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Article

Multiple Protein Phosphatases Are Required for Mitosis in *Drosophila*

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Summary

Background: Approximately one-third of the *Drosophila* kinome has been ascribed some cell-cycle function. However, little is known about which of its 117 protein phosphatases (PPs) or subunits have counteracting roles.

Results: We investigated mitotic roles of PPs through systematic RNAi. We found that G_2-M progression requires Puckered, the JNK MAP-kinase inhibitory phosphatase and PP2C in addition to string (Cdc25). Strong mitotic arrest and chromosome congression failure occurred after Pp1-87B downregulation. Chromosome alignment and segregation defects also occurred after knockdown of PP1-Flapwing, not previously thought to have a mitotic role. Reduction of several nonreceptor tyrosine phosphatases produced spindle and chromosome behavior defects, and for *corkscrew*, premature chromatid separation. RNAi of the dual-specificity phosphatase, Myotubularin, or the related Sbf "anti-phosphatase" resulted in aberrant mitotic chromosome behavior. Finally, for PP2A, knockdown of the catalytic or A subunits led to bipolar monoastral spindles, knockdown of the Twins B subunit led to bridged and lagging chromosomes, and knockdown of the B′ Widerborst subunit led to scattering of all mitotic chromosomes. Widerborst was associated with MEI-S332 (Shugoshin) and required for its kinetochore localization.

Conclusions: We identify cell-cycle roles for 22 of 117 *Drosophila* PPs. Involvement of several PPs in G_2 suggests multiple points for its regulation. Major mitotic roles are played by PP1 with tyrosine PPs and Myotubularin-related PPs having significant roles in regulating chromosome behavior. Finally, depending upon its regulatory subunits, PP2A regulates spindle bipolarity, kinetochore function, and progression into anaphase. Discovery of several novel cell-cycle PPs identifies a need for further studies of protein dephosphorylation.

Introduction

Together with cell-cycle-dependent transcription and ubiquitin-mediated protein degradation, reversible protein phosphorylation provides the cell with a capacity to coordinate progression through the cell division cycle. Levels of cellular protein phosphorylation are controlled both by protein kinases (PKs) and protein phosphatases (PPs). The significance of PKs in cell-cycle progression has long been appreciated. In metazoans, the major phases of the cell cycle are set by a family of cyclin-dependent kinases (Cdks). Functions within each of these phases are also regulated by other specific protein kinases. Thus for example, Cdk1 and its cyclin B partner are required for entry into mitosis and to set the mitotic state by phosphorylating histone H1 and chromatin-associated proteins to facilitate chromosome condensation, lamins to promote nuclear-envelope breakdown, and numerous other components of the mitotic apparatus [1]. In the mitotic cell, the Polo and Aurora-like kinases augment Cdk1 functions to regulate centrosome maturation and separation, microtubule nucleation and dynamics, proper chromosome attachment to the spindles, sister-chromatid separation, and progression through anaphase into cytokinesis [2, 3]. Protein phosphorylation is also used in the surveillance checkpoints to ensure that the cell has fulfilled the necessary requirements for proceeding to the next cell-cycle stage. Thus, the Chk1 and Chk2 kinases monitor defects in DNA replication and DNA damage before permitting mitotic entry, and the BubR1 kinase regulates the correct attachment and alignment of kinetochores on the mitotic spindle before permitting anaphase [4, 5]. Therefore, it is logical to assume that PPs must play equally important reciprocal roles in the control of cell division as PKs. PPs are typically classified into three structurally distinct superfamilies: the PPP and PPM superfamilies that encode protein serine/threonine protein phosphatases and the protein tyrosine phosphatase (PTP) superfamily comprising both tyrosine-specific and dual-specificity phosphatases (DSP) (Figure S1 in

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the Supplemental Data available online) [6]. However, in contrast to the extensive studies of cell-cycle PKs, little is known of their antagonistic PPs. In *Drosophila* for instance, only a few members of the PPP and PTP have been described for their cell-cycle roles, but none of the PPM family has been implicated in this process. Genetic evidence in *Drosophila* has indicated that PP1 and PP2A (PPP) regulate spindle organization and sister-chromatid segregation [7–9]; PP4 (PPP) has been implicated in centrosome maturation [10, 11]; and the DSP Cdc25/String triggers mitosis [12]. Moreover, it is becoming clear that the diverse functions of a small number of PPP catalytic subunits are accomplished through interactions with a wide variety of regulatory subunits [6], and thus reflects the need for further dissection of their cell-cycle roles by study of their regulatory subunits.

In an attempt to define a complete list of PPs and their regulatory subunits required for cell-cycle progression, we used *Drosophila melanogaster* as a model system because of the following advantages. The annotated sequence of the *Drosophila* genome allows us to identify all of the predicted PPs. Moreover, the low level of genetic redundancy in this organism coupled with the very high efficiency of RNA interference (RNAi) in S2 cells facilitates the detection of functional requirements for the assayed gene products [13, 14]. Finally, the high degree of evolutionary conservation of cell-cycle functions in metazoans indicates that our study would provide a significant contribution to the understanding and treatment of proliferative diseases in man.

We surveyed cell-cycle functions of the 117 PPs or their regulatory subunits in the *Drosophila* genome by a combination of three different assays: flow cytometry, mitotic-index measurement, and quantitative assessment of mitotic defects. By using this combination of independent assays, we identified all the previously known *Drosophila* cell-cycle PPs identified by classical genetics and thus validated our approach. We report novel cell-cycle functions for PP5 and PP6 (PP2A-like) of the PPP superfamily and for PP2C of the PPM superfamily. In addition, we revealed cell-cycle roles for 11 PTPs, consistent with the fact that tyrosine phosphorylation plays a critical role in the control of a wide array of signaling pathways that are involved in the regulation of cell proliferation [15].

Results and Discussion

Global View of Protein Phosphatases Required for Cell-Cycle Progression

We used RNAi to silence 117 known and predicted *Drosophila* genes encoding PPs or their regulatory subunits in S2 cells. This was followed by flow cytometry for monitoring changes in DNA content and cell size, and mitotic-index analysis for identifying the proportion of cells in mitosis. We found that downregulation of 29 genes led to a cell-cycle phenotype. Two recent reports have demonstrated the possibility of sequence-dependent off-target effects associated with long dsRNA in *Drosophila* [16, 17]. In order to minimize the number of false positives that can potentially arise from such off-target effects, we re-examined the flow-cytometry profiles and mitotic indices by using a second set of dsRNAs targeting a distinct region of each positive candidate identified in our first screen. As shown in Table S1, we confirmed the phenotypic effects for the majority of the positive candidates, although not for seven, which were therefore discarded from further analysis. In one case (*sbf*), however, we observed similar flow-cytometry profiles but not mitotic indices when two dsRNAs were used. Thus in summary, depletion of 18 gene products altered the proportions of cells at different cell-cycle stages (in some cases associated with aneuploidy, cell death, and increased cell size) as revealed by flow cytometry (Figure 1), and 11 showed abnormal mitotic indices (Table 1; see also Experimental Procedures). In total, 22 genes (19% of the genes tested) possessed cell-cycle functions by these criteria. We cannot be certain to have identified all PP genes required for cell-cycle progression in our survey because we did not, and in most cases cannot, confirm that RNAi did indeed reduce levels of protein products as a result of the limitation of antibodies available. However, this appears not to be a major problem in cell-based RNAi screens in *Drosophila* [17].

