



Drosophila protein phosphatases 2A B' Wdb and Wrd regulate meiotic centromere localization and function of the MEI-S332 Shugoshin

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Proper segregation of chromosomes in meiosis is essential to prevent miscarriages and birth defects. This requires that sister chromatids maintain cohesion at the centromere as cohesion is released on the chromatid arms when the homologs segregate at anaphase I. The Shugoshin proteins preserve centromere cohesion by protecting the cohesin complex from cleavage, and this has been shown in yeasts to be mediated by recruitment of the protein phosphatase 2A B' (PP2A B'). In metazoans, delineation of the role of PP2A B' in meiosis has been hindered by its myriad of other essential roles. The *Drosophila* Shugoshin MEI-S332 can bind directly to both of the B' regulatory subunits of PP2A, Wdb and Wrd, in yeast two-hybrid experiments. Exploiting experimental advantages of *Drosophila* spermatogenesis, we found that the Wdb subunit localizes first along chromosomes in meiosis I, becoming restricted to the centromere region as MEI-S332 binds. Wdb and MEI-S332 show colocalization at the centromere region until release of sister-chromatid cohesion at the metaphase II/anaphase II transition. MEI-S332 is necessary for Wdb localization, but, additionally, both Wdb and Wrd are required for MEI-S332 localization. Thus, rather than MEI-S332 being hierarchical to PP2A B', these proteins reciprocally ensure centromere localization of the complex. We analyzed functional relationships between MEI-S332 and the two forms of PP2A by quantifying meiotic chromosome segregation defects in double or triple mutants. These studies revealed that both Wdb and Wrd contribute to MEI-S332's ability to ensure accurate segregation of sister chromatids, but, as in centromere localization, they do not act solely downstream of MEI-S332.

meiosis | sister-chromatid cohesion | centromere | chromosome segregation | spermatogenesis

The reduction of chromosome number to produce haploid gametes, the crucial consequence of meiosis, results from two rounds of chromosome segregation that are not punctuated by DNA replication. In the first meiotic division, the homologous copies of each chromosome pair and segregate whereas the replicated sister chromatids do not segregate until the second meiotic division. Deferral of sister-chromatid segregation until meiosis II requires that cohesion between the sister chromatids be maintained at the centromere until the metaphase II/anaphase II transition. This is accomplished by the Shugoshin (Sgo) family of proteins that protect the cohesin complex at the centromere, ensuring that it is retained as cohesin along the chromosome arms is cleaved and removed at the metaphase I/anaphase I transition (1).

The founding member of the Sgo family, the *Drosophila* MEI-S332 gene, was recovered as a mutant that exhibited premature loss of sister-chromatid cohesion in late meiosis I, resulting in chromosome loss and nondisjunction in meiosis II (2–4). The MEI-S332 protein was shown to localize to centromeres from prometaphase I until the metaphase II/anaphase II transition, corresponding to release of centromeric sister-chromatid cohesion (5, 6). MEI-S332 has been shown to maintain the SMC1 cohesin subunit and SOLO, another cohesin protein, on meiotic centromeres until anaphase II (7). Although MEI-S332 is not essential for mitosis, it localizes to mitotic centromeres and contributes

to sister-chromatid attachment when cohesion along the chromosome arms is compromised (8). Rather than a Sgo, the Dalmatian protein recently has been shown to be essential to protect centromere cohesion in mitosis in *Drosophila* (9).

Analysis of the function of Sgo protein family members in vertebrates revealed distinct mechanisms by which Sgo proteins protect sister-chromatid cohesion at the centromere in mitosis and meiosis (1, 10). In mitosis, Sgo1 protects the cohesin complex against removal by Wapl whereas, in meiosis, Sgo2 retains centromere cohesion by blocking cleavage of a meiosis-specific subunit of the cohesin complex. Despite the different mechanisms of cohesin removal, in both mitosis and meiosis, centromere protection by Sgo is mediated by recruitment of one form of protein phosphatase 2A (PP2A) B' (1, 10). PP2A B' has a catalytic subunit, the A structural subunit, and the B' form of the regulatory subunits (11). In mitosis, PP2A B' dephosphorylates cohesion, as well as the Wapl inhibitor Sororin, to stabilize cohesin (12–14). In yeasts, as well as mouse, the meiosis-specific Rec8 subunit of cohesin requires phosphorylation for Separase cleavage, and cohesin is thus protected at the centromere by the action of PP2A B' (15–19). Support for a crucial meiotic role for PP2A is provided by the observation that the PP2A inhibitor, I2PP2A, is required for separation of sister chromatids in meiosis II in mouse oocytes (20).

These studies highlight the significance of the PP2A phosphatase in chromosome segregation. PP2A plays a myriad of cellular roles, and its function is essential. This has limited delineation of the

Significance

Meiosis is the specialized cell division that generates haploid sperm and eggs. Proper segregation of chromosomes in meiosis is required to prevent pregnancy loss and birth defects. This requires that the replicated copies of each chromosome remain attached as the homologous copies of each chromosome segregate in the first meiotic division. The replicated copies of each chromosome then segregate in the second meiotic division. The Shugoshin proteins protect attachments between the replicated chromosome copies in meiosis I. This paper shows that, in *Drosophila* meiosis, a phosphatase and the Shugoshin MEI-S332 reciprocally regulate each other's localization to centromeres and together they thus function to ensure accurate segregation.

