The Protein Phosphatase 2A regulatory subunit Twins stabilizes Plk4 to induce centriole amplification

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Centriole duplication is a tightly regulated process that must occur only once per cell cycle; otherwise, supernumerary centrioles can induce aneuploidy and tumorigenesis. Plk4 (Polo-like kinase 4) activity initiates centriole duplication and is regulated by ubiquitin-mediated proteolysis. Throughout interphase, Plk4 autophosphorylation triggers its degradation, thus preventing centriole amplification. However, Plk4 activity is required during mitosis for proper centriole duplication, but the mechanism stabilizing mitotic Plk4 is unknown. In this paper, we show that PP2A (Protein Phosphatase $2A^{Twins}$) counteracts Plk4 autophosphorylation, thus stabilizing Plk4 and promoting centriole duplication. Like Plk4, the protein level of PP2A's regulatory subunit, Twins (Tws), peaks during mitosis and is required for centriole duplication. However, untimely Tws expression stabilizes Plk4 inappropriately, inducing centriole amplification. Paradoxically, expression of tumor-promoting simian virus 40 small tumor antigen (ST), a reported PP2A inhibitor, promotes centrosome amplification by an unknown mechanism. We demonstrate that ST actually mimics Tws function in stabilizing Plk4 and inducing centriole amplification.

Introduction

Centrosomes are the major microtubule-nucleating centers in higher eukaryotic cells and are important in organizing mitotic spindle poles. Mitotic cells must establish a bipolar spindle to accurately segregate chromosomes and cortical cell fate determinants. Central to this is the precise duplication of the centrosome, a tiny organelle containing a pair of barrel-shaped centrioles, the duplicating elements of this organelle (Strnad and Gönczy, 2008). A centriole pair normally duplicates once per cell cycle, before mitosis, ensuring that only two centrosomes are present to guide bipolar spindle assembly (Tsou and Stearns, 2006). If this process goes awry, overproduction of centrosomes (i.e., centrosome amplification) can lead to transient multipolar spindle formation, with consequent errors in chromosome segregation and aneuploidy (Ganem et al., 2009; Silkworth et al., 2009). Aneuploidy is a known driving force for tumor formation (Weaver et al., 2007), and many human tumors contain cells with elevated centrosome numbers and aneuploidy (Lingle et al., 2002; Pihan et al., 2003). Furthermore, centrosome amplification in *Drosophila melanogaster* stem cells confers tumor-forming potential by inducing abnormal stem cell division and, consequently, altering polarity and cell fate (Basto et al., 2008; Castellanos et al., 2008). Notably, the mechanisms limiting centriole duplication to one event per cell cycle are unclear, and the molecular alterations that promote centriole amplification in cancerous cells are ill defined.

Centriole duplication is governed by Plk4/Sak (Polo-like kinase 4/Snk-akin kinase), a conserved master regulator and initiator of centriole assembly (Fode et al., 1994; Bettencourt-Dias et al., 2005; Habedanck et al., 2005). Although it is not clear how Plk4 initiates centriole duplication, Plk4 localizes to a "parent" centriole as a single asymmetric spot and modifies this site, making the parent competent for assembly of a procentriole (or "daughter" centriole; Kleylein-Sohn et al., 2007). Plk4 loss of function leads to a failure in centriole duplication (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). Conversely, Plk4 overexpression induces centriole amplification

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Abbreviations used in this paper: DRE, downstream regulatory element; dsRNA, double-stranded RNA; KD, kinase domain; Mts, microtubule star; OA, okadaic acid; PI, propidium iodide; PLP, pericentrin-like protein; SBD, Slimb-binding domain; SBM, Slimb-binding mutation; ST, small tumor antigen; Tws, Twins; wt, wild type.

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and can do so via three different pathways: (1) centriole reduplication (arising from the repeated separation and duplication of centriole pairs; Rogers et al., 2009; Dzhindzhev et al., 2010), (2) multiple daughter centriole assembly (manifested as a single duplicating mother centriole assembling several daughters near simultaneously; Habedanck et al., 2005; Kleylein-Sohn et al., 2007), or (3) de novo centriole assembly (Peel et al., 2007; Rodrigues-Martins et al., 2007). Plk4 overexpression is oncogenic in *Drosophila*, promoting multipolar spindle formation in neural stem cells and conveying tumor-forming potential (Basto et al., 2008; Castellanos et al., 2008). Intriguingly, Plk4 also acts as a tumor suppressor, as Plk4 heterozygotic mice display a high incidence of spontaneous liver tumors containing dividing cells with supernumerary centrosomes and multipolar spindles (Ko et al., 2005). However, because Plk4 also functions in cytokinesis in mammalian cells, these particular phenotypes are likely the result of cytokinesis defects (Rosario et al., 2010). Interestingly, expression of several viral oncoproteins can also induce centrosome amplification, including SV40 small tumor antigen (ST), human papillomavirus E7, and hepatitis B oncoprotein X (Duensing et al., 2000; Yun et al., 2004; Kotadia et al., 2008). How these viral proteins alter the centriole duplication machinery is not known, but given the importance of Plk4 in this process and its link to cancer, it seems possible that some viral oncoproteins directly influence Plk4 activity.

Understanding Plk4 regulation is critical in gaining insight into both the fidelity of centriole duplication and the etiology of centriole amplification during tumorigenesis. Plk4 activity is primarily regulated by cell cycle control of its protein turnover rate (Fode et al., 1996; Rogers et al., 2009). During interphase, Plk4 is expressed but then maintained at low levels because of the activity of the SCF (Skp/Cullin/F-box) E3 ubiquitin ligase complex, which recognizes phosphorylated Plk4 via the F-box protein Slimb (β-TrCP in humans; Cunha-Ferreira et al., 2009; Korzeniewski et al., 2009; Rogers et al., 2009). Slimb localizes to centrioles throughout the cell cycle and is properly poised to target Plk4 for proteasome-mediated degradation, thus blocking Plk4 accumulation on centrioles and the ensuing centriole amplication that would occur (Rogers et al., 2009). During mitosis, however, Plk4 protein levels peak, decorating each centriole as a single asymmetric spot and endowing the centriole with the ability to duplicate (Rogers et al., 2009). Remarkably, Plk4 accumulates on mitotic centrioles in spite of centriole-associated Slimb. Although our understanding of Plk4 down-regulation is increasing, it is not known how Plk4 is stabilized, nor is it clear how Plk4 regulation is altered during oncogenesis. In this study, we investigate the mechanism that stabilizes Plk4 and explore a causal link between viral oncogene expression, Plk4 misregulation, and centriole amplification.

