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POLO Kinase Regulates the *Drosophila* Centromere Cohesion Protein MEI-S332

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Summary

Accurate segregation of chromosomes is critical to ensure that each daughter cell receives the full genetic complement. Maintenance of cohesion between sister chromatids, especially at centromeres, is required to segregate chromosomes precisely during mitosis and meiosis. The *Drosophila* protein MEI-S332, the founding member of a conserved protein family, is essential in meiosis for maintaining cohesion at centromeres until sister chromatids separate at the metaphase II/anaphase II transition. MEI-S332 localizes onto centromeres in prometaphase of mitosis or meiosis I, remaining until sister chromatids segregate. We elucidated a mechanism for controlling release of MEI-S332 from centromeres via phosphorylation by POLO kinase. We demonstrate that POLO antagonizes MEI-S332 cohesive function and that full POLO activity is needed to remove MEI-S332 from centromeres, yet this delocalization is not required for sister chromatid separation. POLO phosphorylates MEI-S332 in vitro, POLO and MEI-S332 bind each other, and mutation of POLO binding sites prevents MEI-S332 dissociation from centromeres.

Introduction

The accurate segregation of chromosomes is essential to prevent aneuploidy following cell division. Cohesion between sister chromatid centromeres is necessary to ensure that the two sister chromatid kinetochores attach stably to microtubules emanating from opposite spindle poles. In mitosis, cohesion at centromeres and along the chromosome arms is maintained between sisters until the metaphase/anaphase transition. In meiosis, chromosome arm cohesion plays an additional role in linking homologs together by stabilizing chiasmata (Buonomo et al., 2000). Arm cohesion is dissolved at the metaphase I/anaphase I transition, permitting homolog segregation, and only centromere cohesion remains between sisters until the metaphase II/anaphase II transition when the sisters finally separate (reviewed in Nasmyth, 2001; Petronczki et al., 2003). Therefore, the maintenance of centromere cohesion is essential to prevent chromosome missegregation in meiosis, yet the molecular mechanisms underlying the control of this cohesive force are poorly understood.

The cohesin complex is required for sister chromatid cohesion. This complex is composed of four subunits, Smc1, Smc3, Scc1 (RAD21), and Scc3, and in meiosis Rec8 replaces the Scc1 subunit. In some organisms, other additional meiotic-specific subunits replace those employed in mitosis (reviewed in Uhlmann, 2003). These proteins are thought to form a ring structure that loops around the two sisters and holds them together (Anderson et al., 2002; Gruber et al., 2003; Haering et al., 2002). At the metaphase/anaphase transition in mitosis, the cysteine protease separase is activated via the anaphase promoting complex/cyclosome (APC/C)-dependent destruction of its inhibitor securin. It then cleaves Scc1 to release cohesion between sisters. In yeast during meiosis, separase becomes activated at the metaphase I/anaphase I transition and cleaves arm cohesin. A portion of the cohesin complex remains uncleaved at centromeres, keeping sister chromatids together until the metaphase II/anaphase II transition, when this persistent cohesin is cleaved (reviewed in Nasmyth, 2001; Page and Hawley, 2003).

Although it is likely that protector proteins prevent cleavage of cohesin at centromeres until the metaphase II/anaphase II transition, their identity remains elusive. The best candidate is the *Drosophila* centromere cohesion protein MEI-S332 because of its striking mutant phenotype and localization pattern. Mutations in *mei-S332* lead to loss of sister chromatid cohesion at the centromere beginning at anaphase I, resulting in chromosome nondisjunction during meiosis II (Davis, 1971; Goldstein, 1980; Kerrebrock et al., 1992). Consistent with a direct role in regulating cohesion, MEI-S332 localizes to meiotic centromeres from prometaphase I to metaphase II, dissociating concomitantly with segregation of sister chromatids (Kerrebrock et al., 1995; Moore et al., 1998). MEI-S332 also localizes to mitotic chromosomes from prometaphase to metaphase and plays a modest role in sister chromatid cohesion during mitosis (LeBlanc et al., 1999; Moore et al., 1998).

Until recently, MEI-S332 was thought to be a unique *Drosophila* centromere protein. It now has been recognized as the founding member of a newly identified family of proteins conserved from yeast to humans. The MEI-S332 homolog Sgo1 was identified in budding and fission yeast by its ability to protect centromere cohesion, its expression during meiosis, and its localization to centromeres in meiosis I (Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). Based on sequence identity/similarity, the Sgo1 protein is present in many other organisms including humans, and a related protein, Sgo2, has been identified in *S. pombe* and *Arabidopsis*. Sgo1 has been proposed to protect the centromeric meiosis-specific subunit Rec8 from separase cleavage at the metaphase I/anaphase I transition. In contrast, in fission yeast Sgo2 promotes mono-orientation of sister chromatid kinetochores to ensure that the two sister chromatids of each homolog migrate to the same pole at anaphase I (Rabitsch et

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al., 2004). Sgo1 affects mitotic segregation in budding yeast, and Sgo2 functions in mitosis in fission yeast (Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004). In vertebrate cells, Sgo1 is needed to maintain centromere cohesion in mitosis and also affects spindle microtubule dynamics (Salic et al., 2004).

A candidate regulator for the MEI-S332 family is the highly conserved POLO kinase. POLO controls many aspects of mitosis, such as the onset of mitosis, spindle formation, the metaphase/anaphase transition, and cytokinesis (reviewed in Blagden and Glover, 2003; Glover et al., 1998; Ohi and Gould, 1999). One crucial POLO substrate is the Scc1/Rad21 cohesin subunit, which accounts for the metaphase arrest and failure to release sister chromatid cohesion seen in *polo* mutants (Sunkel and Glover, 1988; Sumara et al., 2002; Alexandru et al., 2001). Phosphorylation of Rad21 by the *Xenopus* homolog of POLO, Plx1, is responsible for the separase-independent removal of the bulk of cohesin along sister chromatid arms in prophase (Losada et al., 2002; Sumara et al., 2002). In budding yeast, phosphorylation of Scc1 by the POLO ortholog Cdc5 near separase cleavage sites enhances its cleavage by separase (Alexandru et al., 2001).

POLO also is critical for unique aspects of meiosis. Cdc5 is required for complete phosphorylation and subsequent cleavage of Rec8 (Clyne et al., 2003; Lee and Amon, 2003). It also regulates kinetochore orientation by phosphorylating Mam1, a meiosis-specific kinetochore protein that is part of a protein complex needed for sister kinetochores to mono-orient toward the same spindle pole in prometaphase I (Toth et al., 2000). In *cdc5* mutants Mam1 fails to localize to kinetochores (Clyne et al., 2003; Lee and Amon, 2003). In addition, Cdc5 may be necessary for the resolution of recombination intermediates into crossovers prior to meiosis I (Clyne et al., 2003).

To understand the mechanism that controls cohesion at centromeres, we investigated how MEI-S332 function and localization are regulated in mitosis and meiosis. We find that POLO kinase plays an essential role in MEI-S332 dissociation from centromeres.

Results

POLO Kinase Antagonizes MEI-S332 Function in Meiosis

Given the role of POLO in promoting release of sister chromatid cohesion in mitosis and meiosis, we tested the relationship between POLO and MEI-S332 by investigating genetic interactions between *polo* and *mei-S332* mutants in *Drosophila*. Although homozygous strong *polo* mutations are lethal (*polo*^{9} and *polo*^{10}; Donaldson et al., 2001) and weak mutations are sterile (*polo*^{1}; Sunkel and Glover, 1988), we tested for dominant enhancement or suppression of the *mei-S332* meiosis II nondisjunction phenotype in the presence of one mutant copy of the *polo* gene. We used the *mei-S332*^{8} allele that results in intermediate levels of chromosome missegregation in males; this mutant form of the protein still localizes to centromeres (Kerrebrock et al., 1992; Tang et al., 1998).