We further investigated the potential mitotic functions of these 22 genes by staining dsRNA-treated cells to detect α- and γ-tubulin and DNA and therefore to visualize the spindle, centrosomes, and chromosomes. Mitotic cells were classified into four main stages (prophase, prometaphase and metaphase, anaphase, and telophase) and mitotic defects in centrosomes, spindles, and chromosomes were quantified by the scoring of 20 morphological parameters (Table S2; see also Quantitative Assessment of Mitotic Defects in Supplemental Experimental Procedures). The delay in progression through a given mitotic stage and the proportion of mitotic cells showing centrosome, spindle, or chromosome defects were compared. The severity of these abnormalities was then ranked by a significance factor, Z score (which is a measure of the separation in standard deviations of the observed test statistic from the mean of the expected distribution). We defined an RNAi phenotype only when the Z score was significantly different from controls at the 99.7% confidence level ($Z > 3$ or < -3). According to this definition, depletion of 12 individual PPs and four regulatory subunits resulted in a mitotic phenotype. Figure 2 provides a summary of the cell-cycle phenotypes after RNAi of PPs. A comprehensive comparison of the cell-cycle phenotypes identified in the current study with previous studies in yeasts and metazoans, where applicable, can be found in Table S3. In most cases, cellular functions for PPs can be suggested either based solely on our results or on some genes upon consideration of the published literature. However, it should be kept in mind that any phenotype observed could have arisen indirectly after the disruption of a distant pathway requiring the phosphatase being assayed.

Surprisingly, we did not clearly identify any phosphatase required for G_1-to-S-phase transition (Figure 1), although RNAi knockdown of several PPs resulted in an increase in cells with DNA content between 2C and 4C, and such an increase could suggest a delay in S phase. These include PPV/PP6, Ptp61F, three members of the PP2A family, and two members of the Myotubularin family (Figures 1A and 1E, arrows; Figure 2).

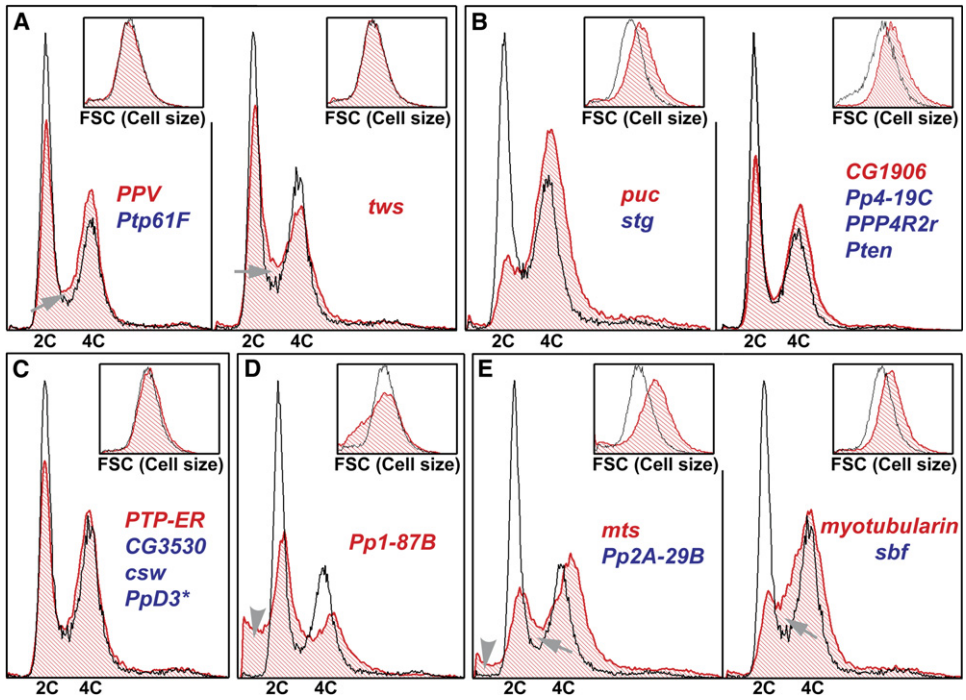

Figure 1. Proportion of Cells at Different Cell-Cycle Stages after RNAi of PPs

Changes in the proportion of cells with subdiploid (cell death), 2C (G_1 phase), 4C (G_2/M phase), and intermediate DNA content between 2C and 4C (S phase or aneuploidy) and in cell size were identified by flow cytometry after downregulation of 18 PPs. Defects fall into five broad clusters (A–E). Each panel shows a control cell histogram (black, cells transfected with dsRNA for green fluorescent protein [GFP]) and an example of an experimental histogram (red) after RNAi for a representative protein phosphatase (the specific protein phosphatase illustrated is indicated in red lettering; the asterisk indicates a weak RNAi phenotype for PpD3). The forward light scatter (FSC) profile reflects cell size. Phenotypes are clustered as follows:

(A) RNAi leading to an increase in cells with intermediate DNA content (indicated by arrows) associated with an increase in 4C cells (left) or not (right).

(B) RNAi leading to an increase in 4C cells associated with an increase in cell size. This group has been subdivided according to the extent of accumulation of 4C cells (left versus right).

(C) RNAi leading to an increase in 4C cells without affecting cell size.

(D) RNAi leading to an increase in subdiploid cells (indicated by arrowhead).

(E) RNAi leading to an increase in 4C cells and in cells intermediate between 2C and 4C (indicated by arrows), and in cell size associated with an increase in subdiploid cells (left, indicated by arrowhead) or not (right).

However, because it is difficult to distinguish S phase delay from aneuploidy solely based on flow-cytometry profiles, further investigation is required so that the potential S phase roles of these PPs can be defined. On the other hand, downregulation of many PPs led to a prolonged G_2 phase or mitotic defects, indicating a strong requirement for PPs in regulating progression into and through mitosis.

Mitotic Entry

In the cell cycle, G_2 is a pivotal phase at which mitotic entry may be delayed until DNA is correctly replicated and repaired and until an appropriate cell size is reached. The G_2-M transition requires removal of the inhibitory phosphorylation of Cyclin-dependent kinase 1 (CDK1) by the tyrosine phosphatase, Cdc25 (String in *Drosophila* somatic cells and Cdc25C in vertebrates), whose activity is opposed by the Wee1 and Myt1 protein kinases. The regulation of this inhibitory phosphorylation has been thought to be essential in governing mitotic entry, and its regulators (Cdc25, Wee1, and Myt1) are tightly modulated by a wide range of pathways such as MAP-kinase and DNA replication and repair checkpoint kinase pathways [18].

