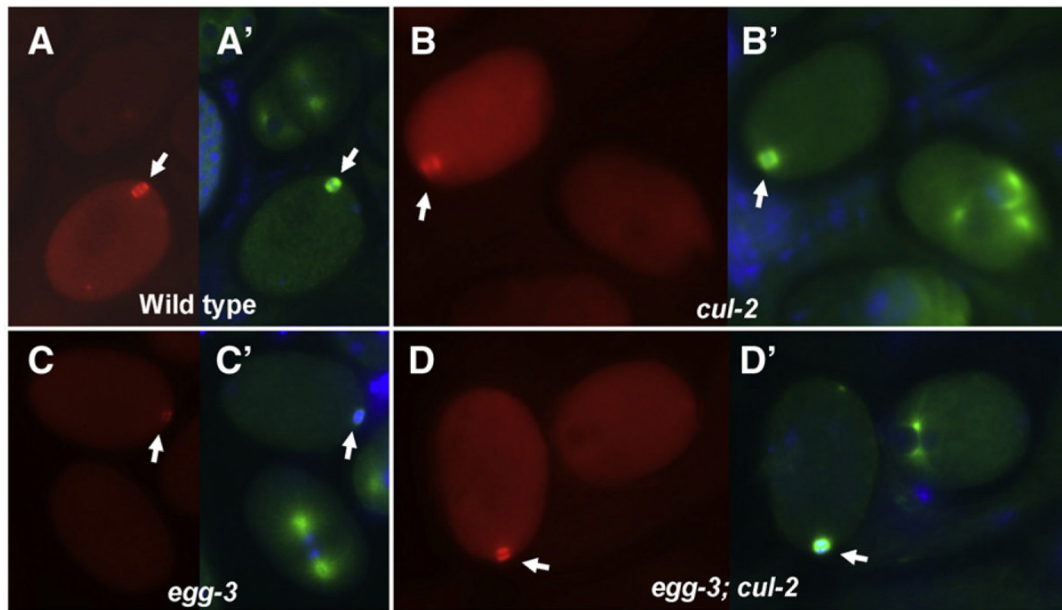
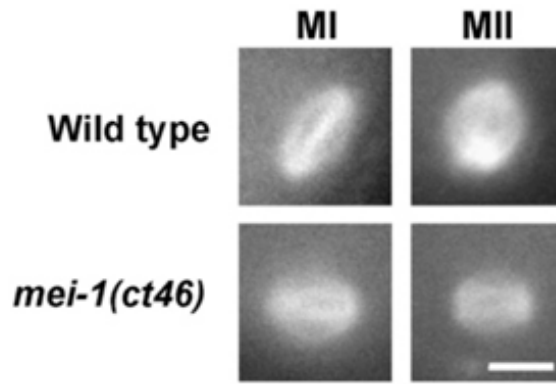


Fig. 3.

Meiotic cytoplasmic MEI-1 levels are not altered in *cul-2* embryos. (A–D) Embryos stained with anti-MEI. (A'–D') Anti-tubulin staining of the same embryos. In each panel, levels in meiotic embryos are compared to the mitotic embryo present in the same field. (A, A') Wild-type cytoplasmic MEI-1 staining is higher in the meiotic embryo than a later-stage embryo. (B, B') *cul-2* has higher levels of cytoplasmic MEI-1 at meiosis than mitosis, and levels at both divisions are higher than corresponding wild-type embryos. (C, C') *egg-3* (*RNAi*) embryos show similar cytoplasmic MEI-1 levels at meiosis and mitosis, indicative of premature MEI-1 degradation. (D, D') *cul-2; egg-3*(*RNAi*) embryos have similar levels of cytoplasmic MEI-1 at meiosis and mitosis, like *egg-3* alone, but cytoplasmic levels at both divisions are increased, as seen with *cul-2* single mutants. Note that in all panels meiotic spindles (arrows) are present and contain MEI-1.