Male flies homozygous for the *mei-S332*^{8} mutation and heterozygous either for a deficiency that removes the *polo* gene, *Df(3L)rdgC-co2*, or for the strong *polo* alleles, *polo*^{9} or *polo*^{10}, were scored for segregation of the X and Y chromosomes and compared to sibling controls mutant only for *mei-S332*^{8}. All three *polo* mutants dominantly suppressed the *mei-S332*^{8} nondisjunction phenotype to degrees proportional to the severity of the *polo* defect (Table 1). The *polo* deficiency suppressed to the greatest extent, decreasing total nondisjunction by 72%. The *polo*^{9} allele suppressed with a 52% decrease and *polo*^{10} with a 46% decrease, correlating with allele strength (Donaldson et al., 2001). In addition, the weak *polo*^{1} allele also dominantly reduced *mei-S332*^{8} nondisjunction, although to a lesser extent. Thus, the effect of the *polo* deficiency and *polo* mutants from three different genetic backgrounds is most likely due to a reduction of POLO activity. The striking ability of decreased *polo* activity to suppress sister chromatid missegregation in *mei-S332* mutants indicates that POLO antagonizes the function of MEI-S332 in promoting sister chromatid cohesion, raising the possibility that POLO inactivates or delocalizes MEI-S332.

POLO Kinase Directs MEI-S332 Dissociation from Meiotic Chromosomes

MEI-S332 localizes to meiotic centromeres from prometaphase I until the metaphase II/anaphase II transition, correlating with its role in maintaining sister chromatid cohesion until segregation in meiosis II (Moore et al., 1998). We tested whether heterozygous or homozygous *polo* mutants affected the centromere localization or delocalization of MEI-S332 in male meiosis. *polo*^{9} and *polo*^{10} were examined as heterozygotes, but homozygous mutants die in the third instar larval stage so spermatocytes could not be recovered. We found, however, that *polo*^{9}/*polo*^{1} transheterozygous animals survived to adulthood but were sterile. MEI-S332 localization was examined in these transheterozygotes.

We observed a striking effect of *polo* mutations on MEI-S332 centromere localization: MEI-S332 remained localized to chromosomes after the metaphase II/anaphase II transition in both heterozygous *polo*^{9}/+ or *polo*^{10}/+ and transheterozygous *polo*^{9}/*polo*^{1} mutant spermatocytes (Figures 1C and 1D, compare to 1B) and failed to delocalize at the proper time. The loss of *polo* activity primarily affected centromere dissociation of MEI-S332 but not association, as foci of MEI-S332 bound to centromeres were clearly visible in the *polo*^{9}/*polo*^{1} cells. Although MEI-S332 was somewhat diffusely spread on chromosomes in the *polo*^{10}/+ telophase II cells, some concentrated foci at centromeres were present (Figure 1C, arrows). MEI-S332 persisted at centromeres in some but not all *polo*^{9}/+ anaphase II cells (data not shown). Some lagging chromosomes were observed in the *polo*^{9}/+ heterozygotes (data not shown), and obvious chromosome segregation defects occurred in the *polo*^{9}/*polo*^{1} transheterozygotes.

The finding that POLO function is needed for delocalization of MEI-S332 from centromeres in meiosis could provide an explanation for the suppression of the *mei-S332*^{8} nondisjunction phenotype. Reduced POLO function may suppress premature sister separation in *mei-S332* mutants either by retention of MEI-S332 longer at centromeres or by increased MEI-S332 activity.

Table 1. *polo* Mutants Suppress Sex Chromosome Nondisjunction in *mei-S332*^{6} Males

Genotype	*mei-S332*^{6}; +	*mei-S332*^{6}; +/ *Df(3L)rdgC-co2*	*mei-S332*^{6}; +	*mei-S332*^{6}; +/ *polo*^{9}	*mei-S332*^{6}; +	*mei-S332*^{6}; +/ *polo*^{10}	*mei-S332*^{6}; +	*mei-S332*^{6}; +/ *polo*^{1}
Regular Sperm								
X	469	282	249	334	296	270	377	402
Y	318	234	181	271	222	242	312	303
Exceptional Sperm								
0	41	9	33	22	41	16	39	31
XX	20	0	13	6	13	9	26	3
XY	1	0	2	4	0	1	0	0
XXY	0	0	0	0	0	0	0	0
Total progeny	849	525	478	637	572	538	754	739
Total NDJ	7.5 ± 2.7	2.1 ± 2.6	11 ± 3.7	5.2 ± 1.3	11.4 ± 5.7	6.2 ± 4.5	8.8 ± 0.5	5.3 ± 2.1
% decrease in NDJ over sibling controls		72%		52%		46%		39%
p value		0.057		0.029		0.2		0.1

Four independent experiments were completed for *polo*^{9}, *polo*^{10}, and the *polo* deficiency, and three experiments were completed for *polo*^{1}, each paired with appropriate *mei-S332*^{6} homozygous sibling controls. Although total numbers of progeny in each class are reported as the sum of the experiments, the percent total nondisjunction is presented as an average of the experiments with standard deviations indicated. A Wilcoxon two-sample rank sum test (Wonnacott and Wonnacott, 1984) was utilized to calculate statistical significance p values (see http://fonsg3.let.uva.nl/Service/Statistics.html).

POLO Kinase Is Required for Proper Delocalization of MEI-S332 from Centromeres in Mitosis

MEI-S332 has a striking localization pattern during the mitotic cell cycle; the protein localizes to centromeres during prometaphase and dissociates at the metaphase/anaphase transition (Moore et al., 1998). To determine whether POLO kinase regulates MEI-S332 in mitosis, the localization of MEI-S332 was examined in *polo* mutant larval brain neuroblasts. This cell type was examined because cells in the larval brain are actively dividing, and the *polo*^{9} and *polo*^{10} mutant phenotype is manifested at this stage of development. These alleles provided the additional advantage that some cells arrest in metaphase with centromeres separated (Donaldson et al., 2001) while others proceed into anaphase.

In both *polo*^{9} and *polo*^{10} heterozygous and homozygous mutant neuroblasts, MEI-S332 localized normally to centromeres during metaphase (Figure 2A, top left and data not shown). In the heterozygotes, MEI-S332 delocalized from centromeres in anaphase as in wild-type (Figure 2A). In homozygous *polo* mutants arrested at metaphase with separated centromeres, MEI-S332 failed to delocalize from centromeres, even when the small fourth chromosomes completely separated and migrated to the poles (Figures 2A, arrows, and 2B, asterisks). These results suggest that POLO kinase is required for the release of MEI-S332 from centromeres in mitosis as in meiosis II.

Cyclin B staining confirmed that some cells with separated centromeres showing MEI-S332 staining were no longer in metaphase, since it is present in metaphase cells but is degraded at the metaphase/anaphase transition and thus absent in anaphase cells (Lehner and O'Farrell, 1990; Parry and O'Farrell, 2001). In contrast to wild-type cells, a significant number of *polo* mutant neuroblasts showing two rows of MEI-S332 staining had low levels of Cyclin B (35% [34/96] in *polo*^{9} mutants and 40% [26/65] in *polo*^{10} mutants) (Figure 2B, left). These cells may be in anaphase, or it is possible that some cells showing no Cyclin B staining had also reached interphase (see below). The presence of localized MEI-S332 in cells lacking Cyclin B, localization never observed in wild-type cells, suggests that POLO kinase activity is needed for MEI-S332 delocalization from centromeres.