Results

Drosophila Plk4 autophosphorylation promotes its degradation

Previously, we found that the Plk4 protein accumulates during mitosis in cultured *Drosophila* S2 cells (Fig. S1; Rogers et al., 2009). At this time, Plk4 associates with centrioles and endows them with the competence to duplicate. During interphase, Plk4 is recognized by SCF^{Slimb} ubiquitin ligase, and its consequent degradation is crucial in blocking centriole reduplication and preventing mother centrioles from assembling multiple daughters (Cunha-Ferreira et al., 2009; Rogers et al., 2009). Phosphorylation of the conserved Slimb-binding domain (SBD) within Plk4 promotes recognition by Slimb, and therefore, we set out to identify the kinases that prime Plk4 for degradation. Recently, it was reported that mammalian Plk4 autophosphorylates a region downstream of its kinase domain (KD; ∼50 amino acids containing the SBD) to promote its destruction by $SCF^{\beta TrCP/Slimb}$ (Guderian et al., 2010; Holland et al., 2010). *Drosophila* Plk4 also contains a serine-rich region surrounding its SBD (denoted here as the downstream regulatory element [DRE]; Fig. 1, A and B). To test whether fly Plk4 utilizes a similar autophosphorylation mechanism, we generated recombinant Plk4 containing the KD and DRE (Plk4-KD). We found that Plk4-KD can autophosphorylate in vitro and can phosphorylate kinase-dead Plk4-KD in trans (Fig. 1 C). As with mammalian Plk4 (Leung et al., 2007; Holland et al., 2010; Sillibourne et al., 2010), the DRE of *Drosophila* Plk4 is a target for autophosphorylation, as Plk4-KD phosphorylates purified DRE (Fig. 1 D). Furthermore, we found that the Plk4 kinase activity is needed for its degradation because kinase-dead Plk4 expressed in S2 cells is more stable than wild-type (wt) Plk4 (Fig. 1 E). These results support the hypothesis that Plk4 autophosphorylation promotes its destruction but do not exclude the possibility that other kinases also regulate Plk4 levels. Therefore, we performed an RNAi-based screen of the *Drosophila* kinome using S2 cells transfected with inducible Plk4-GFP and measured Plk4 levels by quantitative anti-GFP immunoblots (Table S2). As with Slimb RNAi treatment, depletion of the kinase responsible for Slimb recognition should cause a dramatic elevation in Plk4 levels. However, no such kinase was identified in our screen (Fig. S2). Collectively, these results demonstrate that Plk4 autophosphorylation of the DRE is an evolutionarily conserved mechanism that regulates Plk4 stability and that it is unlikely that other kinases influence Slimb binding.

PP2A (Protein Phosphatase 2A) and its regulatory subunit, Twins (Tws), are required for centriole duplication

We next sought to determine the mechanism that stabilizes Plk4 during mitosis. Because Slimb levels are constant throughout the cell cycle (Rogers et al., 2009), and Plk4 autophosphorylation promotes Slimb binding, we hypothesized that a serine/threonine phosphatase might counteract this activity to stabilize mitotic Plk4 and promote centriole duplication. Inhibition of the phosphatase should promote constitutive Plk4 degradation and prevent centriole duplication. Notably, knockdown of PP2A was reported to reduce mitotic centrosome numbers in S2 cells (Chen et al., 2007a), suggesting that PP2A promotes centriole duplication. Centrioles were not directly examined in that study, and the findings could be explained by the inability of mitotic centrioles in PP2A-depleted cells to recruit pericentriolar material proteins (Dobbelaere et al., 2008). Nevertheless, PP2A plays numerous mitotic functions (De Wulf et al., 2009), and

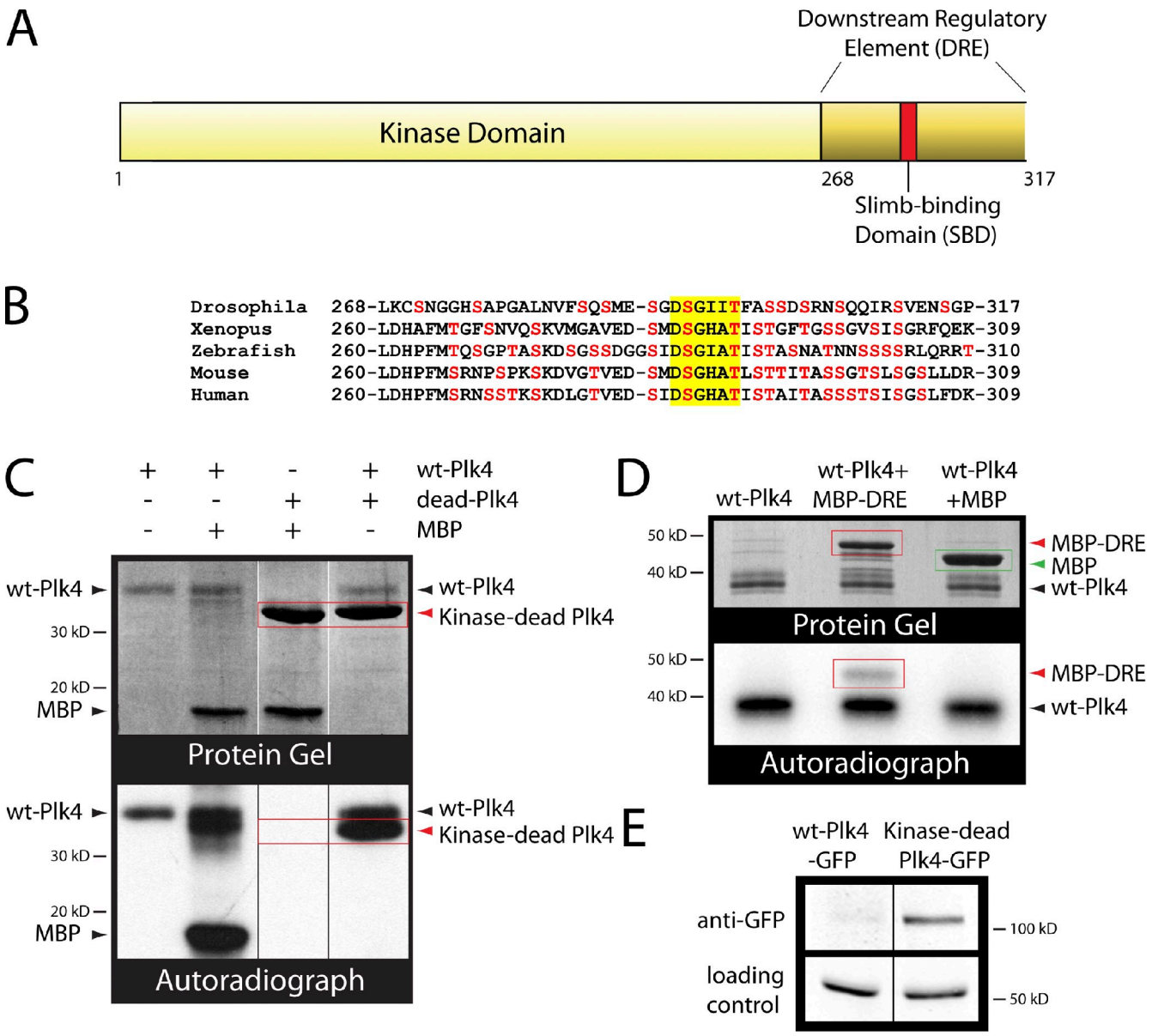

Figure 1. ***Drosophila* Plk4 autophosphorylation promotes its own degradation.** (A) Linear map of *Drosophila* Plk4 amino-terminus encoding the KD and the DRE. The red bar indicates the position of the conserved SBD (DSGXXT). (B) Lineup of the 50 amino acid DRE encoded by Plk4 family members. Serine and threonine residues are shown in red. Yellow box highlights the SBD. (C) Purified recombinant Plk4 kinase domain + DRE (wt-Plk4) but not kinase-dead (D156N point mutation of wt) Plk4 autophosphorylates in vitro. Coomassie-stained gel (top) and corresponding autoradiograph (bottom) are shown. (lanes 1 and 2) Plk4 phosphorylates itself and purified bovine myelin basic protein (MBP). (lane 3) Kinase-dead Plk4 lacks kinase activity. (lane 4) wt-Plk4 phosphorylates kinase-dead Plk4 in trans. (D) wt-Plk4 phosphorylates purified DRE fused to maltose-binding protein (MBP-DRE) in trans but does not phosphorylate purified maltose-binding protein. (E) A kinase-dead mutation in Plk4 inhibits its degradation in S2 cells. Anti-GFP immunoblot of S2 cell lysates shows that full-length kinase-dead Plk4-GFP is more stable than wt-Plk4-GFP, which is degraded and nearly undetectable. Inducible Plk4 constructs were cotransfected into S2 cells with Nlp-GFP (used as a loading control and driven by its endogenous promoter). Black/white lines indicate that intervening lanes have been spliced out.