Cells accumulating with a 4C DNA content can represent G_2 phase cells, mitotic cells, or binucleated G_1 cells that have failed cytokinesis. However, defective

Table 1. RNAi of PPs Leading to Abnormal Mitotic Indices

	K-S Value	
Gene Name	First Screen	Second Screen
Pp1-87B	1.00000	1.00000
flw	0.77612	0.85341
mts	1.00000	1.00000
Pp2A-29B	1.00000	1.00000
wdb	0.71416	0.71334
Pp4-19C	0.53333	0.78596
PPP4R2r	0.44167	0.83728
PPV	0.88095	1.00000
CG9311	0.56429	0.62955
csw	0.69523	0.70126
ssh	0.44318	0.65575

Mitotic indices were analyzed with the Kolmogorov-Smirnov (K-S) test (see Supplemental Experimental Procedures). Candidate genes whose downregulation showed significantly different distributions from *GFP* RNAi controls at the 0.01 level in both screens involving distinct dsRNAs are listed. The corresponding average K-S values are also shown. The average x-fold change in mitotic index after RNAi of these genes can be found in Figure 2.

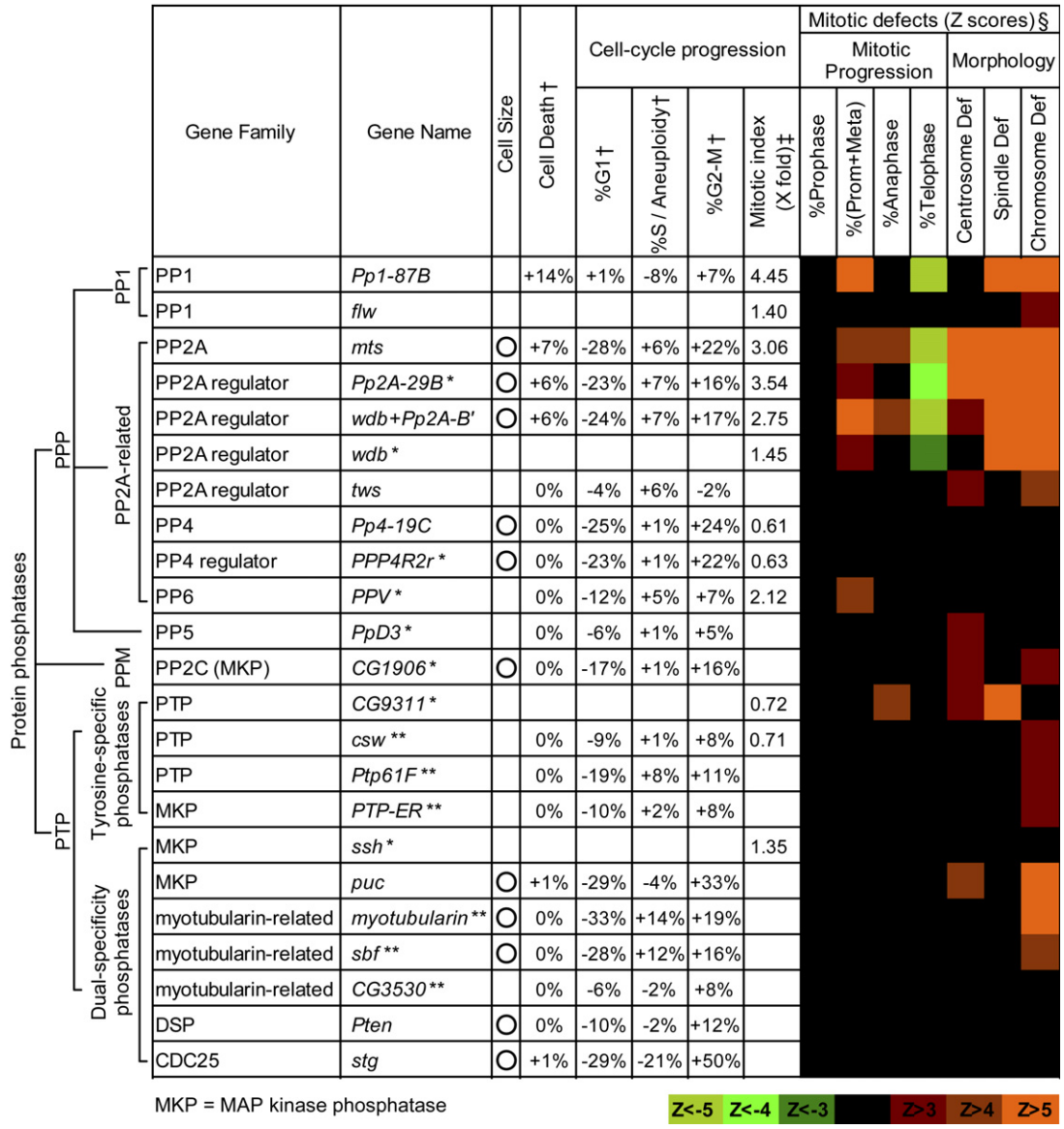

Figure 2. Cell-Cycle Functions of PPs Identified in Current Study Grouped by Families

**Indicates novel cell-cycle regulators.

*Indicates novel *Drosophila* cell-cycle regulators whose counterparts in other organisms have been implicated in the cell-cycle control (see Table S3 for details).

○ Indicates an increase in cell size.

†Increases (+) or decreases (−) in cell death or the proportion of cells in a given phase of the cell cycle are shown when flow-cytometric data are significantly different from control values.

‡Average x-fold change in the mitotic index is shown when mitotic-index data are significantly different from control values.

§Defects in mitotic progression and morphology were quantified and ranked by Z score (see Supplemental Experimental Procedures). A Z score of three or more standard deviations from the mean was used as a threshold for selecting positive candidates. "Centrosome Def," "Spindle Def," and "Chromosome Def" refers to the percentage of mitotic cells showing defects in centrosome number or positioning, spindle assembly, and chromosome condensation, alignment, or segregation, respectively.

cytokinesis often also results in an increase in polyploid cells with DNA content of 8C arising from re-entry of the binucleated cells into the cell cycle. Consistent with its mutant phenotype [12], *string/Cdc25* RNAi led to a 50% increase in 4C cells with no increase in the mitotic index or 8C cells, suggesting a pronounced G_2 arrest (Figures 1B and 2). These cells showed an increase in size in anticipation of a delayed mitotic entry, reminiscent of the *cdc25* mutant phenotype in fission yeast [19].