To investigate whether MEI-S332 remained localized to centromeres in interphase in *polo* mutants, we used the presence of phosphorylated histone H3 to mark cells in mitosis and, conversely, its absence to identify cells in interphase (Hendzel et al., 1997). MEI-S332 is not normally detected on chromosomes in interphase cells. In contrast, in *polo* mutant neuroblasts with MEI-S332 staining, 33% (49/150) of *polo*^{9} cells and 28% (37/133) of *polo*^{10} cells had no phospho-histone H3 staining (Figure 2C). These results indicate that MEI-S332 is unable to be released from centromeres in *polo* mutants.

MEI-S332 Is Phosphorylated at the Metaphase/Anaphase Transition in Embryos

Because POLO kinase may regulate the function of MEI-S332 directly through phosphorylation, we first asked if MEI-S332 was phosphorylated in embryo extracts. Embryo extracts were utilized because large quantities

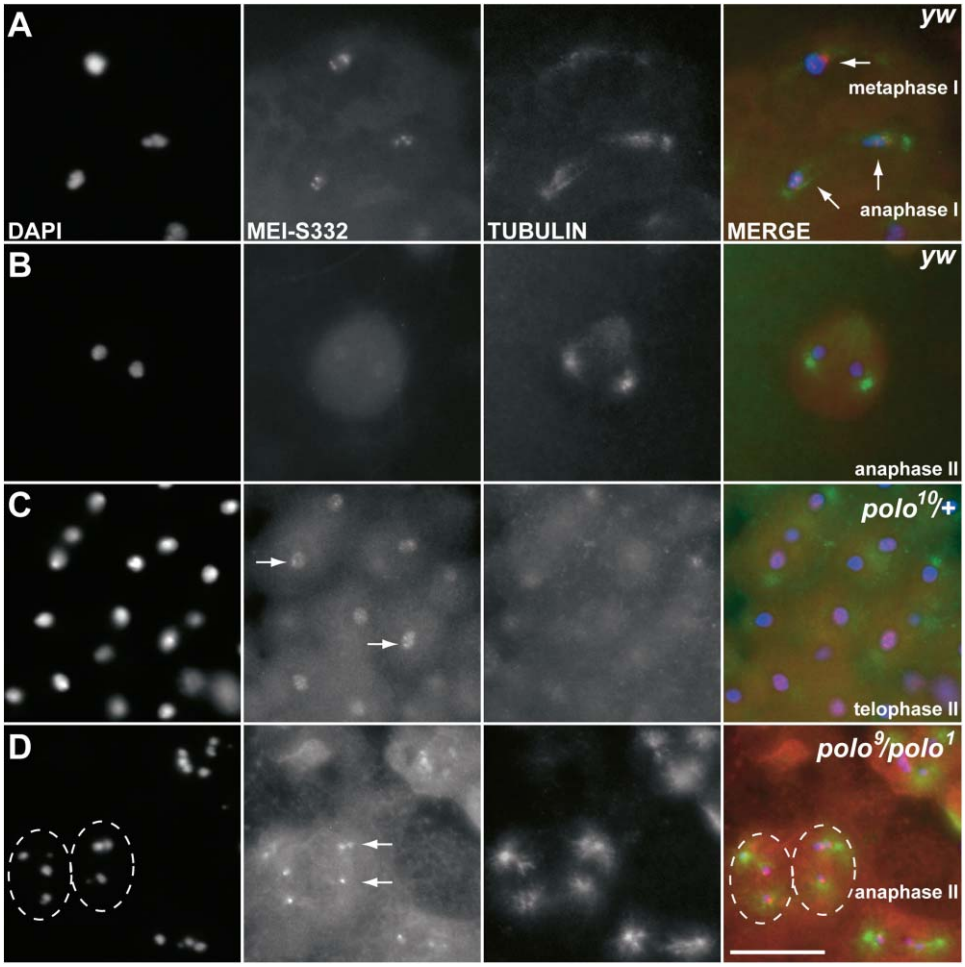

Figure 1. MEI-S332 Persists on Centromeres after Anaphase II of Meiosis in *polo* Mutants

Spermatocytes from *yw*, *polo*^{10}/+, and *polo*^{9}/*polo*^{1} males were stained for MEI-S332 (red), α-tubulin (green), and DAPI (blue).
(A) Metaphase and anaphase I cells in wild-type *(yw)* show MEI-S332 centromere staining.
(B) MEI-S332 is delocalized from centromeres in late anaphase II in wild-type.
(C) MEI-S332 persists on centromeres (foci, arrows) and chromosomes (diffuse staining) in *polo*^{10}/+ heterozygotes into telophase II. All cells are in telophase II and part of a larger cyst.
(D) MEI-S332 persists at centromeres in *polo*^{9}/*polo*^{1} transheterozygotes into late anaphase II (arrows). Based on spindle length and cross shape, the two nuclei (in dashed ovals) are in anaphase II and chromosomes nondisjoined probably in meiosis I as there are only four chromosomes (half the normal number) segregating in each nucleus. Scale bar equals 20 μm.

of protein from cells in mitosis could be easily isolated and analyzed biochemically. Because MEI-S332 resolves into a doublet of bands by SDS-PAGE analysis (LeBlanc et al., 1999), we determined whether this doublet was due to phosphorylation. Wild-type embryo extracts were treated with lambda protein phosphatase and the migration pattern of MEI-S332 was analyzed on Western blots. MEI-S332 shifted to the slower migrating form upon phosphatase treatment, and this conversion was blocked by incubation with sodium vanadate and sodium phosphate, lambda phosphatase inhibitors (Figure 3A). By contrast, sodium fluoride, a weak inhibitor of lambda phosphatase, failed to block this shift. These results indicate that MEI-S332 is a phosphoprotein and that the faster migrating form is the phosphorylated form. Although it is more common for phosphoproteins to have a reduced electrophoretic mobility, there are precedents for phosphorylated protein forms migrating faster (Grasser and Konig, 1992). From this analysis, it cannot be determined if there are multiple phosphorylation events responsible for the faster migrating form of MEI-S332, especially given that there are at least 53 possible phosphorylation sites in MEI-S332 (Blom et al., 1999).

We examined whether MEI-S332 centromere localization correlated with phosphorylation state by preparing extracts from embryos in specific stages of mitosis. Early embryos undergoing rapid S/M cycles were isolated, fixed, stained with DAPI to visualize the chromosomes, and micromanipulated to gather embryos with nuclei in interphase, metaphase, and anaphase, and then extracts were prepared from the staged embryos.