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requirements for PP2A B' in metazoan meiosis, including defining its dependency on Sgo, as well as the identification of potential roles for PP2A B' independent of Sgo. The importance of defining the relationship and dependency between Sgo and PP2A B' in meiosis is heightened further by the observations that Sgo family members can interact with multiple proteins to control chromosome segregation by distinct mechanisms (1, 10). Examples include the Chromosome Passenger Complex and MCAK.

We chose to investigate the meiotic role of PP2A B' and its relationship to MEI-S332 in *Drosophila* (4, 5, 21–24), as we identified the *Drosophila* B' subunits as the predominant MEI-S332 interactors in a yeast two-hybrid screen. *Drosophila* encodes single PP2A catalytic and A subunits, but has four B-type subunits (25). There are two B' subunits that are expressed throughout development, including in adult testes and ovaries (26). One of these, encoded by the *widerborst* (*wdb*) gene, is essential (27). The other B' subunit is called Well Rounded (*Wrd*), and null mutations in the *wrd* gene are viable but affect neuromuscular junctions (28). In *Drosophila* cell culture, *Wrd* is not required for mitosis but exhibits some redundancy with *Wdb*, which was shown to be required for normal levels of MEI-S332 centromere localization (25). In addition to the availability of mutants, *Drosophila* spermatogenesis provides the experimental advantages for meiotic analysis that sister-chromatid cohesion and centromere localization of the MEI-S332 Sgo can be visualized directly throughout all meiotic stages and chromosome segregation accuracy can be quantified (29). Using these approaches, here, we identify reciprocal dependency between the MEI-S332 Sgo and the PP2A B' phosphatases for centromere localization and shared roles in sister-chromatid segregation.

Results

MEI-S332 Interacts with both *Drosophila* PP2A B' Subunits *Wdb* and *Wrd*. To identify proteins that could participate with MEI-S332 in controlling chromosome segregation, we used a yeast two-hybrid approach. Ovary cDNA libraries were used to enrich for potential meiotic partners of MEI-S332. Although ~25 independent interactors were identified, the proteins for which clones were repeatedly recovered were the two B' regulatory subunits of the PP2A phosphatase, *Wrd* and *Wdb*. These results are consistent with the observation that, when ectopically expressed in mitotic S2 cells, MEI-S332 can coimmunoprecipitate the *Wdb* B' form of PP2A (25) but extend the results by demonstrating that this interaction can be via direct binding to *Wdb*, as well as the other B' subunit, *Wrd*.

***Wdb* Colocalizes with MEI-S332 at Centromeric Regions of Spermatocytes.** Given the physical binding of both PP2A B' subunits to MEI-S332, we wanted to determine whether these regulatory subunits localize to meiotic chromosomes, as has been previously demonstrated for a *Wdb*-GFP fusion protein on mitotic centromeres (25). Immunostaining of testes with anti-*Wdb* antibodies (30) produced striking punctate staining consistent with centromere localization (Fig. 1). To test whether *Wdb* indeed localized to the centromere, we costained the spermatocytes with anti-CID antibodies. This additionally served as a positive control for the staining procedure as this antibody gives consistent staining and localizes to centromeres in 100% of spermatocytes. *Wdb* was first detected in midprophase I (stage 4) spermatocytes where it localized to both the arms and centromeric regions of the chromosomes (Fig. 1). This pattern of localization was maintained until the transition to early prometaphase I when most of the arm localization was lost and *Wdb* was concentrated in the region closer to the centromeres. In prometaphase I and metaphase I, *Wdb* was solely present in the pericentromeric region, displaying a broader localization than CID (Fig. 1 and Fig. S1), and remained there until anaphase I when homologous chromosomes separate. At telophase I, *Wdb* was undetectable on the chromosomes but relocalized to the centromeric region at prometaphase II. This localization was maintained through metaphase II and was lost by anaphase II when sister chromatids separated. To determine the localization profile of *Wrd*, we stained spermatocytes with antibodies generated against this B' subunit (*SI Materials and Methods*). Unfortunately, despite testing multiple fixation conditions