therefore, we explored a possible role for PP2A in stabilizing mitotic Plk4 and promoting centriole duplication.

PP2A is a holoenzyme composed of three subunits: a catalytic C subunit (*microtubule star* [*Mts*] in *Drosophila*), a structural A subunit (*Drosophila* PP2A-29B), and a regulatory B subunit encoded by one of four fly genes (*Tws/PR55*, *Widerborst*, *Well rounded*, or *PR72*; Mayer-Jaekel et al., 1992; Uemura et al., 1993; Snaith et al., 1996; Hannus et al., 2002; Li et al., 2002; Viquez et al., 2006). To test whether PP2A is involved in centriole duplication, we used RNAi to deplete each protein individually (when antibodies were available, we confirmed depletion by immunoblotting; Fig. S3 A) and measured centriole numbers in S2 cells. Depletion of Mts or PP2A-29B resulted in loss of centrioles (Fig. 2 A), significantly increasing the percentage of cells with less than two centrioles compared with controls (Fig. 2 B). This effect was phenocopied by treating cells with the PP2A inhibitor okadaic acid (OA; Fig. 2, A and B). Depletion of only one regulatory subunit, Tws, also significantly decreased centriole number (Fig. 2 B). Prolonged Tws RNAi had no effect on cell cycle progression (Fig. S3 B) or mitotic index

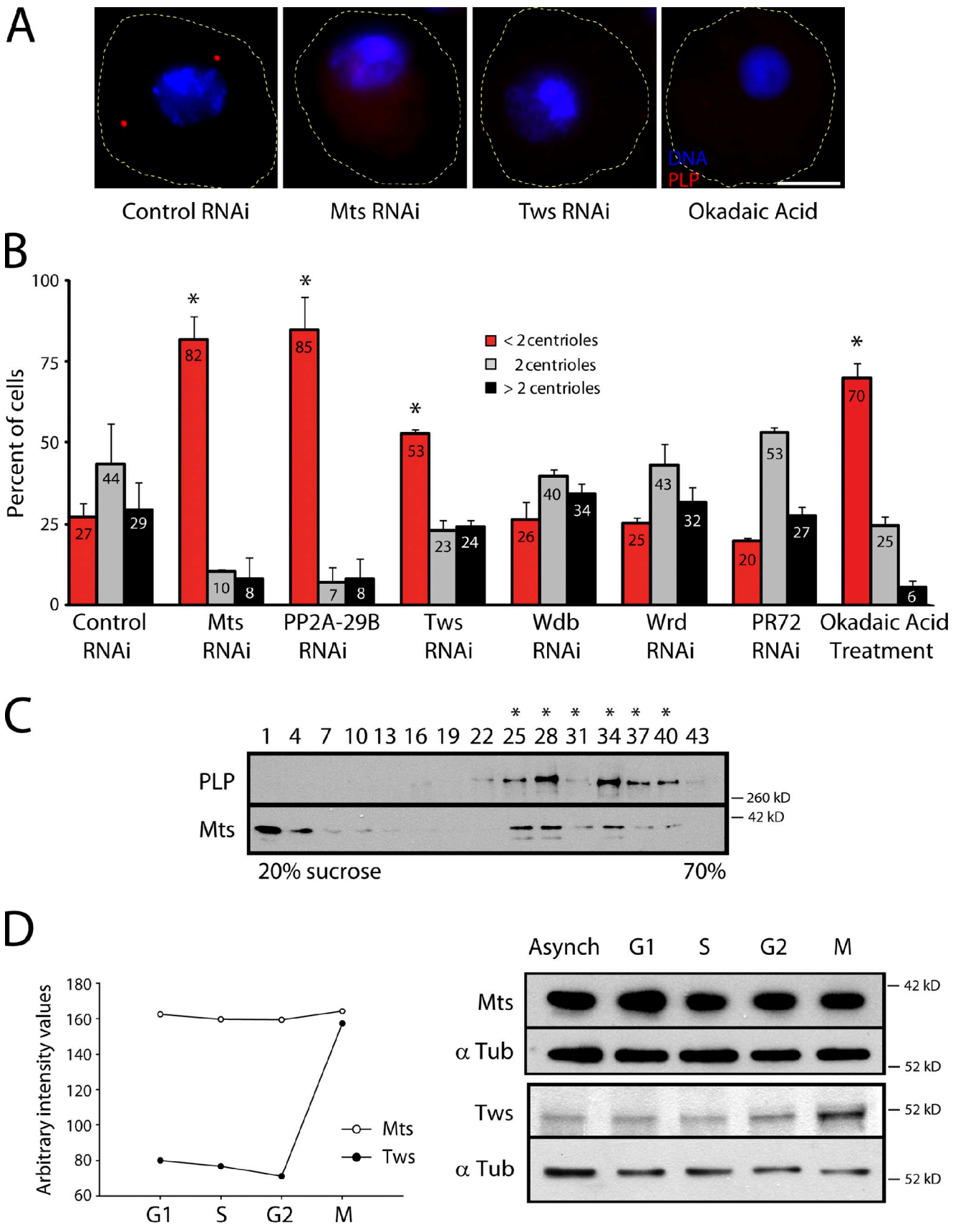

Figure 2. **PP2A^{Tws} is required for centriole duplication.** (A) 5-d RNAi-treated or 24-h OA-treated S2 cells were immunostained for PLP to mark centrioles and Hoechst stained to label DNA. Cell borders are traced with dashed lines. Bar, 5 µm. (B) PP2A inhibition by Mts, 29B, or Tws RNAi leads to centriole loss. After RNAi treatment, the number of PLP-immunostained centrioles per cell was measured. Graph shows the percentage of cells with the indicated number of

(control RNAi = 2.0%; Tws RNAi = 2.7%) but dramatically elevated the frequency of mitotic spindles lacking centrosomes (similar experiments with prolonged Mts or PP2A-29B RNAi led to significant cell death; Fig. S3 C). Consistent with a role in centriole duplication, Mts and PP2A-29B localize to mitotic centrioles in S2R+ cells (Dobbelaere et al., 2008). Likewise, we found a fraction of Mts copurified with centrioles isolated from mitotic S2 cells using sucrose gradient centrifugation (Fig. 2 C). Together, these results suggest that a $PP2A^{Tws}$ complex is required for centriole duplication and is appropriately positioned on mitotic centrioles.