Entry into mitosis can be delayed by activation of stress-response pathways [20, 21]. Two classes of stress-activated protein kinases (SAPK) have been identified: c-JUN amino-terminal kinases (JNKs) and p38 MAPKs. They are each returned to their inactivated state through dephosphorylation by MAPK phosphatases (MKP). RNAi knockdown of Puckered (Puc), the single phosphatase known to inhibit *Drosophila* JNK MAP-kinase [22–24], also led to a severe accumulation (33%)

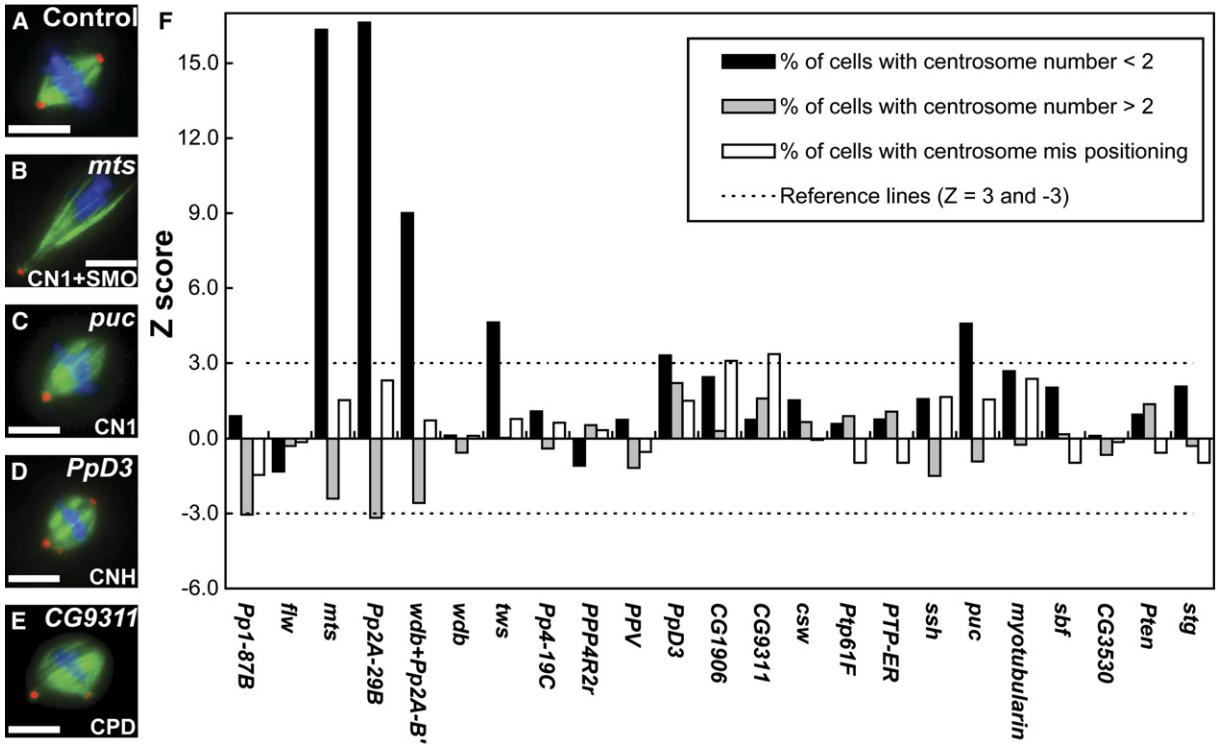

Figure 3. Centrosome Functions of PPs

(A–E) Examples of cells showing defects in centrosome number (B–D) or mispositioned centrosomes that are detached from the spindle pole (E) after RNAi for PPs.

(F) Quantification by Z score of these defects. The 99.7% confidence interval (CI) is indicated. Cells were stained for detecting α-tubulin (green), γ-tubulin (red), and DNA (blue). Abbreviations for phenotypes are as follows: CN1, centrosome number 1 (only one pole with γ-tubulin staining); CNH, centrosome number high; CPD, centrosome positioning defects; SMO, monopolar spindle. Scale bars represent 5 μm.

of large G_2 cells (Figures 1B and 2). This would be in accordance with the MAP-kinase pathways being able to either inhibit String activity or promote the activity of its antagonistic tyrosine kinases Wee1/Myt1 [18]. JNK kinases participate in several signaling roles in addition to the stress response and have also been previously implicated in cell-cycle regulation in *Drosophila*. In *Drosophila* follicle cells, JNKs and Puc phosphatase regulate the transition from mitotic to endoreplication cell cycles [25]. Lack of JNK activity results in the initiation of premature endocycles, and loss of Puckered leads to loss of endocycles. Although this may reflect a specific requirement for this highly specialized cell-cycle transition, it does point to opposing cell-cycle roles for JNK and Puc. Interestingly, we also found a high proportion of cells with reduced copies of centrosomes after *puc* RNAi (Figures 3C and 3F). In light of the fact that human JNK has been reported to localize to centrosomes where it is active from early S through late anaphase [26], this points toward possible antagonistic functions for JNK kinase and Puc phosphatase either in regulating centrosome duplication in S phase or separation in mitosis.

An alternative perspective for the increased proportion of cells in G_2 could be accelerated G_1 progression. This might arise after RNAi of *CG1906* (PP2C), which has been recently suggested to be the *Drosophila* MKP that inhibits Ras signaling [27]. *CG1906* RNAi knockdown led to a substantial increase in the proportion of 4C but not mitotic or 8C cells and was associated with increased cell size (Figures 1B and 2). In agreement with the phenotypes seen in S2 cells depleted for CG1906, activation of Ras in *Drosophila* wing cells increased cell size and promoted the G_1-S transition [28]. In addition, PP2C has been implicated in checkpoint inactivation after double-strand DNA breakage in *S. cerevisiae* [29, 30]. Thus, inability to turn off such checkpoint signaling operating as a normal part of DNA-replication surveillance could also account for the accumulation of G_2 cells.

Finally, we unexpectedly found that knockdown of either the catalytic or regulatory subunit of PP4 led to an accumulation of large 4C nonmitotic cells (Figures 1B and 2). This enzyme has been reported to be required for the recruitment of γ-tubulin to centrosomes in syncytial *Drosophila* embryos [10, 11]. However, although western-blot analysis indicated that Pp4-19C protein had been efficiently knocked down after its RNAi (Figure S3), we were not able to observe any visible mitotic defects or a gross reduction in the staining for γ-tubulin or Polo kinase after RNAi for this protein in S2 cells (Figure 2; data not shown). A role in regulating G_2 progression suggested by our present results would not have been seen in the rapid S-M cycles of syncytial embryos. Consequently, this G_2 requirement for PP4 activity may mask any other role at the centrosome upon mitotic entry in S2 cells. Alternatively, PP4 may be required for mitotic centrosome function in embryos but not in S2 cells.

Centrosome Duplication, Maturation, and Separation

The centrosome is the major microtubule-organizing center (MTOC) and thus plays a critical role during mitosis where it contributes to spindle bipolarity, spindle positioning, chromosome segregation, and cytokinesis. Centrosomal roles for PPs could be expected to counteract the large number of PKs that regulate centrosome functions [31, 32]. RNAi in *Drosophila* S2 cells has previously revealed the respective centrosomal roles of the Polo and SAK/Plk4 protein kinases. However, a quantitative approach has proved to be important in such experiments because some 20%–30% of control mitotic S2 cells show centrosomal defects (see also [33]).