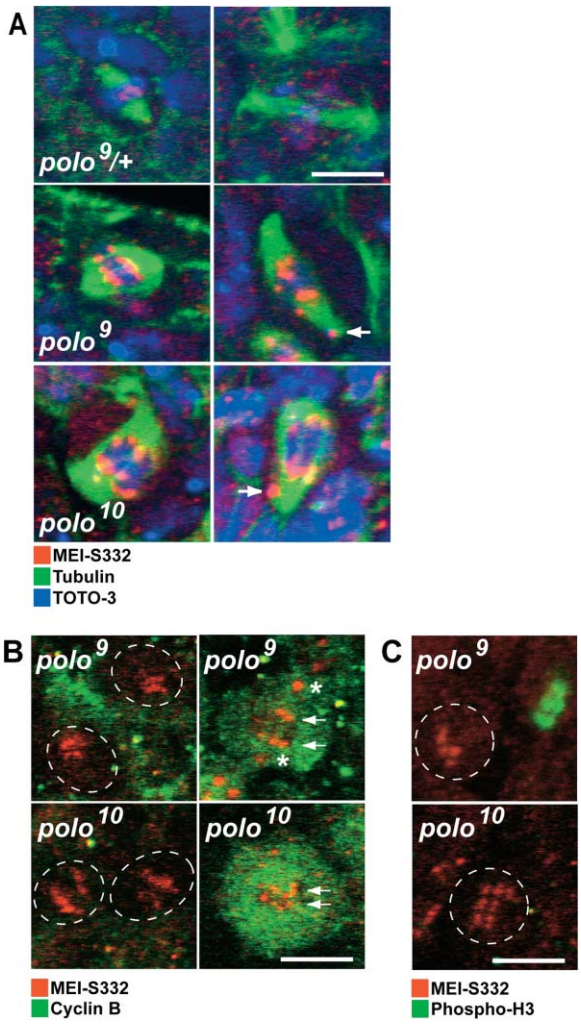

Figure 2. MEI-S332 Remains on Centromeres in *polo* Mutants in All Stages of Mitosis

(A) Third instar larval brains from *polo*^{9}/+, *polo*^{9}, and *polo*^{10} animals were stained for MEI-S332 (red), α-tubulin (green), and TOTO-3 to mark the DNA (blue). In control *polo*^{9}/+ heterozygotes (top), MEI-S332 localizes to centromeres during metaphase (left) and delocalizes from centromeres at the metaphase/anaphase transition (right). In *polo*^{9} (middle) and *polo*^{10} (bottom) mutants, MEI-S332 persists on centromeres in cells with separated centromeres (left) even when the small fourth chromosome has segregated to the poles (right panels, arrows). The fourth chromosome at the other pole is out of the plane of focus.

(B) Third instar larval brains from *polo*^{9} and *polo*^{10} animals were stained for MEI-S332 (red) and Cyclin B (green). In *polo* mutants, there are nuclei that retain MEI-S332 centromeric foci despite the absence of Cyclin B (left), but not in all cells (right). White circles indicate nuclear boundaries, arrows indicate two probable anaphase rows of MEI-S332 staining, and asterisks indicate MEI-S332 staining on the fourth chromosome migrating to the poles.

(C) Third instar larval brains from *polo*^{9} and *polo*^{10} animals were stained for MEI-S332 (red) and for the mitotic marker phospho-histone H3 (green). MEI-S332 localizes to centromeres in cells showing no phospho-histone H3 staining, revealing that MEI-S332 remains on centromeres during interphase. Dashed circles indicate nuclear boundaries. Scale bars equal 5 μm.

This analysis revealed that MEI-S332 was in the apparent phosphorylated state in interphase, the dephosphorylated state in metaphase, and the phosphorylated state in anaphase (Figure 3B). Therefore, MEI-S332 appears to be in the dephosphorylated state when it is localized to centromeres and phosphorylated when it is not at centromeres. This correlation suggests that MEI-S332 could be phosphorylated at the metaphase/anaphase transition. Our analysis cannot distinguish whether the anaphase and interphase phosphorylated forms of MEI-S332 are the same. Indeed, it seems unlikely that the identical phosphorylation state of MEI-S332 persists from anaphase until interphase of the next cell cycle.

To determine if MEI-S332 must be localized to centromeres in order for phosphorylation to occur, we utilized *mei-S332*^{6} mutant embryos in which the mutant protein fails to localize to centromeres during mitosis (Tang et al., 1998). Homozygous *mei-S332*^{6} early embryos were micromanipulated according to cell cycle stage and compared to wild-type extracts. Phosphorylation of MEI-S332^{6} protein was still detectable as in wild-type embryos (Figure 3C), indicating that centromeric localization is not required for the phosphorylation of MEI-S332.

To show directly that MEI-S332 can be phosphorylated, we immunoprecipitated GFP- and myc-tagged forms of the protein previously shown to be functional in vivo (Tang, 1999; Moore et al., 1998). Radiolabeled phosphate was added to the immunoprecipitates, and we examined whether MEI-S332 could be phosphorylated. In immunoprecipitates, a ^{32}P-labeled protein band was observed and confirmed to be the MEI-S332-GFP or myc fusion protein by Western blotting (Figure 3D). These results suggest that a kinase that associates with MEI-S332 is capable of phosphorylating MEI-S332 in vitro.

POLO Kinase Is Required for Proper MEI-S332 Phosphorylation In Vivo

The observations that POLO antagonizes MEI-S332 and is needed for its delocalization from the centromere coupled with the finding MEI-S332 becomes phosphorylated in anaphase suggested that POLO phosphorylates MEI-S332 at the metaphase/anaphase transition. Therefore, we tested whether MEI-S332 phosphorylation was affected in the *polo* mutants. Protein extracts from *polo*^{9} and *polo*^{10} homozygous and heterozygous third instar larval brains were analyzed for the MEI-S332 phosphorylation state by Western blotting. These hypomorphic mutant alleles are lethal at this stage, when no maternal protein pools persist. In homozygous mutants, a high percentage of the cells in brains are arrested in metaphase. In both the heterozygous and homozygous *polo*^{9} mutants, MEI-S332 was predominately in the dephosphorylated, slower migrating form of the protein (Figure 3E). The levels of phosphorylated MEI-S322 were markedly reduced in *polo*^{9} homozygous mutant neuroblasts, and levels were reduced to a lesser extent in the *polo*^{9} heterozygote. It is a possibility that MEI-S332 is mostly dephosphorylated because a large percentage of cells are in metaphase in *polo*^{9} mutants. In the weaker *polo*^{10} heterozygous and homozygous mutants, MEI-S332 was

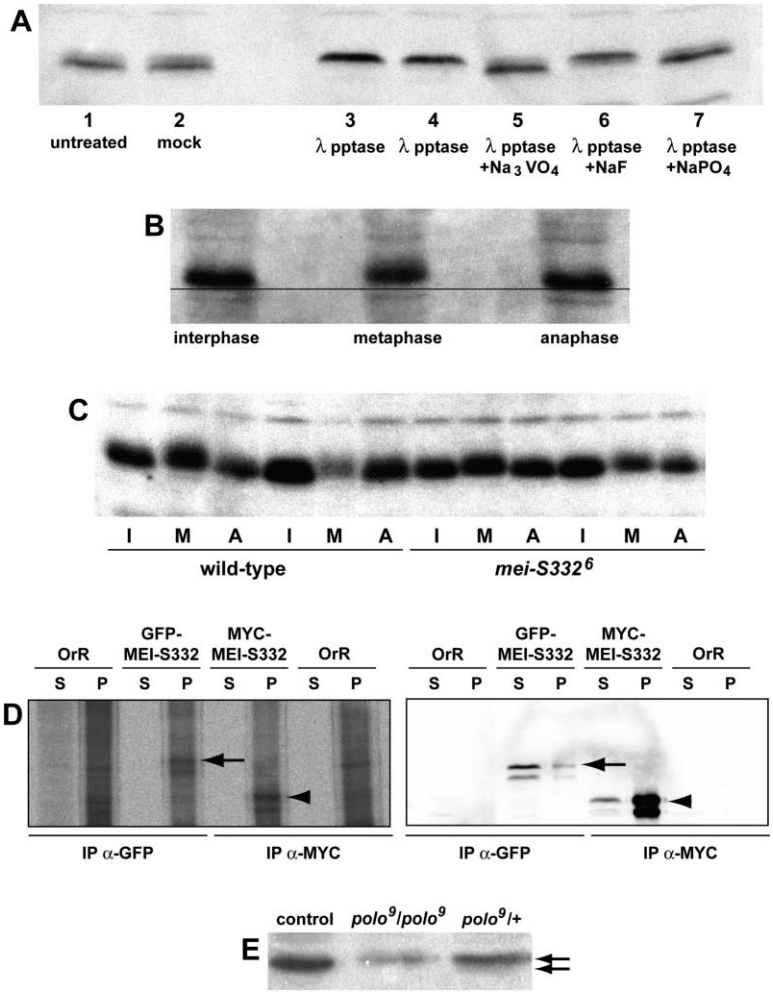

Figure 3. MEI-S332 Is a Phosphoprotein and Its Phosphorylation State Is Correlated with the Cell Cycle and POLO Function but Not with Chromosomal Localization