Tws protein levels peak during mitosis

Because Plk4 protein levels peak during mitosis (Rogers et al., 2009), we examined protein levels of PP2A subunits to determine whether they display similar cell cycle–dependent changes. S2 cells were chemically arrested during each cell cycle stage, and lysates were immunoblotted for PP2A catalytic (Mts) and regulatory (Tws) subunits. Although Mts protein levels were abundant and uniform throughout the cell cycle, Tws levels fluctuated and were strikingly similar to Plk4 (Rogers et al., 2009): low during interphase but high during mitosis (Fig. 2 D). These findings suggest the possibility that increasing Tws levels during mitosis activates a $PP2A^{Tws}$ complex responsible for stabilizing Plk4.

$PP2A^{Tws}$ is required to stabilize Plk4

We next tested whether Tws is involved in the pathway regulating Plk4 stability. Depletion of Slimb increases both Plk4 levels and the percentage of cells with greater than two centrioles (Cunha-Ferreira et al., 2009; Rogers et al., 2009). Therefore, if loss of centrioles after $PP2A^{Tws}$ depletion is caused by Plk4 degradation, this effect should be rescued by codepletion of Slimb because Slimb mediates Plk4 degradation. Indeed, we found that whereas Tws RNAi reduced centriole number, Slimb/Tws co-RNAi (which effectively depletes both proteins; Fig. 3 A) completely reversed the effect and significantly decreased the percentage of cells containing less than two centrioles while increasing cells with greater than two centrioles (Fig. 3 B). Therefore, instead of being required for downstream steps in centriole assembly, $PP2A^{Tws}$ likely acts upstream of Plk4 and Slimb.

We tested for a genetic interaction between $PP2A^{Tws}$ and Plk4 by depleting $PP2A^{Tws}$ in S2 cells expressing a Plk4 construct harboring a Slimb-binding mutation (SBM; a nonphosphorylatable alanine [S293A/T297A] mutation), which prevents Slimb binding (Cunha-Ferreira et al., 2009; Rogers et al., 2009). Like Slimb RNAi, Plk4-SBM expression increased the percentage of cells containing greater than two centrioles (Fig. 3 C; Rogers et al., 2009). We hypothesized that if $PP2A^{Tws}$ dephosphorylates and stabilizes Plk4, expression of Plk4-SBM would rescue the loss of centrioles after Tws depletion (Fig. 3 C). We found this to be the case, as Plk4-SBM expression restored centriole numbers to control levels (Fig. 3 C). Together, these results suggest that $PP2A^{Tws}$ promotes centriole duplication by stabilizing Plk4 and, furthermore, may work by dephosphorylating key residues in the Plk4 SBD to block Slimb binding.

To determine directly whether PP2A is required to stabilize Plk4, we overexpressed Plk4-GFP in S2 cells treated with OA. As described previously (Rogers et al., 2009), Plk4-GFP levels rise in mitotic cells (Fig. 3 D, first and second lane). However, PP2A inhibition dramatically prevented this increase in mitotic Plk4-GFP and, strikingly, eliminated Plk4-GFP in these cells (Fig. 3 D, third and fourth lane). To test whether this effect was simply caused by an inhibition of translation upon OA treatment, we induced the expression of GFP alone and then treated cells with OA. OA had no effect on GFP levels (unpublished data), suggesting that PP2A activity is specifically required for Plk4 stabilization.

PP2A dephosphorylates Plk4

Plk4 from OA-treated cells also has a distinct electrophoretic shift compared with control-treated cells (Fig. 3 E), suggesting that Plk4 is more phosphorylated after PP2A inhibition. It was necessary to use the stable Plk4-SBM-GFP transgene for this experiment: although the SBM prevents phosphorylation within the SBD, multiple serines that flank this site are likely phosphorylated (Holland et al., 2010) and may be recognized by PP2A. Thus, Plk4 is likely a bona fide PP2A substrate and can be phosphorylated on multiple residues outside the SBD. Lastly, we examined whether PP2A can dephosphorylate Plk4 in vitro using purified components. When human PP2A-A and -C subunits (sharing 75 and 94% identity with their fly homologues) were incubated with autophosphorylated fly Plk4-KD (containing the DRE), PP2A dephosphorylated Plk4-KD in a dose-dependent manner and was inhibited by addition of OA (Fig. 3 F). Collectively, these results indicate that Plk4 is a PP2A substrate, and this interaction has a profound effect on Plk4 stability.

Tws overexpression is sufficient to stabilize Plk4 and induce centriole amplification

Because Tws protein levels peak during mitosis when Plk4 levels are highest (Fig. 2 D), we tested whether overexpression of this regulatory subunit alone is sufficient to stabilize Plk4 during periods when Plk4 is normally degraded. Tws-GFP (or GFP) and Plk4-GFP were coexpressed in S2 cells, and their levels were monitored with GFP immunoblots. Although control GFP overexpression had no effect on Plk4-GFP levels, Tws-GFP overexpression dramatically increased Plk4-GFP levels in asynchronous cells in a dose-dependent manner (Fig. 4, A and B).

centrioles per cell. Each number in a bar is the percent mean for two experiments (n = 500 cells/treatment). Asterisks mark significant differences (relative to control) for comparisons mentioned in the text ($P < 0.02$). Error bars indicate SD. (C) Mts cosediments with centrioles purified from mitotic S2 cells on a 20–70% sucrose gradient. Fractions (numbered) were immunoblotted for the indicated proteins. Asterisks mark the major centriole-containing fractions. (D) Tws protein is maximal during mitosis. (left) Graph of normalized endogenous Mts and Tws levels in asynchronous cells (Asynch) or after a 24-h drug-induced cell cycle arrest. Plotted values were determined from the anti-Mts and Tws immunoblots (right) shown. The graph and blots are representative examples of three independent experiments, all with similar results. α-Tubulin (α Tub) was used as a loading control. Wdb, Widerborst; Wrd, Well rounded.