Aberration of centrosome number was found after downregulation of *PpD3/PP5*. Its RNAi resulted in not only an increase in cells with reduced centrosome numbers but also a marginal increase ($Z = 2.2$) in the number of cells with extra centrosomes (Figures 3D and 3F) and thus was possibly indicative of a role in centrosome separation in mitosis.

The localization of proteins at the centrosome has been taken as an indicator of their potential function. PTP-BL, a human phosphatase closely related to fly CG9311, has been reported to be highly enriched at centrosomes during early mitosis [34]. We found that when *CG9311* was downregulated, cells had mispositioned centrosomes detached from spindle poles, suggesting the possibility of a conserved relationship between the localization and function of this enzyme (Figures 3E and 3F).

Mitotic Functions of PP1 Isoforms

Evidence from both yeast and animal cells suggests that PP1 phosphatases oppose both the Aurora A- and B-type kinases [35]. This would implicate PP1 in regulating a range of processes including the establishment of the spindle, microtubule-kinetochore interactions, the mitotic phosphorylation of histone H3 and its centromeric counterpart, CENP-A (CID in *Drosophila*), and cytokinesis.

We observed a striking increase in the mitotic index after knockdown of Pp1-87B, one of the four *Drosophila* type 1 protein serine/threonine phosphatases (PP1s). This protein phosphatase has previously been described to have essential mitotic functions [7]. As observed in mutant neuroblasts, *Pp1-87B* RNAi led to an accumulation of cells in metaphase, defective spindle organization, abnormal sister-chromatid segregation, and aneuploidy (Figures 1D, 2, and 4J–4L). In addition, we found an increase in cells with spindles of metaphase length containing uncongressed chromosomes and cells with abnormally elongated anaphase-like spindles showing lagging chromosomes and unequal segregation (Figures 4D and 4E). These phenotypes led us to ask whether sister chromatids were separated in those anaphase-like cells. To this end, we examined the distribution of the *Drosophila* MEI-S332 protein, which is present on centromeres of adjoined but not separated chromatids [36]. In control cells, MEI-S332 staining was lost from centromeres after the metaphase-anaphase transition (Figure 4M). In contrast, its signal was still detectable at centromeres in comparable *Pp1-87B* RNAi cells (Figure 4N). Moreover, the presence of the checkpoint component BubR1 at kinetochores in such cells further suggested that, despite the long anaphase-like spindles, chromosomes had failed to congress (Figures 4O and 4P). Consistently, cyclin B had also failed to be degraded in such cells (Figures 4Q and 4R). Taken together, these data indicated new mitotic functions for Pp1-87B in regulating chromosome congression, perhaps, as has been suggested in yeast [37], by facilitation of the attachment of sister kinetochores to opposite spindle poles. In addition, we found that knockdown of Pp1-87B led to a failure to dephosphorylate phosphohistone H3 (P-H3) upon mitotic exit (see below).

Although not as dramatic as the downregulation of Pp1-87B, RNAi knockdown of another PP1 family member encoded by *flapwing* (*flw*) also led to an increase in a mitotic index with cells showing an increased frequency of metaphase cells with uncongressed chromosomes and anaphase cells with lagging chromosomes (Figures 2, 4H, and 4K). This was surprising because this isoform has previously only been implicated in the dephosphorylation of the regulatory myosin light chain [38]. However, examination of larval neuroblasts from a *flw* P-element insertion mutant also revealed tangled, unevenly condensed chromosomes and lagging chromosomes and chromosome bridges at anaphase, and such a finding confirms a requirement for this PP1 isoform in mitosis (Figures S4B, S4E, and S4F).

In summary, many of the various mitotic phenotypes observed after knockdown of PP1s in *Drosophila* cells can be accounted for an inability to oppose either A- or B-type Aurora kinases. Although this is consistent with other studies, we cannot discount a greater role for PP1 in dephosphorylating protein substrates of other mitotic protein kinases. Indeed in mammalian cells, PP1 has also been proposed to counteract the activity of the Nek2 mitotic kinase [35].

Mitotic Functions of PTPs

Downregulation of several tyrosine-specific phosphatases resulted in mitotic defects: *CG9311* RNAi cells displayed disorganized spindles, and spindle poles tended to be unfocused (Figures 4F and 4J); and knockdown of two cytoplasmic PTPs, Ptp61F and Corkscrew (Csw), led to marked chromosome alignment/segregation defects (Figures 4G and 4K). In the case of *csw*, its RNAi also resulted in a significantly reduced mitotic index (Figure 2). Consistent with these phenotypes, we saw a low mitotic index in larval neuroblasts of a *csw* P-element insertion mutant, and interestingly, orcein-stained chromosomes from squashed preparations of such *csw* mutant cells revealed metaphases with prematurely separated chromatids; this finding implicates a role for protein tyrosine phosphorylation in maintaining sister-chromatid cohesion until anaphase onset (Figure S4C).

We also observed mitotic defects after knockdown of several dual-specificity phosphatases, most notably Myotubularin-related family members (Figures 2, 4I, and 4K). Several of the Myotubularin-related protein family genes have been implicated in regulating cell proliferation [39]. Although sequence comparison and in vitro assays suggest that Myotubularin is a dual-specificity phosphatase, recent studies indicate that phosphatidylinositol 3-phosphate (PI(3)P), a lipid second messenger, is a primary substrate in vivo [39]. It is thus possible that