(A) Wild-type Oregon R embryo extracts were treated as described followed by Western blot analysis probing for MEI-S332. Lane 1, untreated; lane 2, mock treated; lanes 3 and 4, lambda protein phosphatase; lane 5, lambda protein phosphatase and 10 mM sodium vanadate; lane 6, lambda protein phosphatase and 50 mM sodium fluoride; lane 7, lambda protein phosphatase and 100 mM sodium phosphate. Two forms of MEI-S332 are present in control extracts (lanes 1 and 2), whereas only the slower migrating, dephosphorylated form is present in lambda protein phosphatase-treated extracts (lanes 2 and 3). Sodium vanadate (lane 5) and sodium phosphate (lane 7) inhibit this dephosphorylation reaction.

(B) Wild-type embryos were manually grouped into interphase, metaphase, and anaphase samples and subjected to Western blot analysis probing for MEI-S332. MEI-S332 is in the dephosphorylated, slower migrating form in metaphase embryos and the phosphorylated, faster migrating form in interphase and anaphase embryos.

(C) Similarly, wild-type (left) and *mei-S332*^{6} (right) interphase (I), metaphase (M), and anaphase (A) embryo samples were analyzed as above. Although MEI-S332^{6} mutant protein fails to localize to mitotic centromeres, the same phosphorylation pattern is observed as in wild-type.

(D) MEI-S332 can be phosphorylated in vitro. MEI-S322 was immunoprecipitated from Oregon R, GFP-MEI-S332, and myc-MEI-S332 embryos and kinase assays were performed with the immunoprecipitates (left) followed by Western blotting (right). Both GFP-MEI-S332 (arrow) and myc-MEI-S332 (arrowhead) are phosphorylated by a coprecipitating kinase in the pellets (P) as shown by ^{32}P incorporation (S indicates supernatant). As a negative control, the Oregon R immunoprecipitates show no ^{32}P incorporation. The identity of the GFP-MEI-S332 and myc-MEI-S332 bands are confirmed by Western blot.

(E) Third instar larval brain protein extracts from control, *polo*^{9}/*polo*^{9}, and *polo*^{9}/+ animals were probed for MEI-S332 phosphorylation state by Western blotting. In control extracts, MEI-S332 is predominately in the phosphorylated, faster migrating form, whereas in *polo*^{9}/*polo*^{9} and *polo*^{9}/+ it is predominately in the slower migrating, dephosphorylated form.

predominately in the phosphorylated form (data not shown). These results are consistent with POLO kinase affecting MEI-S332 phosphorylation in vivo, but we cannot exclude the possibility that electrophoretic variants of MEI-S332 are present in the *polo* mutant strains.

MEI-S332 Is Phosphorylated in Mitotic *Xenopus* Egg Extracts in a Plx1-Dependent Manner

We utilized an in vitro *Xenopus* egg extract system to test whether POLO could phosphorylate MEI-S332 directly. In vitro transcribed and translated (IVT) MEI-S332 protein underwent multiple mobility shifts when incubated with mitotic extracts as compared to interphase extracts (Figure 4B, lanes 1 and 2). These mitotic extracts have active APC/C and thus are in a state equivalent to anaphase (McGarry and Kirschner, 1998), when MEI-S332 is expected to be phosphorylated. These events were due to the phosphorylation of MEI-S332, because treatment with lambda protein phosphatase abolished all mobility shifts (Figure 4B, lane 3). Phosphorylation in vitro shifts MEI-S332 to a slower migrating form, whereas in vivo MEI-S332 is shifted to a faster migrating form. Despite this difference, which is discussed below, these results indicate that MEI-S332 is phosphorylated in a cell cycle-dependent manner in vitro, similar to what we observed in *Drosophila* embryos.

We took advantage of these in vitro observations to determine if the *Xenopus* POLO kinase, Plx1, was responsible for MEI-S332 phosphorylation in the extracts. We immunodepleted the *Xenopus* interphase and anaphase extracts with antibodies against Plx1 or a control antibody and then incubated MEI-S332 IVT protein in these depleted extracts. Importantly, depleting for Plx1 in *Xenopus* extracts does not affect Cdc2 or Aurora B kinase activities (Losada et al., 2002). All of the mitotic MEI-S332 phosphorylation mobility shifts were present in the control-depleted sample, but the slowest migrating form of phosphorylated MEI-S332 was no longer

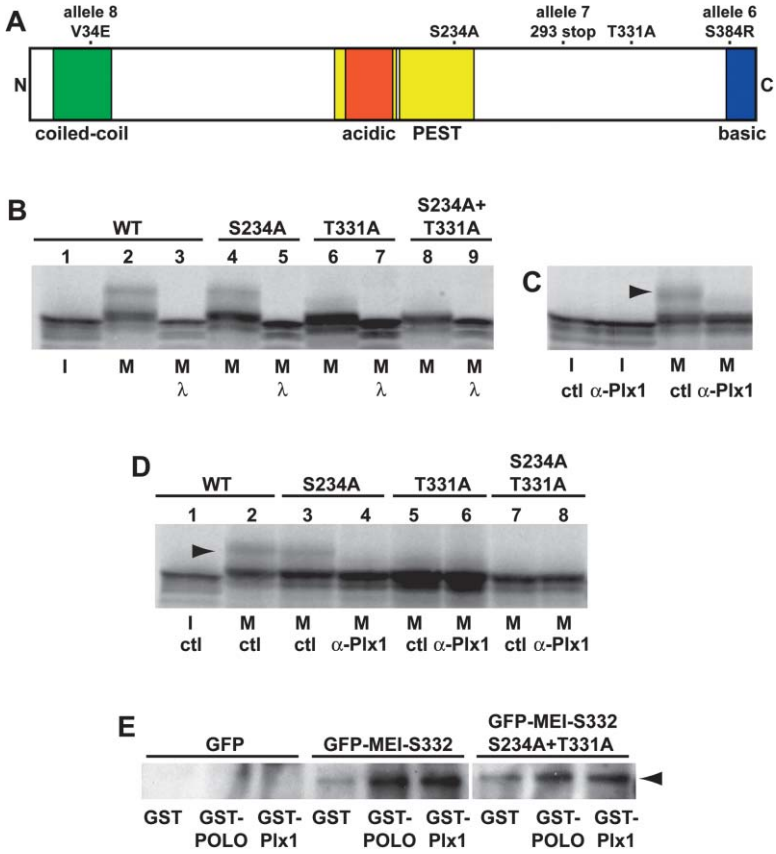

Figure 4. MEI-S332 Is Phosphorylated in *Xenopus* Mitotic Extracts in a Plx1-Dependent Manner, and a Mutation in One of the MEI-S332 PBD Binding Sites Abolishes This Phosphorylation

(A) A scaled representation of the 401 amino acid MEI-S332 protein and its important motifs is diagrammed with relevant alleles. The N-terminal coiled-coil domain (green) extends from residue 13 to 44. Two PEST sequences are located at residues 167–200 and 202–242 (yellow). An acidic domain lies within the first PEST sequence from residue 173 to 198 (red) and a C-terminal basic domain (blue) is found from residue 385 to 401. Two PBD binding sites exist in MEI-S332, SSP from residue 233 to 235, and STP from residue 330 to 332.

