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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.

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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 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

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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/deneddylation 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. Immunolocalization 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)*, *rfl-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 (ctgaattcattacagtccagatggaggtgg) 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 resuspended 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 μg/ml 4′,6-diamidine-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 μm 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.

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