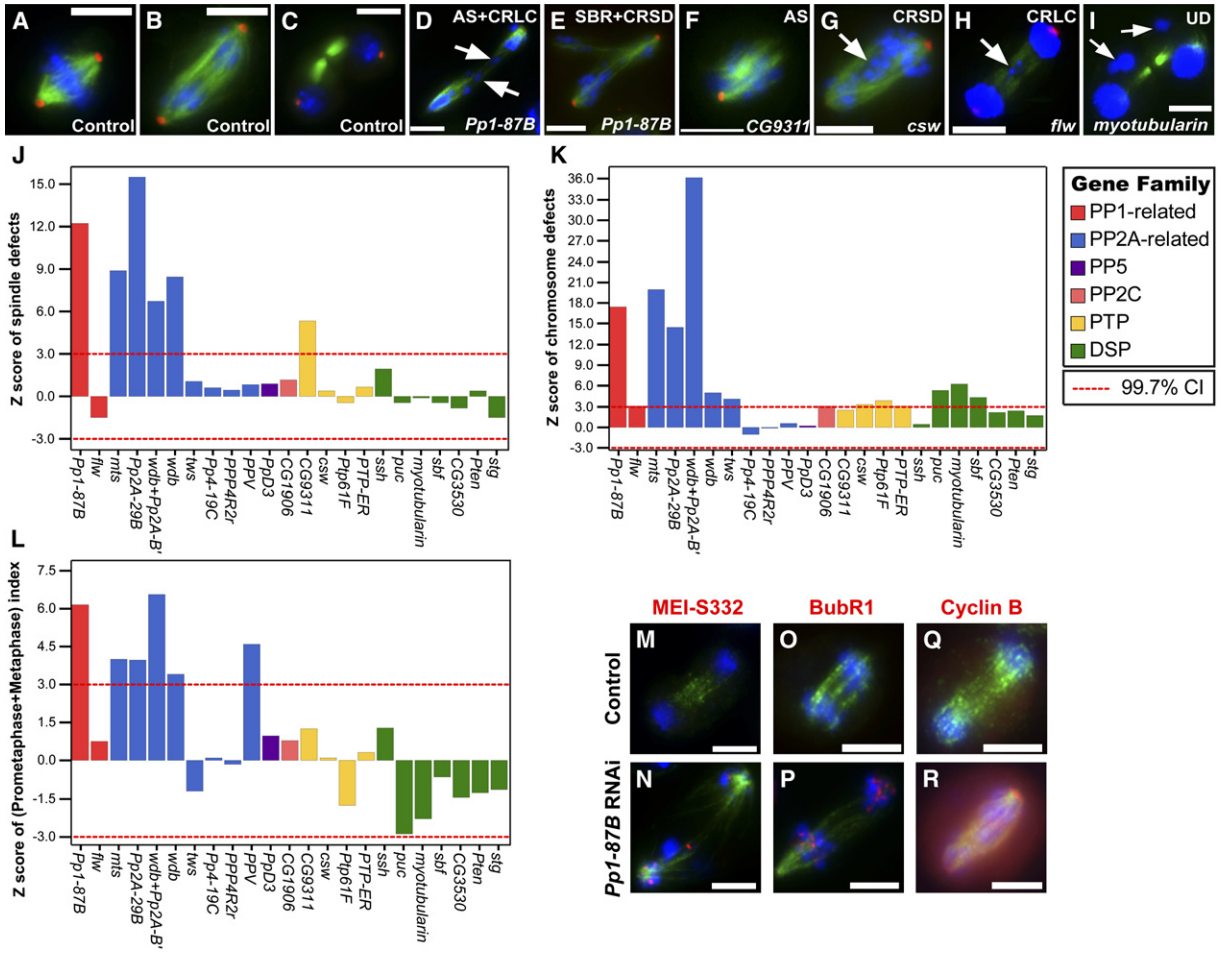

Figure 4. Mitotic Defects after RNAi for PPs

(A–I) Examples of cells showing defects in spindle assembly or chromosome behavior after RNAi for PPs. Cells were stained for detecting α-tubulin (green), γ-tubulin (red), and DNA (blue). Abbreviations for phenotypes are as follows: AS, abnormal spindle; SBR, branched spindle; CRSD, chromosome segregation defect; CRLC, lagging chromosomes; UD, unevenly divided DNA.

(J) Quantification by Z score of the percentage of mitotic cells showing spindle defects.

(K) Quantification by Z score of the percentage of mitotic cells showing chromosome defects.

(L) Quantification by Z score of the proportion of prometaphase and metaphase cells relative to the number of total mitotic cells. Indicated are 99.7% confidence intervals (CI).

(M–R) *Pp1-87B* RNAi led to defects in chromosome congression. Cells were stained for detecting α-tubulin (green), DNA (blue), and indicated proteins (red). In control cells, MEI-S332 (M) and BubR1 (O) dissociated from the kinetochores, and cyclin B (Q) was degraded after anaphase onset. In comparable *Pp1-87B* RNAi cells, however, MEI-S332 (N) and BubR1 (P) remained associated with the centromere or kinetochore moving toward the spindle poles and cyclin B (R) was not degraded. Scale bars represent 5 μm.

Myotubularin-related proteins may regulate the cell division cycle via lipid-mediated signaling pathways. Notably, RNAi of *SET domain binding factor* (*sbf*), which encodes a Myotubularin-related but catalytically inactive "antiphosphatase," also resulted in defects in segregation and lagging chromosomes at anaphase (Figures 2 and 4K). As has been suggested in mammalian cells, this protein may function by binding to and protecting substrates from dephosphorylation by Myotubularin-related phosphatases [40]. Taken together, these findings point toward cooperation between Myotubularin-related proteins in the control of cell-cycle progression.

PP2A Displays Multiple Mitotic Roles

The Catalytic and A Subunits

PP2A is a heterotrimeric serine/threonine phosphatase composed of invariant catalytic (C) and structural (A) subunits together with one member of a family of B regulatory subunits thought to direct the AC core to different substrates [41]. The *Drosophila* gene for the catalytic subunit of type 2A protein serine/threonine phosphatase (PP2A) is known as *microtubule star* (*mts*) because mutant embryos show multiple individual centrosomes with disorganized astral arrays of microtubules [8]. In agreement with this mutant phenotype, we found that S2 cells depleted for Mts (PP2A-C) displayed aberrant elongated arrays of microtubules with a high proportion (5- to 10-fold increase over the control) of bipolar monoastral spindles or monopolar spindles emanating from a single centrosomal mass (Figure 3B). This phenotype is also consistent with the observations in *Xenopus* egg extracts where mitotic microtubules grow longer and bipolar spindles can not be assembled after inhibition of PP2A by low concentrations of okadaic acid (OA)

[42]. We speculate that the monopolar spindle phenotype after *mts* dsRNA treatment is a consequence of the spindle collapse rather than a failure in centrosome duplication or separation because most of the RNAi-treated cells showed well-separated centrosomes during prophase (data not shown). In support of this view, spindle bipolarity can be rescued by restoration of microtubule dynamics in OA-treated *Xenopus* egg extracts [42].

In *Drosophila*, as in many other eukaryotes, mitosis-specific phosphorylation of histone H3 requires Aurora B activity, but the identity of the opposing phosphatase remains unclear [43, 44]. Because P-H3 (Ser 10) levels were used for monitoring the mitotic index in our analysis, it is possible that a high mitotic index observed after RNAi for PPs may also reflect a defect in dephosphorylating P-H3 in the absence of PPs upon mitotic exit. We therefore examined the phosphorylation state of this histone after RNAi for PPs that displayed a significant increase in the mitotic index in our screen. The immunostaining of control cells showed that P-H3 signals began to decrease at early telophase and then disappeared completely at late telophase (Figures S5A–S5C). After RNAi knockdown of Mts (PP2A-C) or Pp1-87B, however, the majority of mitotic cells were arrested at prometaphase (Figure 4L), but we could occasionally found late telophase figures showing an abnormal accumulation of P-H3 on decondensed chromosomes (Figures S5D and S5E). To better assess the effect of depletion of these two PPs on P-H3 dephosphorylation, we inactivated the spindle-assembly checkpoint by simultaneously knocking down BubR1. We found that this rescued the prometaphase arrest of cells simultaneously depleted for Mts or Pp1-87B and allowed us to study telophase cells (Figure S5F). P-H3 was present in the majority of such telophase cells compared to control cells (Figure S5G), indicating that both PPs are required for P-H3 dephosphorylation. These results are in accordance with previous studies showing that reduction of PP1 activity can partially suppress defects in the mitotic histone H3 phosphorylation in yeast and *C. elegans* [45].