(B) In mitotic extracts, wild-type and S234A MEI-S332, but not T331A mutant or double mutant IVT proteins, are significantly shifted to several slower migrating forms compared to interphase extracts. Mitotic extract samples were first incubated with ^{35}S-IVT proteins, then treated with lambda protein phosphatase (M + λ). All mitotic shifts (M) return to the fastest migrating form as in interphase (I), indicating that all shifts are due to phosphorylation. Although all extracts were immunodepleted with control rabbit serum, the same results were obtained with untreated extracts.

(C) Immunodepletion of Plx1 abolishes one of the MEI-S332 mitotic phosphorylation shifts. Control- and Plx1-depleted extracts were tested for the ability to phosphorylate MEI-S332. One of the mitotic phosphorylation shifts is Plx1 dependent and is absent in the Plx1-depleted mitotic sample (arrowhead). Other phosphorylated forms are unaffected.

(D) The MEI-S332-T331A mutant abolishes the Plx1-dependent mitotic phosphorylation shift. Wild-type MEI-S332 IVT protein was incubated with control-depleted interphase (lane 1) or mitotic extracts (lane 2). Similarly, three MEI-S332 mutant proteins were incubated with control-depleted (lanes 3, 5, and 7) or Plx1-depleted (lanes 4, 6, and 8) mitotic extracts. The MEI-S332-S234A mutant protein is phosphorylated as in wild-type (compare lanes 2 and 3). The MEI-S332-T331A mutant protein lacks the Plx1-dependent mitotic phosphorylation shift (arrow, compare lanes 2 and 5).

(E) The PBD of *Drosophila* POLO and *Xenopus* Plx1 bind MEI-S332 in vitro. GST-POLO PBD and GST-Plx1 PBD when incubated with GFP-MEI-S332-transfected S2 cell lysates bind GFP-MEI-S332 3-fold over the GST control. The GST PBDs do not bind as well to the GFP-MEI-S332 S234A+T331A double mutant protein (50% reduction for POLO PBD and 24% for Plx1 PBD). Quantification of binding was normalized based on transfection efficiencies.

present in the Plx1-depleted sample (Figure 4C). The immunodepleted extracts were probed by Western blot for Plx1, confirming the extent of the depletion (Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/8/1/53/DC1/). These results demonstrate that *Xenopus* Plx1 kinase is required for one phosphorylated form of *Drosophila* MEI-S332 in an anaphase state in vitro. The other in vitro mitotic MEI-S332 phosphorylation events likely result from one or more kinases other than Plx1. It remains to be seen whether these additional phosphorylation events are relevant in vivo.

Plx1-dependent phosphorylation of MEI-S332 results in a slower migrating form of the protein, whereas in vivo the POLO-dependent phosphorylation of MEI-S332 results in a faster migrating form. Because it appears that other kinases are phosphorylating MEI-S332 in vitro, these modifications may alter the mobility of MEI-S332 IVT protein as compared to the in vivo form. Additionally, other posttranslational modifications, such as ubiquitination, may be differentially present on MEI-S332 between the in vitro and in vivo forms, thereby affecting the mobility of the protein. These possibilities are supported by the fact that the unphosphorylated MEI-S332 IVT protein has a significantly slower mobility than both protein forms in vivo (data not shown). The in vitro *Xenopus* extract system demonstrates that a vertebrate POLO kinase is required for phosphorylation of *Drosophila* MEI-S332 in an anaphase state.

A POLO Box Domain Binding Site in MEI-S332 Is Required for Plx1-Dependent Phosphorylation in *Xenopus* Egg Extracts

A consensus motif of Ser-pSer/pThr-Pro/X defines a POLO box domain (PBD) binding site on POLO substrates (Elia et al., 2003a, 2003b). The PBD is conserved in POLO-like kinases from many organisms, and it is proposed to be critical for proper binding of POLO to the substrates and release of inhibition of the POLO kinase domain. In substrates, the central serine/threonine residue of the PBD binding site must be phosphorylated to ensure proper binding of POLO. We found this motif in two sites in MEI-S332: SSP with the central

serine at residue 234 and STP with the central threonine at residue 331 (Figure 4A). To test the relevance of these two PBD binding motifs in MEI-S332, we mutated the central residue in each motif to an alanine to make single and double mutants.

To determine if Plx1-dependent phosphorylation of MEI-S332 was likely to be the result of a direct interaction between POLO and MEI-S332, these PBD binding site mutant constructs were transcribed and translated in vitro, and resulting proteins were incubated with control- and Plx1-immunodepleted *Xenopus* mitotic extracts. Mitotic shifts of all three mutant proteins were demonstrated to be due to phosphorylation by treatment with lambda protein phosphatase (Figure 4B, lanes 4–9). We observed that the Plx1-dependent phosphorylation was unaffected by the S234A mutation but was abolished with the T331A and double mutations (Figure 4D, arrow, compare lanes 2, 3, 5, and 7). Thus, at least the T331 residue and possibly the combination of the T331 and S234 are required for Plx1-dependent phosphorylation to occur in mitotic extracts.

The PBD of POLO and Plx1 Binds MEI-S332 In Vitro

To test more directly if MEI-S332 could be a substrate of POLO kinase, we asked whether the *Drosophila* POLO PBD and the *Xenopus* Plx1 PBD interact with MEI-S332. We carried out GST pull-down experiments using purified GST, GST-POLO PBD (residues 298–576), and GST-Plx1 PBD (Elia et al., 2003a) (residues 317–598) proteins expressed in bacteria and lysates from transfected S2 cells. N-terminal GFP-tagged MEI-S332 was set under the control of the constitutive *armadillo* promoter (Vincent et al., 1994) and transiently expressed in *Drosophila* S2 cells. GFP-MEI-S332 showed strong binding to both the GST-POLO and GST-Plx1 PBD compared to GST alone (Figure 4E, approximately 3-fold above background).

We next asked if the binding of the PBD to MEI-S332 was affected in T331A, S234A, and double mutant-transfected S2 lysates. The double mutant protein was decreased in binding to POLO PBD by 50% and to Plx1 PBD by 24% (Figure 4E). The S234A and T331A mutant proteins also showed a decrease in binding to POLO and Plx1 PBDs, but to a lesser extent (data not shown). Input lysates for GST pull-down experiments are shown in Supplemental Figure S2. Together, these results suggest that MEI-S332 is a direct substrate of POLO kinase in vivo and that the interaction is mediated through the PBD of POLO kinase.

MEI-S332 Chromosomal Dissociation at the Metaphase/Anaphase Transition Requires a POLO Box Domain Binding Site

We determined whether the S234 and T331 sites were required in vivo for proper MEI-S332 localization. N-terminal GFP-tagged MEI-S332 wild-type and mutant constructs were transiently expressed in S2 cells. MEI-S332 localization was monitored by GFP fluorescence in interphase and mitotic cells to determine if MEI-S332 was localized properly during the cell cycle. As expected, wild-type MEI-S332 associated with chromosomes in metaphase and delocalized in anaphase (Figure 5A). All three mutant proteins localized normally to metaphase chromosomes in comparison to wild-type (Figures 5A–5C, center rows and data not shown). However, the T331A and the double mutant proteins remained localized to chromosomes in anaphase, telophase, and interphase in a higher percentage of cells than in wild-type transfected cells (Figures 5B–5D and Supplemental Table S1).