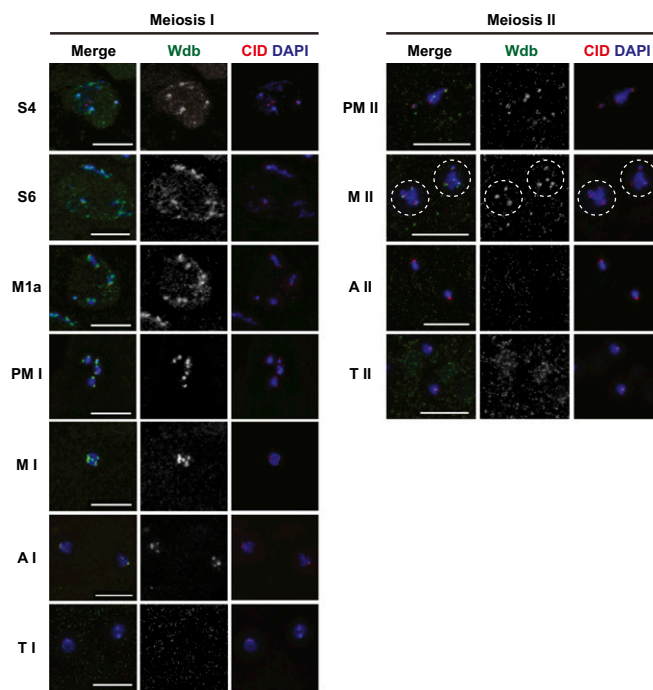


Fig. 1. Localization of *Wdb* in male meiosis. Meiotic stages (38) are labeled on the left of the Meiosis I and Meiosis II panels: A I, anaphase I; A II, anaphase II; M I, metaphase I; M II, metaphase II; M1a, early prometaphase I; PM I, prometaphase I; PM II, prometaphase II; S4, midprophase I; S6, late-prophase I; T I, telophase I; T II, telophase II. Merged panels show *Wdb* in green, CID in red, and DAPI in blue. Split channels are shown for *Wdb* alone and CID with DAPI. Dashed circles in M II demarcate individual M II spermatocytes. (Scale bars: 20 μ m.) *Wdb* was detected on the arms and centromeres of chromosomes in 45% of S4 ($n = 92$) and 38% of S6 ($n = 430$) spermatocytes. In 92% of early and late PM I, *Wdb* localization was restricted to the centromeric region ($n = 52$), and it was detected only at the centromeres in 93% of M I ($n = 15$) and 100% of A I ($n = 4$) spermatocytes. *Wdb* was not present on the chromosomes in T I ($n = 19$, 100%). *Wdb* was again detected at the centromeres in 72% of PM II spermatocytes ($n = 25$) and 90% of M II spermatocytes ($n = 10$), lost by A II ($n = 10$, 100%), and absent from T II centromeres ($n = 9$, 100%).

and staining procedures, we were unable to detect *Wrd* on chromosomes by immunofluorescence.

The timing of the pericentromeric localization of *Wdb* from prometaphase I to telophase II is almost identical to that of MEI-S332 (5, 22, 23). To assess whether *Wdb* colocalizes with MEI-S332, we examined the localization of this B' subunit in spermatocytes from larvae expressing a functional MEI-S332-GFP fusion protein under the control of the endogenous promoter (5). *Wdb*, but not MEI-S332, was present on chromosome arms and centromeres in prophase I (Fig. 2). From prometaphase I through the metaphase II/anaphase II transition, MEI-S332 and *Wdb* colocalized in the centromere region, showing the temporal localization pattern described above for *Wdb*.

These data demonstrate that *Wdb* colocalizes with MEI-S332 in meiosis but that, unexpectedly, PP2A-*Wdb* localizes to the centromere before detection of MEI-S332 (Fig. 1). With two different antibodies against MEI-S332, as well as the GFP fusion protein, MEI-S332 was not detectable before prometaphase I (5, 6, 22, 23). Additionally, the two proteins differ in when in meiosis they are present along chromosome arms. *Wdb* is present on chromosome arms before centromere localization in prophase I whereas MEI-S332 is present on chromosome arms during a brief window of anaphase I (23), but we did not detect *Wdb* on the arms during this stage.

MEI-S332 Is Required for Centromere Localization of *Wdb* in Meiosis. The chromosomal localization patterns observed for *Wdb* and MEI-S332 in spermatocytes argue against a simple model that