Downregulation of *Pp2A-29B*, the structural A subunit, revealed almost identical aberrant phenotypes to those observed after *mts* (*PP2A-C*) RNAi (Figure 2 and Figure S6). Consistently, knockdown of Pp2A-29B (PP2A-A) led to a reduction of the protein level of Mts (PP2A-C) (Figure S6A; see also [46, 47]).

The B Subunits

The *Drosophila* genome contains 4 B-type PP2A regulatory subunits, *twins/tws/aar* (B sub-type), *widerborst/wdb* (B′ sub-type), *Pp2A-B′* (B′ sub-type), and *Pp2A-B″* (B″ sub-type), but mitotic defects have so far only been reported for mutants of *tws*. Consistent with the phenotype of *tws* mutants [9], we observed that RNAi for this gene led to an increased proportion of anaphase figures showing lagging chromosomes and chromosome bridges (Figures 5A and 5B).

In metazoans, the B′ regulatory subunits of PP2A have evolved into two related subclasses with conserved central regions and diverged amino and carboxy termini. The protein encoded by *widerborst* (*wdb*) is more closely related to the human α and ε subunits (79%–80% identity) than to the β, γ, or δ subunits (69%–75% identity). Whereas RNAi for *tws* led to lagging chromosomes, *wdb* RNAi led to dramatic scattering of chromosomes throughout the spindle (Figures 5A and 5B). We considered whether this dramatic effect of *wdb* RNAi on chromosome segregation reflected any particular subcellular localization of this regulatory subunit. To this end, we expressed a GFP-tagged Wdb in S2 cells (Figure 5D). During interphase and prophase, Wdb::GFP partially colocalized with the centromeric marker CID (CENP-A). After spindle formation, Wdb::GFP was found adjacent and external to the centromeres. Although less pronounced, this distribution remained during chromosome segregation at anaphase. Because MEI-S332 (*Drosophila* Shugoshin) is a dynamic centromeric marker [36], we examined its distribution in *wdb* RNAi cells. In control cells, MEI-S332 localized in a band between each pair of the centromeres at metaphase (Figure 5E). After downregulation of *wdb*, however, we found greatly reduced MEI-S332 staining on the metaphase chromosome (Figures 5E and 5F). In contrast, depletion of MEI-S332 by RNAi did not affect the normal localization of the Wdb B′ PP2A subunit (Figure S7). Thus, we conclude that the Wdb B′ subunit is required for correct localization of MEI-S332 but not vice versa. We next asked whether the two proteins existed in the same complex. To address this, we expressed a Protein A (PrA)-tagged form of MEI-S332 in S2 cells to purify potential protein complexes and identify its components by mass spectrometry. As shown in Figure 5C, we identified the catalytic C (Mts), the structural A (PP2A-29B), and the regulatory B′ (Wdb) and B (Tws) subunits of PP2A associated with MEI-S332. Three recent studies also identified PP2A complexed to the B′ subunit bound to Shugoshin (Sgo) in human and yeast cells, where they are thought to protect centromeric cohesin subunits from phosphorylation that would promote premature sister-chromatid separation [48–50]. As with the archetypal family member, *Drosophila* MEI-S332, the Shugoshins function primarily to protect sister chromatids from separation in the first meiotic division but are also present in mitotic divisions [51]. Consistent with our observations in *Drosophila* S2 cells, Tang et al. [50] and Kitajima et al. [48]. both found that depletion of PP2A in human cells led to premature dissociation of Shugoshin 1 (Sgo1) from the kinetochore and loss of mitotic centromere cohesion. The finding of Shugoshin complexed to PP2A/B′ in yeast and human, and now in *Drosophila*, points to a highly evolutionally conserved role for this particular PP2A heterotrimer in regulating sister-chromatid cohesion. Interestingly, we also recovered Tws B regulatory subunit associated with MEI-S332. How this subunit of PP2A might function with MEI-S332 should be the subject of future investigations.

Only a moderatedly elevated mitotic index (by approximately 10%) was observed after downregulation of the second *Drosophila* B′ regulatory subunit (Pp2A-B′/B56-1) (Figure S6). However, when this second B′ subunit was simultaneously knocked down with Wdb, this led to similar phenotypes seen in Mts (PP2A-C) or Pp2A-29B (PP2A-A)-depleted cells (Figure 2 and Figure S6). Western-blot analysis showed that the Mts (PP2A-C) level decreased after simultaneous knockdown of both B′ subunits, suggesting that this phenotype could be partially due to the loss of PP2A catalytic