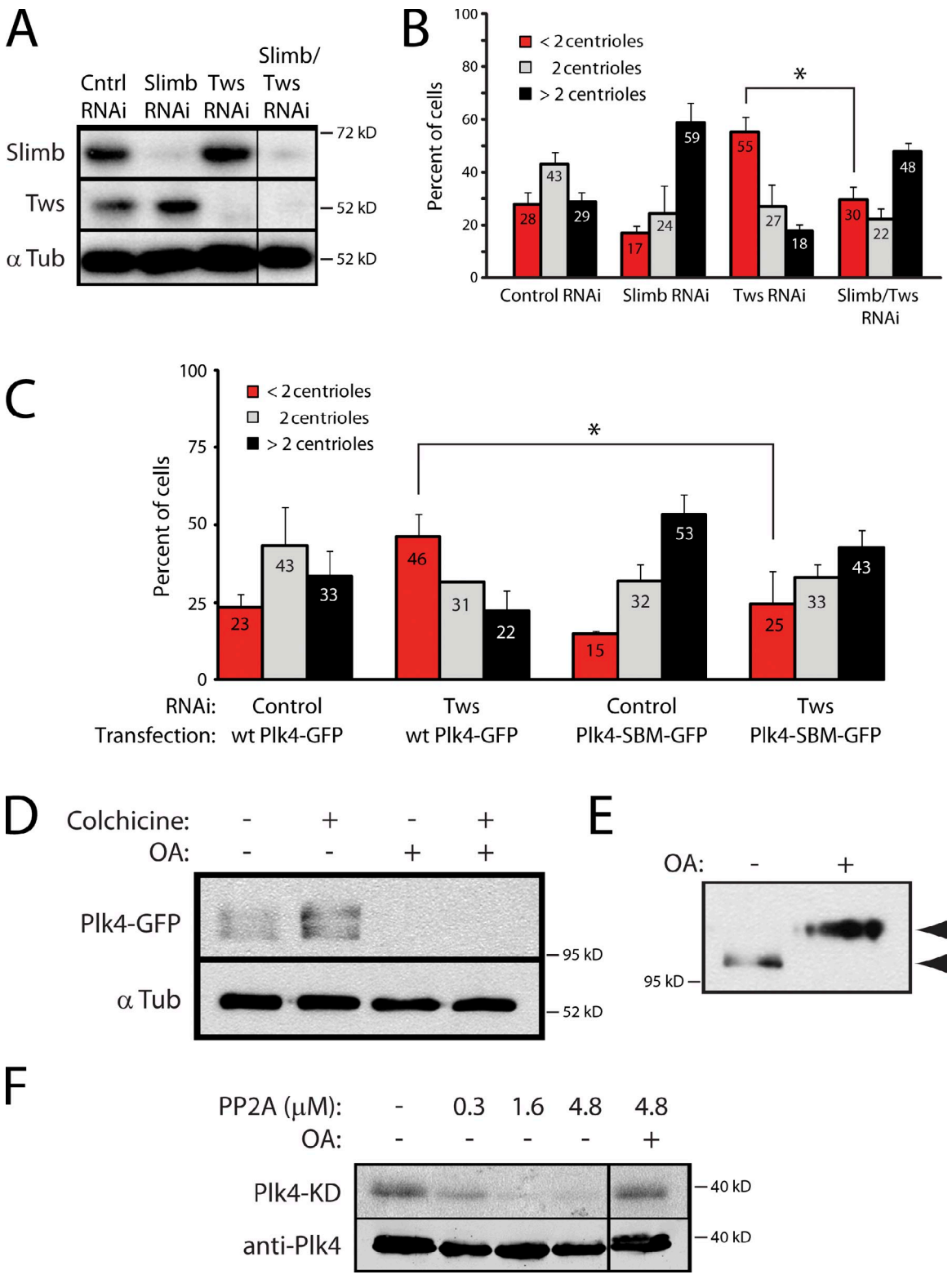

Figure 3. **PP2A^{Tws} stabilizes Plk4 to promote centriole duplication.** (A) Immunoblots of 6-d RNAi-treated S2 cells demonstrating knockdown of target proteins. α Tub, α-tubulin; Cntrl, control. (B) Loss of centrioles by Tws RNAi is rescued by codepletion of Slimb. Each mean percentage of cells (numbers) is derived from two experiments (n = 598 cells/treatment). Asterisk indicates significant difference ($P < 0.02$) between compared treatments mentioned in the text. (C) Low expression of nondegradable Plk4-SBM-GFP also rescues the centriole loss by Tws-RNAi. Each mean percentage (numbers) is derived from three experiments (n = 900 cells/treatment). *, $P < 0.04$. (D) PP2A is required to stabilize Plk4. S2 cells overexpressing Plk4-GFP were treated with colchicine or OA for 24 h as indicated, and lysates were probed for GFP. (E) PP2A dephosphorylates Plk4-SBM-GFP in cells. S2 cells expressing Plk4-SBM-GFP were treated with OA for 24 h, and their lysates were immunoblotted for GFP. Note the clear shift in mobility of Plk4-SBM after OA treatment (arrowheads), consistent with Plk4-SBM being hyperphosphorylated after PP2A inhibition. (F) PP2A dephosphorylates Plk4 in vitro. Human PP2A dephosphorylates autophosphorylated Plk4-KD + DRE-$(His)_6$ protein (Plk4-KD) but is inhibited by OA. (top) Autoradiogram; (bottom) Plk4 immunoblot. Black lines indicate that intervening lanes have been spliced out. Error bars indicate SD.