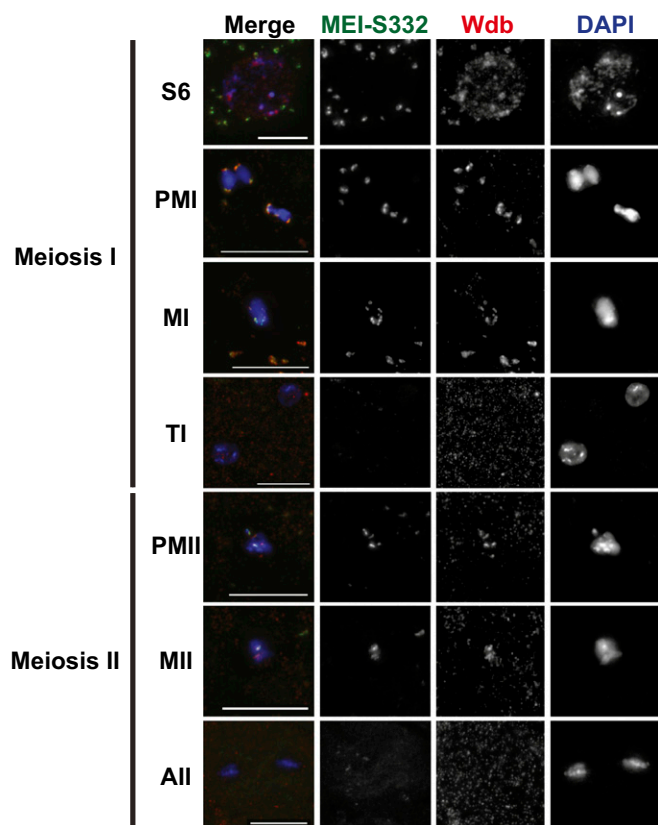


Fig. 2. Localization of MEI-S332 and Wdb during male meiosis. Merged panel shows localization of MEI-S332-GFP in green, Wdb in red, and DAPI in blue. Split channels are shown for MEI-S332-GFP, Wdb, and DAPI. Labels and scale bars are as in Fig. 1. (Scale bars: 20 μ m.) As noted previously, MEI-S332-GFP was present in puncta in the cytoplasm of primary spermatocytes (5). Wdb was present in late prophase I (S6) spermatocytes in which MEI-S332 was undetectable on the chromosomes ($n = 13$, 77%). Wdb and MEI-S332 colocalized at centromeres in prometaphase I (PMI) ($n = 44$, 98%) and metaphase I (MI) spermatocytes ($n = 27$, 100%). Both proteins were undetectable at the centromeres in telophase I (TI) ($n = 2$, 100%). They relocated to the centromeres in prometaphase II (PMII) ($n = 33$, 100%) and metaphase II (MII) ($n = 18$, 100%) and were lost from centromeres in anaphase II (All) ($n = 7$, 100%).

MEI-S332 solely directs localization of Wdb in meiosis. In mitotic *Drosophila* S2 cells, RNAi against *wdb* reduced MEI-S332 centromere localization, but depletion of MEI-S332 did not reciprocally affect localization of Wdb-GFP (25). It has been reported in mammalian cells that PP2A can be necessary for Sgo localization (31). Consequently, having defined colocalization of Wdb and MEI-S332 on meiotic centromeres, but differences in timing, we set out to delineate dependency relationships. We first examined Wdb localization by immunostaining *mei-S332* mutant spermatocytes, using the *mei-S332^d/mei-S332⁷* null allelic combination (4). Centromeres were marked by costaining with CID antibodies. In midlate prophase I, Wdb localized to the chromosomes comparably in WT and *mei-S332* mutant spermatocytes (Fig. 3). In contrast, whereas 96% of CID-positive WT spermatocytes showed foci of Wdb on the chromosomes in prometaphase I (PMI) and metaphase I (MI), none of the CID positive spermatocytes of the same stage displayed Wdb signal in the *mei-S332* mutant. Similarly, in prometaphase II and metaphase II, no CID-positive spermatocytes showed Wdb staining in the *mei-S332* mutant, compared with 65% of WT CID-positive spermatocytes. Thus, in contrast to previous results in cultured mitotic cells, MEI-S332 is required to maintain the centromeric localization of Wdb from prometaphase I onward.

Both PP2A B' Subunits Are Required for MEI-S332 Localization. We next tested whether the PP2A B' phosphatases are required to localize MEI-S332 to meiotic centromeres. Because *wdb* is an essential gene, we could not test homozygous loss-of-function mutants. In addition, *wdb* is cell-lethal, making clonal analysis not possible (27). RNAi against *wdb* did not eliminate the protein in spermatocytes (Fig. S2). *Drosophila* homozygous for a deletion of *wrd* are viable; thus, we first examined this B' subunit. MEI-S332 centromere localization was not affected in spermatocytes lacking the *wrd* gene and WT for the *mei-S332* gene (Fig. S3). We reasoned that use of an allele of *mei-S332* that compromised function might yield a threshold at which effects on MEI-S332 protein localization or function could be detected.

The *mei-S332^s* mutation provided several important experimental advantages for delineating the relationship with the PP2A B' phosphatases. This allele reduces *mei-S332* function but does not eliminate centromere localization of the protein (32). The mutation does not reduce the levels of MEI-S332 protein (32). In humans, the B' subunit interacts with a conserved coiled-coil domain at the N terminus of Sgo1 (13). The *mei-S332^s* mutation changes V35 on the interface of the predicted coiled coil of MEI-S332 to Glu and weakens dimerization, which should reduce PP2A B' binding (13, 32). The MEI-S332^s protein does not have dominant negative or gain-of-function properties because the *mei-S332^s* allele is completely recessive (4). By examining *mei-S332^s* in trans to the genetic and protein null *mei-S332^d* mutation (5, 32), we could test solely the sensitized MEI-S332^s protein form.

We examined MEI-S332 localization in spermatocytes from *mei-S332^s/mei-S332^d* flies that lack the Wrd B' subunit and compared it with localization in the *mei-S332^s/mei-S332^d* sibling controls to control for background effects in the stocks. In this background, the control *mei-S332^s/mei-S332^d* flies had 95% of spermatocytes with normal MEI-S332 localization (Fig. 4). In the *mei-S332 wrd* double mutants, however, the number of spermatocytes with normal MEI-S332 staining was reduced to 58%. These results show that Wrd facilitates localization of MEI-S332 on the centromeres in meiosis I. We used the yeast two-hybrid system to evaluate how MEI-S332^s affects interaction with Wrd (Fig. S4). Interactions were weaker between Wrd and MEI-S332^s than WT MEI-S332. Thus, this B' subunit may depend on the coiled coil or its dimerization for interaction with MEI-S332, and it could promote MEI-S332 centromere localization via a direct physical interaction.