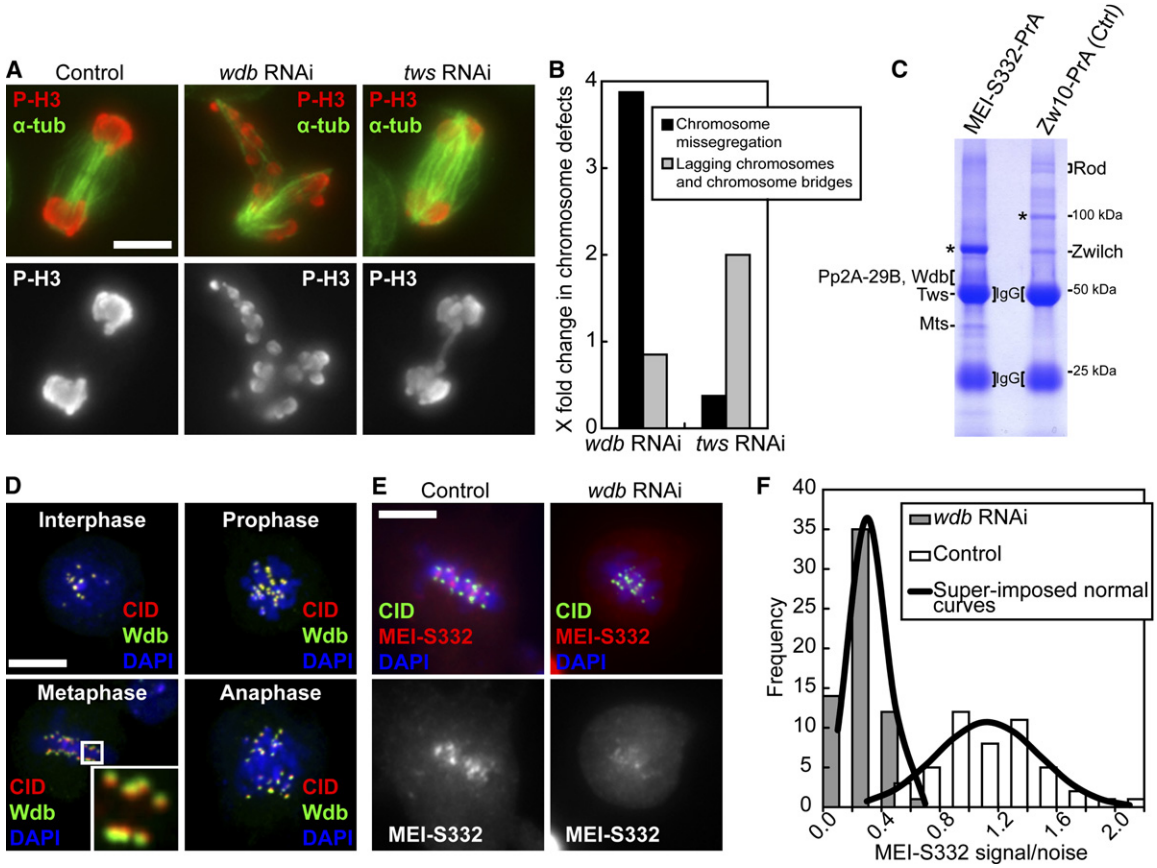

Figure 5. PP2A Displays Multiple Mitotic Roles

(A) RNAi for *tws* encoding the B subunit of PP2A led to lagging chromosomes, whereas RNAi for *wdb* encoding one of the B′ subunits of PP2A led to missegregation of multiple chromosomes. The scale bar represents 5 μm.

(B) Quantification of lagging chromosomes and chromosome missegregation after RNAi for *tws* and *wdb*.

(C) Wdb associates with MEI-S332. Extracts from stable cell lines expressing protein A-tagged MEI-S332 or protein A-tagged Zw10 (control) were subjected to affinity purification on IgG beads. Purified proteins were resolved on SDS-PAGE, bands were excised, and proteins were identified by mass spectrometry. The gel was stained with colloidal Coomassie blue. Tagged protein baits are indicated by the asterisks. The locations of the catalytic subunit (Mts), scaffolding A subunit (Pp2A-29B), regulatory B′ (Wdb) and B (Tws) subunits of PP2A, Rod, and Zwilch are indicated. Bands corresponding to IgG heavy and light chains are also indicated. These PP2A subunits were specifically identified in association with MEI-S332 and never in a great number of similar purifications with a wide range of mitotic proteins, including the kinetochore component Zw10.

(D) Wdb::GFP (B′ subunit) partially colocalized with the centromeric marker CID (CENP-A) at all phases of the cell cycle. Note that Wdb::GFP appeared adjacent and external to the centromeres at metaphase. The scale bar represents 5 μm.

(E) Downregulation of *wdb* led to dissociation of MEI-S332 from the metaphase chromosomes. Note that MEI-S332 staining on the metaphase chromosomes was greatly reduced after RNAi for *wdb*. The scale bar represents 5 μm.

(F) Histogram of the signal intensity of MEI-S332 on the metaphase chromosomes showing that the signal intensity was remarkably reduced after RNAi for *wdb* (n = 62 pairs of centromeres from 23 cells) compared with RNAi for *GFP* (control, n = 48 pairs of centromeres from 16 cells). See Supplemental Experimental Procedures for details.

subunit, although we cannot exclude the possibility that the two B′ subunits share partially redundant mitotic functions (Figure S6).

Conclusion

Cell-cycle kinases represent a large family of enzymes governing the cell division cycle [14]. It is therefore not surprising that a considerable number of counteracting cell-cycle phosphatases (19% of the genes for tested) were identified in the current study. In addition to finding all the well-known PPs required for cell-cycle progression in *Drosophila* (Mts, Tws, String, Pp4-19C, and Pp1-87B), we have identified the *Drosophila* counterparts of some eight PPs implicated in cell-cycle functions from studies on other organisms together with six PPs for which we ascribe novel cell-cycle roles. We validated our results by confirming the observed phenotypes with a second nonoverlapping dsRNA. In two cases (*flw* and *csw*), we also confirmed their mitotic roles through the analysis of phenotypes in mutant larval neuroblasts. We were also able to validate the RNAi phenotypes of catalytic subunits by observing similar phenotypes after downregulation of the corresponding regulatory subunits (e.g., Pp4-19C and PPP4R2r, Mts/PP2A-C and Pp2A-29B/PP2A-A, and simultaneous RNAi of the two PP2A-B′ regulatory subunits). Although a recent large-scale RNAi screen based solely on flow

cytometry in *Drosophila* S2 cells identified many regulators of the cell cycle, cell size, and cell death (488) [52], this study showed a very low degree of overlap with our analysis (only six, Table S1), reflecting the need for more sensitive small-scale screens that can examine the functional requirements of assayed proteins in greater detail. Our results have provided novel insights into the cell-cycle functions of the *Drosophila* PPs, and it is likely that, in many cases, these functions have been conserved in other metazoans including humans. Our study should guide future work aimed at elucidating the significance and mechanisms of the balanced activities of PKs and PPs in regulating the cell division cycle. The challenge ahead will be to match up the functions of the PPs we have identified with their corresponding counteracting PKs and to identify their common key substrates.

Experimental Procedures

RNAi

Drosophila S2 cells were cultured and transfected with 20 μg of dsRNA and 20 μl of Transfast (Promega) in six-well plates as described [53]. The cells were incubated for 96 hr in the presence of dsRNA to allow for the depletion of targeted gene products. (See Supplemental Experimental Procedures for dsRNA production.)

Flow Cytometry

Flow cytometry was carried out as described [14]. Results were analyzed with Summit (Dako Cytommation) and Multicycle (Phoenix Flow Systems). At least two independent experiments were performed for each dsRNA.

Immunofluorescence Microscopy and Western blotting

Cells were fixed, permeabilized, and stained as described [14]. Mitotic-index measurement and quantitative assessment of mitotic defects are described in Supplemental Experimental Procedures. Standard procedures were used for western blotting. (See Supplemental Experimental Procedures for details of antibodies used in immunofluorescence and western blotting.)

Expression Vectors and Stable Cell Lines

A plasmid expressing an N-terminal EGFP-tagged version of Wdb was generated with Gateway (Invitrogen) technology as described [54]. For protein-complex purification, Gateway plasmids, pAc5-TEV/PrA, and pMT-TEV/PrA were created to allow the expression of fusion proteins containing a C-terminal Protein A tag. (See Supplemental Experimental Procedures for details). Stable cell lines were generated as described [54].

Protein A Affinity Purifications and Mass Spectrometry

Protein A fusion proteins were affinity purified with rabbit IgG-conjugated DynaBeads (Invitrogen). Protein complexes were resolved by SDS-PAGE on a Novex Tris-Glycine gel (Invitrogen). Protein bands were revealed by Coomassie blue, excised, and analyzed by mass spectrometry with a LC-MS/MS. Proteins were identified by the MASCOT search engine. (See Supplemental Experimental Procedures for details.)

Fly Stocks and Cytology

The Canton S stock was used as the wild-type. The P element insertion mutants flw^{G0172} and csw^{G0170} are described in [55]. Preparations of neuroblast mitotic chromosomes are described in [56].

Supplemental Data

Supplemental Data include additional Experimental Procedures, eight figures, and four tables and are available with this article online at http://www.current-biology.com/cgi/content/full/17/4/293/DC1/.

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