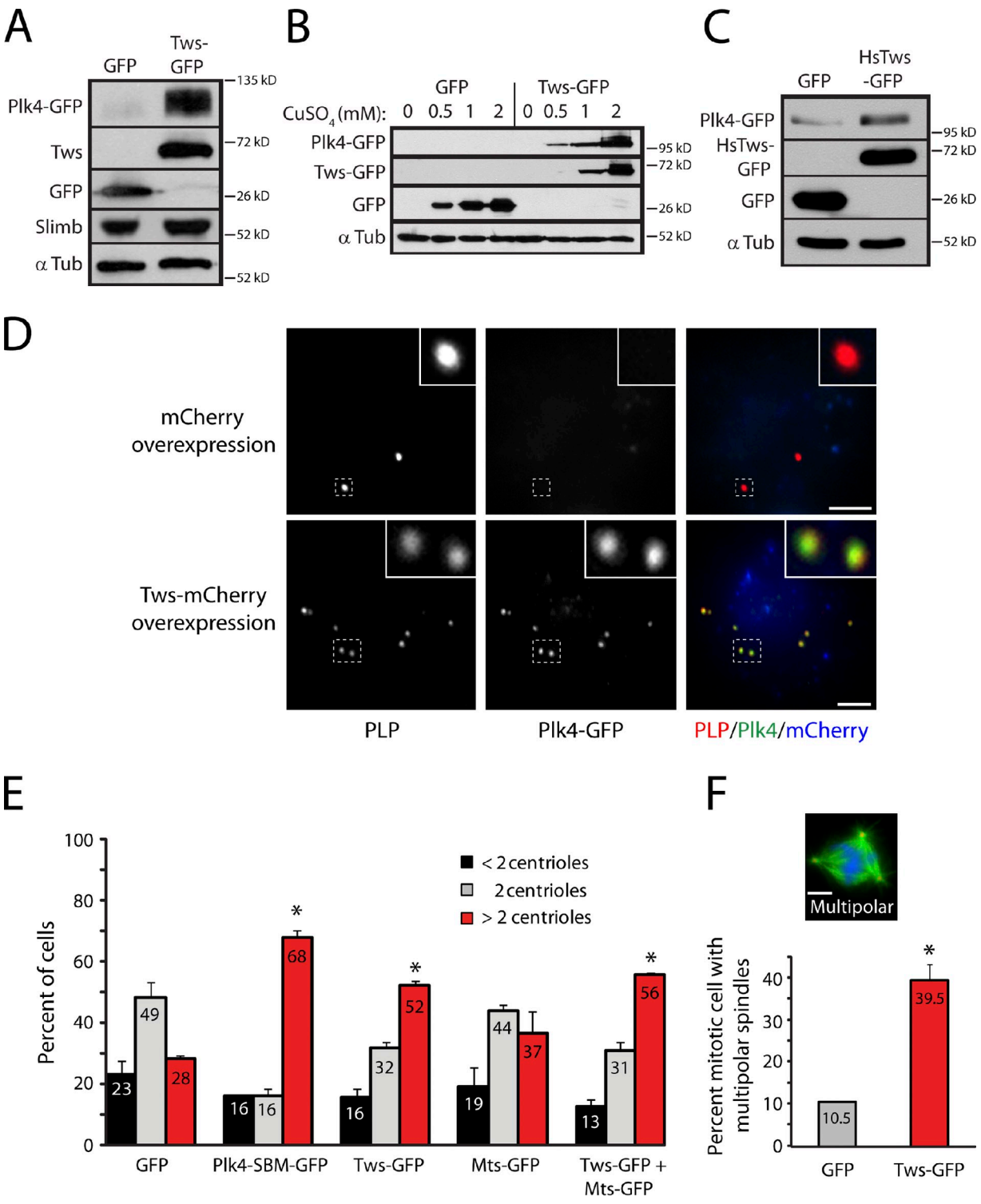

Figure 4. **Ectopic Tws expression is sufficient to stabilize Plk4 and promote centriole amplification.** (A) Immunoblots of S2 cell lysates showing that Tws-GFP overexpression stabilizes Plk4-GFP without affecting Slimb levels. S2 cells were cotransfected with inducible Plk4-GFP and inducible GFP (first lane) or Tws-GFP (second lane) expression constructs. After a 24-h recovery period, gene expression was induced with 1 mM $CuSO_4$ for 20 h. Cell lysates were then prepared and immunoblotted for GFP, endogenous Slimb, and α-tubulin (α Tub; loading control). (B) The extent of Plk4-GFP stabilization is dose dependent on Tws-GFP. S2 cells were cotransfected with Plk4-GFP (driven by the weak, constitutive *Drosophila* SAS-6 promoter) and either GFP (negative control) or Tws-GFP, each controlled by the copper-inducible metallothionein promoter. Cells were incubated with 0, 0.5, 1, or 2 mM $CuSO_4$ for 24 h, and cell lysates were probed by GFP immunoblotting. (C) Immunoblot of S2 cell lysates showing that Plk4-GFP is also stabilized by human Tws (HsTws; PR55-α) overexpression. (D) Tws-mCherry expression drives abnormal accumulation of Plk4-GFP on centrioles (anti-D-PLP) in interphase S2 cells. Insets show centrioles (dashed boxes) at higher magnification. Bars, 5 µm. (E) Tws-GFP overexpression promotes centriole amplification. Graph shows the percentage of transgene-expressing cells containing the indicated number of centrioles; means (numbers) derived from three experiments (n = 600 cells/treatment). *, $P < 0.003$ (treated conditions compared with GFP control). (F) Tws-GFP overexpression increases the frequency of multipolar spindles. S2 cells expressing GFP or Tws-GFP were immunostained for centrioles (anti-PLP, red) and α-tubulin (green). DNA (blue) is Hoechst stained. Bar, 2.5 µm. Graph shows mean percentages (numbers) of mitotic cells with multipolar spindles (two experiments; n = 76 cells/treatment). *, $P < 0.01$. Error bars indicate SD.

The effect was not caused by mitotic arrest (mitotic index: GFP, 2.7%; Tws-GFP, 3.2%) nor caused by a decrease in Slimb levels, which remained unchanged (Fig. 4 A). A similar result was observed in S2 cells overexpressing human Tws (which shares 79% amino acid identity with fly Tws), in which Plk4-GFP levels increased by fourfold (Fig. 4 C), suggesting $PP2A^{Tws}$ may function analogously in human cells to stabilize Plk4.

If $PP2A^{Tws}$ stabilizes Plk4, several predictions follow. First, Tws overexpression should drive abnormal accumulation of Plk4 on interphase centrosomes. Plk4-GFP localization was examined in interphase S2 cells overexpressing Tws-mCherry and was observed to be associated with centrioles, whereas this was not observed in cells expressing mCherry (Fig. 4 D). Second, Tws overexpression should drive centriole amplification. When centriole numbers were measured in Tws-GFP–overexpressing interphase cells, we found a significant increase in the percentage containing greater than two centrioles as compared with controls, similar to the expression of Plk4-SBM (Fig. 4 E). Notably, overexpression of the catalytic subunit Mts did not increase centriole number and was not synergistic with Tws coexpression (Fig. 4 E), suggesting that endogenous Mts is not limiting for $PP2A^{Tws}$ activity. Furthermore, Tws overexpression increased the frequency of multipolar spindles in dividing cells by fourfold (Fig. 4 F). Therefore, Tws overexpression is sufficient to stabilize Plk4 during cell cycle stages when Plk4 levels are normally low and, thus, induces centriole amplification and multipolar spindle formation.

SV40 ST expression induces centriole amplification in a PP2A-dependent manner

Based on these findings, we propose that centriole duplication is regulated by a dynamic mechanism governing Plk4 stability: Plk4 autophosphorylation of its DRE promotes SCF^{Slimb}-mediated degradation during interphase to block centriole amplification, but the phosphorylated state is reversed by mitotic $PP2A^{Tws}$ to stabilize Plk4 and enable centriole duplication. As a proof of principle study, we examined whether perturbation of this mechanism could account for centrosome amplification observed during oncogenic transformation by using the ST of the tumor-promoting virus SV40 (Kotadia et al., 2008). ST can act as a potent PP2A inhibitor (Arroyo and Hahn, 2005). By directly binding to the PP2A structural A subunit, ST competes with and displaces endogenous regulatory subunits, thereby preventing the dephosphorylation of PP2A substrates (Chen et al., 2007b; Cho et al., 2007). Not surprisingly, given their high degree of conservation, ST also binds the *Drosophila* structural subunit PP2A-29B (Kotadia et al., 2008). However, regarding ST's effect on centriole duplication, the notion that ST inhibits PP2A is paradoxical because ST expression promotes centrosome amplification in human U2OS cells, cultured fly Kc cells, and fly embryos (Kotadia et al., 2008), whereas PP2A depletion actually eliminates centrosomes in fly cells (Chen et al., 2007a). To resolve this issue, we tested the hypothesis that ST does not inhibit all PP2A functions but can instead act as a surrogate regulatory subunit to mimic the activity of Tws by stabilizing Plk4 and, thus, drive centriole amplification. We found that ST-GFP overexpression in S2 cells increased the percentage of cells containing greater than two centrioles (Fig. 5, A and B). Notably, the effect is PP2A dependent because OA treatment of ST-GFP–expressing cells blocks centriole amplification (Fig. 5 B). Therefore, the centriole amplification that follows ST expression requires PP2A activity and suggests that ST does not inhibit all PP2A functions. This result is surprising given ST's reported inhibitory effects on PP2A activity.