We tested for dominant effects of a mutation of *wdb*, *wdb^{IP}* (27), on MEI-S332 localization in the background of *mei-S332^s/mei-S332^d* transheterozygotes. The *wdb^{IP}* allele, which is recessive for the characterized functions of Wdb, contains a stop codon that eliminates the C-terminal third of the protein, including the region shown to interact with Sgo1 (13, 27). In the *mei-S332^s/mei-S332^d* sibling controls, MEI-S332 localized normally to the centromeres in 77% of spermatocytes observed (Fig. S5). In the remaining 23% of spermatocytes, MEI-S332 localization was reduced, with some chromosomes lacking MEI-S332 foci (Fig. S5). In the *mei-S332^s/mei-S332^d; wdb^{IP}/+* mutants, the percentage of spermatocytes displaying normal MEI-S332 localization was reduced to 31%. Thus, full Wdb function is required for localization of MEI-S332 to the centromere region in meiosis I, even when Wrd is unaffected. Yeast two-hybrid binding experiments showed that Wdb binds more weakly to WT MEI-S332 than Wrd, but the interaction with Wdb is strengthened by the MEI-S332^s mutation (Fig. S4). This interesting observation remains to be understood at a mechanistic level, but it suggests that Wdb either may bind another domain on MEI-S332 or not require dimerization of the coiled coil. Taken together, both Wdb and Wrd are necessary for centromere localization of MEI-S332, but their mechanisms of ensuring localization may differ. Wrd could tether MEI-S332 through binding the coiled coil whereas Wdb could bind through another domain or possibly affect MEI-S332 localization in an indirect manner.

Wrd Does Not Play an Essential Role in Meiosis. Given the role of both the PP2A B' subunits in proper localization of MEI-S332 to meiotic centromeres, we tested whether loss of function of the B'

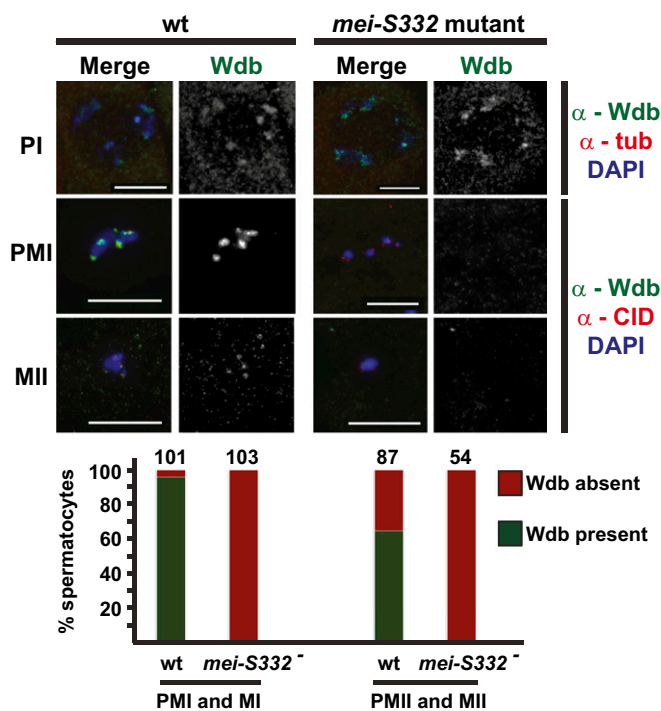


Fig. 3. Effect of loss of MEI-5332 on Wdb localization during male meiosis. (Top) In the merged panel, localization of Wdb in wild-type (wt) and *mei-S332* null mutant spermatocytes is shown in green, DAPI is in blue, and CID is shown in red in the lower panels. In the prophase I (PI) panels α -tubulin is shown in red (α -tub). Wdb was present on the DNA in wt and *mei-S332* mutant PI spermatocytes. Unlike in wt, however, Wdb was no longer detected at prometaphase I (PMI) and metaphase II (MII) in the *mei-S332* mutant spermatocytes. (Scale bars: 20 μ m.) (Bottom) Quantification of Wdb localization in wt and *mei-S332* mutant spermatocytes in meiosis I and II. Green indicates presence of Wdb, and red indicates absence of Wdb. The number of spermatocytes scored for each genotype in three experiments is indicated above each bar. MI, metaphase I; PMII, prometaphase II.

subunit genes resulted in meiotic chromosome nondisjunction. We were able to examine the consequences of complete loss of the B' subunit Wrd but could analyze only heterozygous *wdb* alleles and the effects of RNAi. In addition to the *wdb*^{IP} truncation

allele, we examined the *wdb*^{dw} allele, which generates a stop codon predicted to terminate the protein nine amino acids from the N terminus (27). Nondisjunction of the XY sex chromosomes was scored by adult visible markers. Sperm lacking both sex chromosomes are indicative of either meiosis I or II nondisjunction or chromosome loss. The production of XY sperm is diagnostic of meiosis I nondisjunction whereas the presence of XX sperm indicates meiosis II nondisjunction. In *mei-S332* mutants, the nondisjunctional gametes are either null for the sex chromosomes or nearly all XX sperm, because precocious loss of sister-chromatid cohesion does not occur until anaphase I. Thus, meiosis I segregation is normal, but sister chromatids segregate randomly in meiosis II (4).