Quantification of these S2 results revealed that the S234A mutant is the least severe in the failure to dissociate MEI-S332. This mutant showed a similar number of anaphase cells with MEI-S332 localized to centromeres to that of wild-type and only a modest increase in the number of telophase and interphase cells with MEI-S332 chromosomal localization. However, T331A and the double mutant showed a more severe effect on MEI-S332 delocalization (Supplemental Table S1). In most cases, these MEI-S332 mutant proteins appeared to localize to entire chromosomes. It is possible that this spreading out of MEI-S332 away from centromeres may be due in part to a metaphase delay, since previously it has been shown that in metaphase-arrested cells, additional MEI-S332 loads onto and persists at noncentromeric sites (Lee et al., 2004; Tang et al., 1998). However, centromeric foci of MEI-S332 are clearly visible in a small number of anaphase cells for all three mutants (Figure 5D and data not shown). Overexpression of MEI-S332, particularly in tissue culture cells, can result in protein localization along the chromosomes not solely at the centromeres (Lee et al., 2004), and this most likely explains the persistence of the wild-type protein on chromosomes in some anaphase and telophase cells. The increased frequencies of chromosomal localization of mutant GFP-MEI-S332 in anaphase and telophase cells relative to wild-type GFP-MEI-S332 is not due to higher levels of expression of the mutant proteins than the wild-type following transfection. In contrast, Western blotting showed that the wild-type protein is more highly expressed than the mutants (Supplemental Figure S3). We cannot exclude the possibility that the mutant forms of GFP-MEI-S332 reload onto chromosomes in telophase, as opposed to persisting through the metaphase/anaphase transition. These results suggest that phosphorylation of at least residue T331 and possibly both T331 and S234 are critical for proper delocalization of MEI-S332 from chromosomes during anaphase. Further, these results are consistent with POLO kinase binding to MEI-S332 via phosphorylated PBD binding sites and helping MEI-S332 to be released from centromeres through phosphorylation.

Discussion

The results demonstrate that POLO kinase regulates MEI-S332 localization and aspects of its function. We have shown that *polo* mutants dominantly suppress the *mei-S332*^{8} nondisjunction phenotype and that wild-type MEI-S332 is retained at centromeres past the metaphase II/anaphase II transition in these *polo* mutants. MEI-S332 appears to be phosphorylated in mitosis at the metaphase/anaphase transition, and in *polo* mutants, MEI-S332 persists on centromeres into interphase, consistent with phosphorylation being a signal for MEI-S332 to delocalize. POLO kinase binds to MEI-S332,

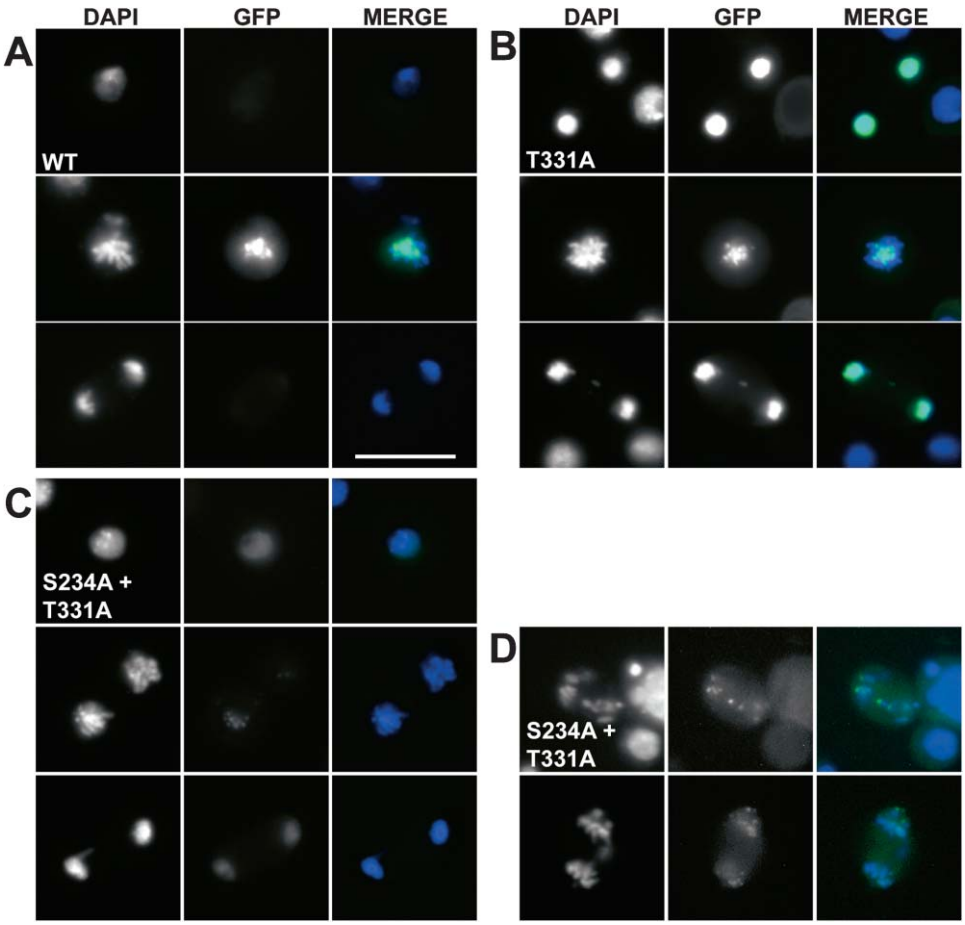

Figure 5. GFP-MEI-S332 Mutant Proteins Remain Localized to Chromosomes in All Stages of Mitosis in *Drosophila* S2 Cells

(A) Wild-type GFP-MEI-S332 localizes normally to centromeres during metaphase (center), but not during interphase (top) and anaphase (bottom).

(B and C) GFP-MEI-S332 mutant proteins remain localized to chromosomes throughout the cell cycle, in metaphase (center rows), anaphase (bottom rows), and late telophase into interphase (top rows).

(D) Centromere foci of GFP-MEI-S332-S234A+T331 mutant protein and the other two mutant proteins (data not shown) are also visible during anaphase. Localization of MEI-S332 was visualized by GFP fluorescence coupled with DAPI staining. Scale bar in (A) equals 20 μm.

and this is partly dependent on two PBD binding site motifs. Furthermore, in vitro phosphorylation of MEI-S332 is dependent on at least one motif and dependent on POLO. These two PBD binding site motifs in vivo are likely required for chromosomal dissociation of MEI-S332, similar to the effects observed in *polo* mutants. Together, these data point to POLO as a key regulator of MEI-S332 centromere localization in both mitosis and meiosis.

MEI-S332 Function and Localization Are Separable

POLO function is required for MEI-S332 delocalization from centromeres in mitosis and meiosis, but the ability of *polo* mutants to dominantly suppress *mei-S332* mutants additionally shows that POLO antagonizes MEI-S332 function. This is important because our results indicate that cohesion can be released even if MEI-S332 remains localized in *polo* mutants. MEI-S332 remains on centromeres after the metaphase II/anaphase II transition in *polo*/+ mutants, yet reasonably normal disjunction of chromosomes occurs during meiosis II in these heterozygotes. In mitosis, MEI-S332 can remain on the centromeres of the fourth chromosomes in *polo* mutants even when sister chromatid cohesion is released and they segregate to the poles. Thus, POLO phosphorylation is necessary for delocalization of MEI-S332, but there must exist a mechanism to inactivate MEI-S332 to release cohesion that is independent of MEI-S332 dissociation (Figure 6A). This pathway is not entirely dependent on POLO, although POLO may contribute.