SV40 ST functionally mimics Tws in stabilizing Plk4

To determine whether abnormal Plk4 hyperstabilization is the causal link between PP2A activation and ST-mediated centriole amplification, ST-GFP and Plk4-GFP were coexpressed in S2 cells, and their levels monitored with GFP immunoblots. Strikingly, we found that ST-GFP expression increased Plk4-GFP levels in asynchronous cells to an extent similar to Plk4-SBM expression (Fig. 5 C). The effect was not caused by mitotic arrest (mitotic index: GFP control, 2.7%; ST-GFP, 3.2%) or a decrease in Slimb levels, which remained unchanged (Fig. 5 C). Like Tws overexpression, the ST effect was dose dependent (Fig. 5 D) and also induced Plk4 localization to centrioles in interphase cells (Fig. 5 E). Lastly, we tested whether ST expression could functionally replace Tws in centriole duplication by overexpressing ST in S2 cells depleted of Tws. Although Tws RNAi treatment of control cells expressing GFP led to centriole loss, ST-GFP expression in similarly treated cells rescued the phenotype and decreased the percentage of cells containing less than two centrioles (Fig. 5 B). ST expression did not rescue centriole loss caused by Plk4 depletion (Fig. 5 B). Therefore, our results suggest that ST does not inhibit all PP2A functions but can mimic the ability of Tws to activate PP2A, thus stabilizing Plk4 at inappropriate cell cycle stages and promoting centriole amplification.

Discussion

Recent studies have increased our understanding of Plk4 inactivation considerably. Plk4 protein is maintained at near-undetectable levels for the majority of the cell cycle by ubiquitin-mediated proteolysis (Fig. 6 A; Fode et al., 1996; Rogers et al., 2009). The ubiquitin ligase SCF^{Slimb} is responsible for Plk4 degradation and recognizes an extensively phosphorylated degron situated immediately downstream of the KD. Slimb is appropriately positioned on centrioles throughout the cell cycle to promote rapid Plk4 destruction, but centrioles are not required for its activity (Rogers et al., 2009). In any case, Plk4 degradation is critical in blocking all pathways of centriole amplification. Unlike other Polo kinase members, Plk4 is a homodimer capable of autophosphorylating its DRE in trans to promote Slimb binding (Leung et al., 2002; Guderian et al., 2010). Our findings demonstrate that autoregulation is a conserved feature of Plk4. Moreover, our RNAi screen of the fly kinome suggests that no other kinase is required for Plk4 degradation. The continuous and efficient degradation of Plk4 indicates that Plk4 is immediately active when expressed and that control of Plk4's protein level is key to regulating its activity.

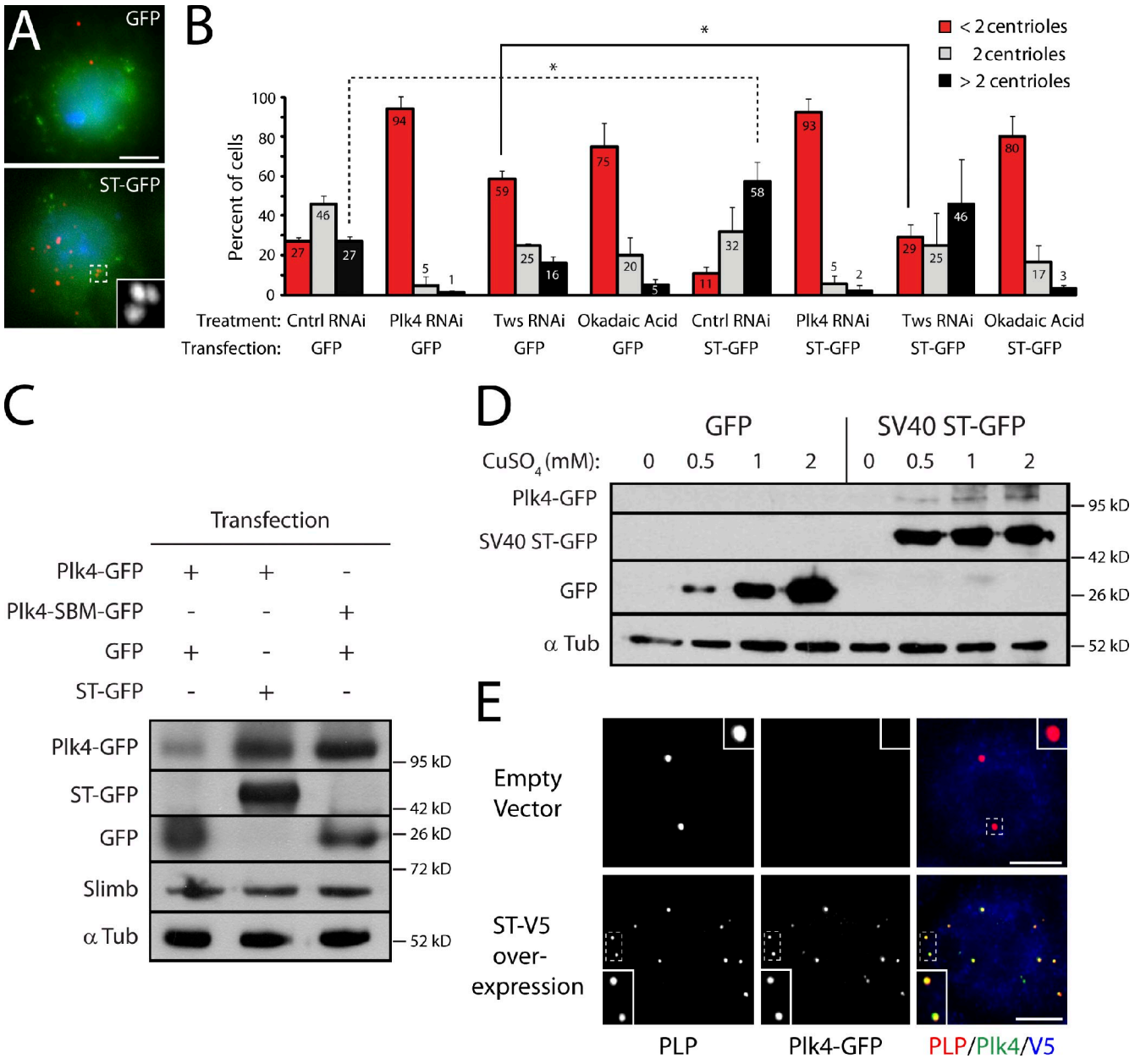

Figure 5. **Tumor-promoting SV40 ST mimics Tws function to stabilize Plk4 and amplify centrioles.** (A) S2 cells overexpressing GFP or ST-GFP (green) immunostained for PLP (red) to mark centrioles. DNA (blue) is Hoechst stained. (B) ST-GFP overexpression increases centriole numbers but not in the presence of OA. ST-GFP expression also rescues centriole loss caused by depletion of Tws but not Plk4. Centrioles were counted after 6-d RNAi or 24-h OA treatment in cells transfected with inducible GFP or ST-GFP (expression was induced during the last 2 d). Mean values (numbers) of two experiments are shown (n = 600 cells/treatment). Asterisks indicate significant differences ($P < 0.05$) between compared treatments mentioned in the text. Error bars indicate SD. Ctnrl, control. (C and D) Immunoblots show that ST-GFP overexpression stabilizes Plk4-GFP in S2 cells to levels similar to Plk4-SBM-GFP, without affecting endogenous Slimb levels (C) and in a dose-dependent manner (D; treatment protocol similar to that described in Fig. 4 B). α Tub, α-tubulin. (E) ST-V5 expression drives accumulation of Plk4-GFP on centrioles (anti-PLP) in interphase S2 cells. Insets show centrioles (dashed boxes) at higher magnification. Bars, 5 µm.