Heterozygous *wdb* alleles, RNAi against *wdb*, or complete loss of *wrd* did not significantly affect meiotic chromosome segregation, even when the null *wrd* alleles were combined with *wdb* mutations (Tables S1 and S2). These data suggest that either complete loss of Wdb or complete loss of both B' phosphatases is needed to disrupt meiotic chromosome segregation.

Wdb and Wrd Cooperate with MEI-5332 in Protecting Centromeric Cohesion. Another approach to address whether the *Drosophila* B' phosphatases participate in the function of MEI-5332 is to test for a genetic interaction between *mei-S332* and *wdb* or *wrd* mutants. In these studies, we analyzed whether mutations in the genes encoding the two PP2A B' subunits enhanced or suppressed the *mei-S332* mutant meiosis II nondisjunction phenotype, using the sensitized allelic combination *mei-S332*⁸/*mei-S332*⁴.

To test for a genetic interaction between *mei-S332* and *wdb* or *wrd*, *mei-S332*⁸/*mei-S332*⁴ males heterozygous for *wdb* or *wrd* mutant alleles were scored for nondisjunction of the sex chromosomes and compared with *mei-S332*⁸/*mei-S332*⁴ sibling controls (Table 1). The sibling controls are needed because strain background influences the extent of nondisjunction. Both *wdb*^{IP} and *wdb*^{dw} significantly dominantly enhanced the *mei-S332*⁸/*mei-S332*⁴ nondisjunction phenotype whereas heterozygous *wrd* did not. As Wdb and Wrd are partially redundant in mitosis (25), we examined chromosome segregation in *mei-S332*⁸/*mei-S332*⁴ flies that were heterozygous for a *wrd* deletion and the *wdb* deficiency, *Df(3R)ED6265* (*mei-S332*⁸/*mei-S332*⁴; *PP2A-B'*^A *Df(3R)ED6265/+*). Heterozygosity for both B' subunits did not significantly elevate nondisjunction levels compared with *mei-S332*⁸/*mei-S332*⁴; *wdb*^{IP}/+ males. In contrast, *mei-S332*⁸/*mei-S332*⁴ males that completely lacked *wrd* displayed significant enhancement of meiosis II nondisjunction compared with *mei-S332*⁸/*mei-S332*⁴ control males (Table 1). These results are

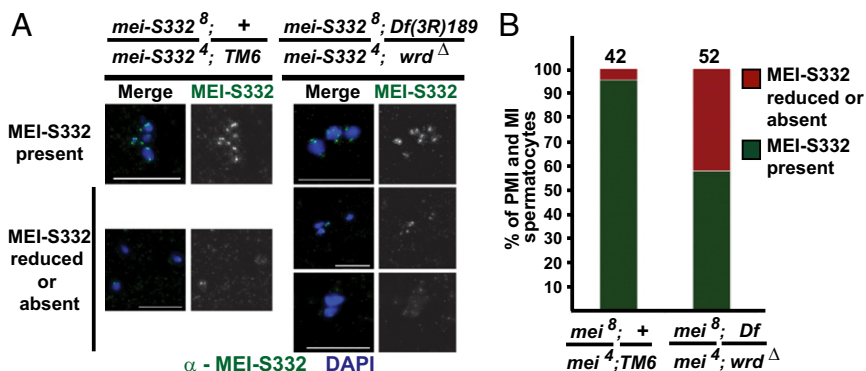


Fig. 4. Localization of the MEI-5332⁸ protein in *wrd* null spermatocytes. (A) Localization of MEI-5332⁸ in prometaphase I (PMI) and metaphase I (MI) spermatocytes from *mei-S332*⁸/*mei-S332*⁴; *TM6*/+ control, and *mei-S332*⁸/*mei-S332*⁴; *wrd*^Δ/*Df(3R)189* mutant males. *wrd*^Δ represents the *PP2A-B'*^A deletion allele of *wrd*. In the merged panel, MEI-5332⁸ localization is shown in green, and DNA stained with DAPI is blue. The Top represents the "MEI-5332 present" category, in which MEI-5332 was detected on all centromeres, whereas the Middle and Bottom represent "MEI-5332 reduced" and "MEI-5332 absent" categories, in which MEI-5332 was absent from some or all chromosomes. (Scale bars: 20 μ m.) (B) Quantification of MEI-5332⁸ localization in control and mutant PMI and MI spermatocytes. Green represents the "MEI-5332 present" category, and red represents the "MEI-5332 reduced" and "MEI-5332 absent" categories. The number of spermatocytes scored in two experiments is indicated above each bar.