The idea that MEI-S332 can remain localized to centromeres without cohesion between sister chromatids is supported by several examples. In *double parked* mutants, MEI-S332 localizes to unreplicated, single chromatids on which cohesion has never been established (Lee et al., 2004). This shows that the presence of sister chromatid cohesion is not a prerequisite for MEI-S332 localization to centromeres. Similarly, MEI-S332 localizes to single sister chromatids in *ord* mutants, in which sister chromatids separate prematurely early in meiosis I (Bickel et al., 1998). Finally, Sgo1 can localize to centromeres in early anaphase II when the 3′ UTR of Sgo1 is disrupted, yet no interference of the

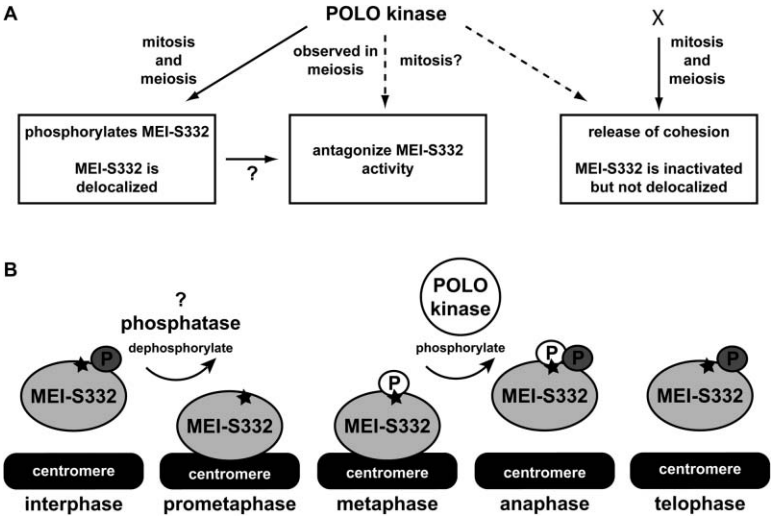

Figure 6. A Model for Regulation of MEI-S332 Centromere Localization by POLO Kinase

(A) MEI-S332 inactivation and delocalization are separable events. POLO kinase phosphorylates MEI-S332 and releases it from centromeres. POLO kinase also functions to antagonize MEI-S332's cohesive activity in meiosis but we have not determined if this regulation occurs in mitosis. The relationship between POLO antagonizing MEI-S332's function and the phosphorylation and release of MEI-S332 from centromeres is unknown. A dashed line indicates that POLO may affect another segregation mechanism, rather than antagonizing MEI-S332 directly. There must exist a second pathway (X) to inactivate MEI-S332 and release centromere cohesion without delocalization of MEI-S332. POLO may contribute to this pathway (dashed line), which could account for the dominant suppression observed.

(B) Model for MEI-S332 phosphorylation from anaphase to prometaphase. A dephosphorylation event by an unknown phosphatase occurs during prometaphase I of meiosis linked to the association of MEI-S332 with centromeres. At the metaphase/anaphase II transition, an unknown kinase phosphorylates T331 (star) and perhaps S234. This allows POLO kinase to bind to and phosphorylate MEI-S332 elsewhere (gray phosphate), resulting in its release from centromeres and concomitant separation of sister chromatids via release of cohesion. Similarly, during mitosis, MEI-S332 remains at the centromere until the metaphase/anaphase transition when it is phosphorylated by POLO kinase. It is unknown if MEI-S332 remains phosphorylated from anaphase until the next cell cycle.

release of sister chromatid cohesion is observed (Rabitsch et al., 2004). This result suggests that Sgo1 can promote sister chromatid cohesion in meiosis and can subsequently be inactivated yet remain at centromeres.

MEI-S332 Localization Is Regulated by Phosphorylation by POLO

We propose that MEI-S332 centromere localization is regulated by phosphorylation. In our model, the assembly of MEI-S332 onto centromeres in prometaphase I is controlled by the action of an unknown phosphatase (Figure 6B). MEI-S332 remains localized to centromeres until the metaphase II/anaphase II transition when POLO kinase binds to MEI-S332, via the phosphorylated T331 PBD binding site (Figure 6B, star), and phosphorylates MEI-S332 elsewhere (gray phosphate), initiating MEI-S332 dissociation from centromeres. Our data suggest that both S234 and T331 contribute to POLO binding and to MEI-S332 centromere dissociation, but in vitro T331 plays the predominant role in Plx1-dependent phosphorylation. Further, we suggest that POLO functions to antagonize MEI-S332 activity in meiosis, thereby affecting the release of sister chromatid cohesion. This may be either through phosphorylation of MEI-S332 or by affecting another component of sister chromatid segregation (Figure 6A). Based on our results, we cannot distinguish whether phosphorylation of MEI-S332 by POLO antagonizes MEI-S332 activity directly.

We propose that POLO directly phosphorylates MEI-S332 because the proteins can bind each other. Importantly, this binding is reduced by disruption of the PBD binding site motifs, and these mutations abolish Plx1-dependent phosphorylation of MEI-S332 and prevent MEI-S332 from dissociating from centromeres in S2 cells. PBD binding sites are required to be phosphorylated in order for POLO to bind to its substrates (Elia et al., 2003a). Given that we disrupted Plx1-dependent phosphorylation of MEI-S332 by mutating this site, it would seem that T331 and S234 need to be phosphorylated prior to POLO binding to MEI-S332, and then subsequent unknown sites on MEI-S332 can be phosphorylated by POLO kinase, thereby dissociating MEI-S332 from centromeres. In support of this idea, it has recently been proposed that once POLO binds to a substrate via an interaction with a PBD binding site, its kinase activity toward that substrate is stimulated (Elia et al., 2003b).

Which kinase is responsible for phosphorylating S234/T331 initially? Candidate kinases are a cyclin-dependent kinase, with specificity for sites with a proline in the +1 position (reviewed in Harper and Adams, 2001) as at the T331 and S234 sites, or a kinase such as Aurora B, which is localized to centromeres at the metaphase/anaphase transition in mitosis (reviewed in Carmena and Earnshaw, 2003). The control of this precise phosphorylation event would effectively prevent POLO from phosphorylating MEI-S332 and releasing it from centromeres until the appropriate time. Correlating with this possibility, during both mitosis and meiosis, POLO is poised at centromeres yet does not act to remove MEI-S332 until the proper time. In mitosis, MEI-S332 becomes localized to centromeres in prometaphase (Moore et al., 1998) and POLO kinase is also localized to centromeres at this time (Arnaud et al., 1998; Logarinho and Sunkel, 1998). In meiosis, from metaphase I to metaphase II POLO localizes to centrosomes and centromeres (Herrmann et al., 1998), yet MEI-S332 is not released until metaphase II/anaphase II (Kerrebrock et al., 1995).

MEI-S332 is the founding member of a family of proteins required for maintaining centromere cohesion between sister chromatids. Here, we define POLO kinase as crucial for delocalization of MEI-S332. We also show that POLO antagonizes MEI-S332 activity. Our results strongly indicate that POLO directly phosphorylates MEI-S332 and that this leads to delocalization. It will be

interesting to identify the anchor for MEI-S332 centromere binding and to decipher how phosphorylation of MEI-S332 affects this interaction. Our results additionally uncover a mechanism distinct from delocalization to inactivate MEI-S332.

Experimental Procedures

For Experimental Procedures as well as Supplemental Figures and Tables, see supplemental data online.

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