	Metaphase I length (μm)	Metaphase II length (μm)	Length at rotation MI + MII (μm)
wild type	8.31 +/- 0.32 n=10	6.93 +/- 0.27 n=10	4.43 +/- 0.62 n=7
<i>mei-1(ct46)</i>	7.52 +/- 0.21 n=12	6.37 +/- 0.22 n=13	4.27 +/- 0.34 n=13

Fig. 4. Decreased MEI-1 degradation during meiosis leads to shorter meiotic metaphase spindles. *C. elegans* meiotic spindles initially maintain a constant length during metaphase I and metaphase II (Yang et al., 2003). However, just prior to anaphase they shorten and rotate from parallel to perpendicular to the cortex. The constant lengths of metaphase I and metaphase II spindles prior to shortening and their lengths at rotation were determined from time-lapse sequences of GFP: tubulin in both wild-type and *mei-1(ct46)* embryos *in utero*. Representative images of metaphase spindles (scale bar, 5 μm) and average metaphase spindle lengths are shown and tabular data are presented +/- the standard deviation. *n* = the number of individual spindles used to determine each average.

Meiosis



Mitosis



Fig. 5. Model of MEI-1 regulation during the meiosis to mitosis transition. Low levels of protein or activity are indicated by lighter shading. During meiosis, CUL-2 (this report) and EGG-3 (Stitzel et al., 2007) keep MEL-26 and MBK-2 activities low, respectively, allowing MEI-1 to accumulate and sever microtubules. It is not known if the interaction between CUL-2 and MEL-26 is direct, and so this interaction is shown with stippled lines. During mitosis the situation is reversed and MEI-1 is degraded, allowing formation of longer microtubules. The kinase that acts in concert with the CUL-3/MEL-26 ubiquitin ligase and the ubiquitin ligase acting with MBK-2 are unknown.

Table 1Decreased levels of *mel-26* compensates for partial loss of *mei-2*.

Genotype ^a	Percent hatching			
	15 °C	20 °C	24 °C	25 °C
<i>mel-26(dn)/+</i>	95	79	8.9	7.8
<i>mei-2(sb39)</i>	84	74	13	1.1
<i>mei-2(sb39) mel-26(dn)/mei-2(sb39) +</i>	— ^b	92	29	4.4
<i>mei-2(sb39)/mei-2(0)</i>	52	39	3.1	—
<i>mei-2(sb39) mel-26(dn)/mei-2(0) +</i>	76	72	10	—
<i>mei-2(ct98)/mei-2(0)</i>	—	—	—	28
<i>mei-1(gf)/+</i>	—	—	—	0
<i>mei-2(ct98) mei-1(gf)/mei-2(0)</i>	—	—	—	45
<i>mbk-2(ts)</i>	92	62	2.6	—
<i>mei-2(sb39); mbk-2(ts)</i>	85	69	17	—
<i>mei-2(sb39)/mei-2(0); mbk-2(ts)</i>	58	35	1.8	—

^a *mel-26(dn)=ct61*, *mei-2(0) =ct102*, which is a strong allele. *mei-2(sb39)* is hypomorphic, with less activity than *mei-2(ct98)*. *mei-1(gf)=ct46*, an allele refractory to MEL-26 degradation, *mbk-2(ts) =dd5*.

^b Not determined.

Table 2
Genetic interactions of *tbb-2* with *mel-26*, *rft-1* and *cul-3*.

Genotype	Percent hatching	
	15 °C	25 °C
<i>mel-26(ct61sb4)</i>	18	0
<i>mel-26(ct61sb4); tbb-2(sb26)</i>	60	40
<i>mei-1(ct46)</i>	23	0
<i>mei-1(ct46); tbb-2(sb26)</i>	96	66
<i>rft-1(or198)</i>	— ^a	0
<i>rft-1(or198) tbb-2(sb26)</i>	—	0
<i>rft-1(or198); mei-2(ct98)</i>	—	0
<i>cul-3(RNAi)</i>	19	11
<i>cul-3(RNAi); tbb-2(sb26)</i>	83	37

^aNot determined.