Table 1. Sex chromosome nondisjunction in *mei-S332⁸/mei-S332⁴* males carrying mutant alleles of *wdb* and *wrd*

Genotype	Regular sperm		Exceptional sperm				Total progeny	Total exceptional progeny (%)
	Y(Y)*	X	Null0-XY (%)	XX (%)	XY(Y) (%)	XX(Y) (%)		
<i>ywly⁺Y; mei-S332⁸/mei-S332⁴; wdb^{IP}/+</i>	683	773	158 (9.4)	53 (3.1)	11 (0.7)	0 (0)	1,677	221 (13.2) [†]
<i>ywly⁺Y; mei-S332⁸/mei-S332⁴; +/+ (sib control)</i>	824	917	86 (4.7)	17 (0.9)	6 (0.3)	0 (0)	1,850	109 (5.9)
<i>ywly⁺Y; mei-S332⁸/mei-S332⁴; wdb^{dw}/+</i>	774	861	135 (7.5)	23 (1.3)	8 (0.4)	0 (0)	1,801	166 (9.2) [†]
<i>ywly⁺Y; mei-S332⁸/mei-S332⁴; +/+ (sib control)</i>	937	925	86 (4.4)	14 (0.7)	9 (0.5)	0 (0)	1,971	109 (5.5)
<i>ywly⁺Y; mei-S332⁸/mei-S332⁴; PP2A-B^{-Δ}/+[‡]</i>	687	805	156 (9.0)	87 (5.0)	2 (0.1)	0 (0)	1,737	245 (14.1)
<i>ywly⁺Y; mei-S332⁸/mei-S332⁴; +/+ (sib control)</i>	754	846	166 (9.0)	74 (4.0)	6 (0.3)	0 (0)	1,846	246 (13.3)
<i>ywly⁺Y; mei-S332⁸/mei-S332⁴; PP2A-B^{-Δ} Df(3R)ED6265/+[§]</i>	439	567	136 (11.4)	50 (4.2)	1 (0.1)	0 (0)	1,193	187 (15.7) [†]
<i>ywly⁺Y; mei-S332⁸/mei-S332⁴; +/+ (sib control)</i>	594	764	75 (5.2)	11 (0.8)	5 (0.4)	0 (0)	1,449	91 (6.3)
<i>ywly⁺Y; mei-S332⁸/mei-S332⁴; PP2A-B^{-Δ}/Df(3R)189[¶]</i>	238	285	145 (20.0)	56 (7.7)	0 (0)	0 (0)	724	201 (27.8) [†]
<i>ywly⁺Y; mei-S332⁸/mei-S332⁴; +/TM6, Tb (sib control)</i>	509	600	40 (3.4)	9 (0.8)	11 (0.9)	0 (0)	1,170	61 (5.2)

*Diplo-Y sperm cannot be distinguished from regular sperm with a single Y chromosome.

[†]Designates significant difference ($P < 0.05$).

[‡]PP2A-B^{-Δ} is a deletion of the *wrd* gene.

[§]Df(3R)ED6265 is a deficiency covering the *wdb* genomic region.

[¶]Df(3R)189 is a deficiency covering the *wrd* genomic region.

consistent with both Wdb and Wrd working with MEI-S332 in protecting centromeric cohesion during meiosis I to ensure accurate meiosis II segregation. The reduction of MEI-S332 centromere localization observed in the *wdb* and *wrd* mutants is consistent with the enhanced meiotic nondisjunction observed in the double and triple mutants.

Discussion

The relationship between the PP2A B' phosphatase and Shugoshin proteins has been extensively analyzed in mitosis, leading to the conclusion that a key function of Shugoshin is to anchor the phosphatase to the centromere to protect cohesin from removal by Wapl. Elucidation of the roles of Shugoshin and PP2A B' in meiosis have been refractory, due to the essential functions of PP2A B' in many processes. The localization studies and genetic function tests reported here indicate that, in contrast to the prevailing model, PP2A B' is not solely downstream of the MEI-S332 Shugoshin. Rather, they reveal a reciprocal functional relationship between the proteins to ensure proper sister-chromatid segregation in meiosis.

The Wdb B' subunit of PP2A localizes initially along meiotic chromosomes, becoming restricted to the centromere by prometaphase I. Thus, Wdb is present on meiotic chromosomes before MEI-S332 whereas, by multiple approaches, MEI-S332 is undetectable at the centromere until nuclear envelope breakdown and prometaphase I (5, 6). Both B' subunits, Wdb and Wrd, are required for centromere localization of MEI-S332, and MEI-S332 is necessary for centromere association of Wdb from prometaphase I until anaphase II. Therefore, these proteins do not show a simple hierarchical relationship with respect to centromere binding but reciprocally contribute to each other's localization. Despite the opposite effects of the V35E mutation on the interaction of MEI-S332⁸ with Wrd and Wdb in the yeast cell, the centromere localization data show that the PP2A B' subunits do not act competitively but rather in parallel for MEI-S332 localization in *Drosophila* spermatocytes.

The localization results presented here for Wdb, combined with previous results on MEI-S332 and chromosome localization patterns of other proteins, reveal a tug of war between localization to the arms versus the centromere. This can be manifest as timing differences, wherein Wdb and the INCENP subunit of the Chromosome Passenger Complex (CPC) initially localize along the arms in *Drosophila* meiosis and then become restricted to the centromere (24). But rather than solely timing differences, these spatial changes could be due to regulation by protein interactions. For example, it is possible that the CPC controls Wdb localization or vice versa.

INCENP is known to restrict MEI-S332 localization to the centromere in meiosis I (24), and Sgo2 is removed from the chromosome arms by phosphorylation by Aurora B/C (33). PP2A activity has been proposed to promote movement of Sorin from chromosome arms to the centromere in mouse meiosis (34). Sgo family members from a number of organisms restrict the CPC to the centromere (1), and, in *Xenopus* mitosis, Sgo2 is needed to localize MCAK to the centromere rather than the arms (35).

The ability to quantify chromosome missegregation in meiosis I and II in *Drosophila* permitted us to assess functional interactions between Wdb, Wrd, and MEI-S332. Reducing the function of Wdb or eliminating Wrd enhanced meiosis II missegregation in *mei-S332* mutants. Importantly, although complete loss of Wrd function did not perturb meiotic chromosome segregation, it significantly increased missegregation in *mei-S332⁸/mei-S332⁴* mutants. Given the level of enhanced chromosome missegregation in the *mei-S332*, *wrd* double mutants, it is puzzling that loss of Wrd alone does not affect meiotic segregation. The simplest explanation is that both B' subunits are redundant in their functional interaction with MEI-S332 even though they appear to have different mechanisms for binding MEI-S332. With WT MEI-S332, Wdb compensates for absence of Wrd. The compromised MEI-S332⁸ protein form demands full function of both Wdb and Wrd for accurate chromosome segregation.

Other Shugoshin protein family members affect multiple aspects of chromosome segregation in mitosis, such as chromosome congression and kinetochore tension (1, 10). If these roles were conserved in meiosis, then chromosome segregation errors would be expected in meiosis I, rather than the meiosis II missegregation resulting from premature loss of sister-chromatid cohesion at anaphase I. Mutants for *mei-S332* exhibit a low level of meiosis I segregation errors: about 10% the frequency of the meiosis II errors (4, 32). Notably, the enhancement of *mei-S332* defects resulting from reduced activity of Wdb and Wrd exclusively affects meiosis II sister-chromatid cohesion. Thus, any additional roles of MEI-S332 in meiosis I likely are not mediated via PP2A. It has been proposed that the additional meiotic functions of mouse Sgo2 beyond protecting centromere cohesin are independent of PP2A (18).

The codependency of MEI-S332 and the two PP2A B' forms for centromere localization and control of sister-chromatid segregation could reflect the fact that they need to be in a complex for centromere binding and cohesin protection. Alternatively, MEI-S332 could serve to localize the two PP2A B' forms directly, and the requirement for Wdb and/or Wrd in MEI-S332 localization and function could be indirect. We previously found that Polo

kinase phosphorylation leads to dissociation of MEI-S332 from the centromeres of mitotic and meiotic chromosomes (21); thus, the two PP2A B' forms could retain MEI-S332 on the centromere by dephosphorylating MEI-S332 to counteract Polo. This role of Plk1 has been proposed for retention of Sgo1 on mitotic centromeres in mammals (31). In either case, the results presented here demonstrate that MEI-S332 does not function solely as a scaffold to localize PP2A B' and is itself dependent on PP2A B' activity. It will be important to explore whether MEI-S332 exerts effects on sister-chromatid cohesion that are independent of the PP2A B' phosphatases, why both Wdb and Wrd are required in meiosis, and whether the PP2A B' phosphatases play roles independently of MEI-S332.

Materials and Methods

Ethics Statement. The production of antibodies in guinea pigs was approved by the Committee for Animal Care at the Massachusetts Institute of Technology.

Yeast Two-Hybrid Screen. *Drosophila* proteins capable of binding MEI-S332 were identified from yeast two-hybrid screens using the Gal4/LexA system with MEI-S332 constructs (32) and ovary cDNA libraries from Finley or the Ovo1b library (36). The entire coding region for MEI-S332 was used as bait. Interaction levels between MEI-S332 and Wdb or Wrd were tested by Hybrigenics Services, as detailed in *SI Materials and Methods*.

Drosophila Stocks. All *Drosophila* stocks and crosses were maintained at 25 °C on standard cornmeal-brewer's yeast-molasses-agar food. The *mei-S332* mutant alleles and functional MEI-S332-GFP fusion transgene lines have

been described previously (4, 5). The following mutant stocks were generously provided by other laboratories: the *wdb* mutant alleles, *wdb^{lP}* and *wdb^{dw}* (Suzanne Eaton, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) (27), *PP2A-B^A* and *PP2A-B^A Df(3R)ED6265* (Anthony Percival-Smith, Western University, London, ON, Canada) (37) that carry deletions of *wrd*, and the *wrd* deficiency, *Df(3R)189* (Aaron DiAntonio, Washington University in St. Louis, St. Louis, MO) (28).

Nondisjunction Assays. Nondisjunction assays for segregation of the sex chromosomes in males were performed as described, with details in *SI Materials and Methods* (4). To determine if nondisjunction frequencies were significantly different, the Wilcoxon two-sample test for two samples (ranked observations, not paired) was used, and a probability of less than 0.05 was scored as significant.

Immunofluorescence Labeling of Spermatocytes. Immunostaining and microscopy (on a Nikon eclipse Ti microscope) of spermatocytes was done as described (23), with modifications and antibodies detailed in *SI Materials and Methods*.

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