However, surprisingly little is known about the converse event: how Plk4 is activated. Our results reveal the existence of a previously unknown facet of the regulation of centriole duplication, a process which transiently stabilizes and activates Plk4 specifically during mitosis. Our study led us to investigate serine/threonine phosphatases as possible effectors to counteract Plk4 autophosphorylation. PP2A is an excellent candidate to fulfill this role as it has important functions in mitosis and localizes to mitotic centrioles in cultured fly cells and centrosomes in dividing *Caenorhabditis elegans* embryos (Schlaitz et al., 2007; Dobbelaere et al., 2008). A previous study found that the number of γ-tubulin foci in mitotic S2 cells was diminished after PP2A RNAi (Chen et al., 2007a), but whether this resulted from a bona fide loss of centrioles or instead reflects a requirement for PP2A for centrosome maturation was not determined. Subsequently, a role for PP2A in centrosome maturation was identified in a genome-wide RNAi screen (Dobbelaere et al., 2008). Our results indicate that PP2A and the regulatory subunit Tws are required for centriole duplication by dephosphorylating and stabilizing Plk4. Without $PP2A^{Tws}$, Plk4 cannot be stabilized,

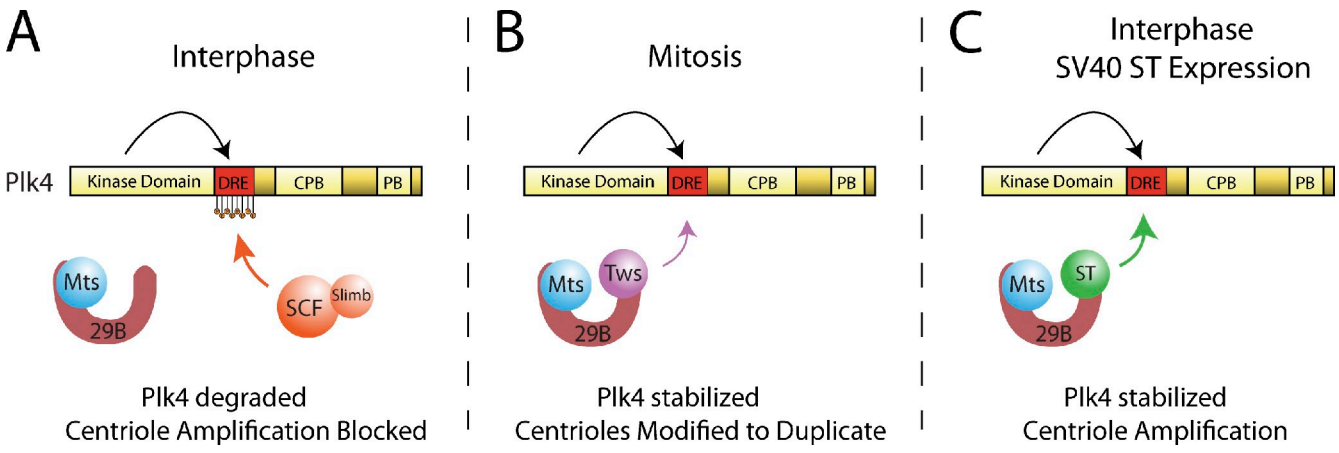

Figure 6. **Model depicting the regulation of Plk4 activity by a counteracting autophosphorylation/dephosphorylation mechanism.** (A) Plk4 is expressed throughout interphase, but continuous Plk4 autophosphorylation of its DRE triggers its own proteasome-mediated degradation by inducing SCF^{Slimb} binding and ubiquitination. As a result, centriole amplification is blocked. Plk4 stabilization can be achieved by PP2A activity, which dephosphorylates Plk4. However, this does not normally occur because its required regulatory subunit, Tws, is present at insufficient levels. (B) During mitosis, Tws protein levels rise, thus activating a $PP2A^{Tws}$ complex, which counteracts Plk4 autophosphorylation. Plk4 is stabilized and modifies centrioles, making them competent to duplicate during the next S phase. Upon mitotic exit, Tws levels decrease, and PP2A is unable to maintain Plk4 stability. (C) This same mechanism is exploited by tumor-promoting SV40 ST. ST binds PP2A and functionally mimics Tws, counteracting Plk4 autophosphorylation. Plk4 is inappropriately stabilized during interphase, localizes to centrioles, and thus, promotes centriole amplification. CPB, cryptic polo box; PB, polo box.

and centrioles fail to duplicate. PP2A is also required for centriole assembly in *C. elegans* embryos but functions downstream in the centriole assembly process (Kitagawa et al., 2011; Song et al., 2011). Although the catalytic and structural PP2A subunits are abundant, regulatory subunits are needed for intracellular targeting and recognition of a myriad of substrates. Tws overexpression is sufficient to stabilize Plk4 in a dose-dependent manner, causing centriole amplification and multipolar spindle formation. Like Plk4, Tws protein levels are low during interphase but rise and peak during mitosis. Accordingly, our results suggest that $PP2A^{Tws}$ stabilizes mitotic Plk4 by counteracting Plk4 autophosphorylation, enabling cells to switch Plk4 activity (and thus centriole duplication) on and off (Fig. 6 B). This mechanism is inherently highly sensitive to the presence of Tws, a rate-limiting component. Moreover, this is likely a conserved mechanism because overexpression of human Tws also stabilizes fly Plk4 in S2 cells. Clearly, an important goal for future studies is to establish whether the regulation of Tws levels and cell cycle control are linked. In addition, our results suggest that up-regulation of Tws could be a means to amplify centrioles in multiciliated cells and that increased Tws activity could be a condition found in cancerous cells.

Centrosome amplification is a hallmark of cancer and is also observed upon expression of DNA tumor virus proteins, which include SV40 ST, human papillomavirus E7, human T cell leukemia virus type-1 Tax, hepatitis B virus oncoprotein X, and human adenovirus E1A (Duensing et al., 2000; De Luca et al., 2003; Yun et al., 2004; Nitta et al., 2006; Kotadia et al., 2008). However, mechanisms for centrosome amplification by viral oncoproteins are not known. SV40 ST has been found to directly bind the highly conserved *Drosophila* catalytic and structural PP2A subunits and to induce centrosome overduplication in cultured fly cells (Kotadia et al., 2008). Notably, ST is a well-established PP2A inhibitor and is known to bind structural PP2A subunits, forcing endogenous PP2A regulatory subunits to be displaced and inhibiting PP2A activity (Arroyo and Hahn, 2005; Chen et al., 2007b; Cho et al., 2007). However, our results demonstrate that ST expression does not inhibit all PP2A activities but, instead, stimulates PP2A stabilization of Plk4 (Fig. 6 C). To our knowledge, this represents the first evidence that ST mimics the function of a PP2A regulatory subunit in cells. It will be important to determine whether ST targets additional PP2A substrates during tumorigenesis and whether other tumorigenic viruses (e.g., human papillomavirus and hepatitis B) known to promote centrosome amplification (Duensing et al., 2000; De Luca et al., 2003; Yun et al., 2004; Nitta et al., 2006) exploit this same mechanism. Intriguingly, human papillomavirus E7 oncoprotein binds PP2A catalytic and structural subunits and prevents PP2A from dephosphorylating Akt (Pim et al., 2005). Although a previous study has suggested that PP2A may function as a tumor suppressor (Janssens et al., 2005), our findings indicate that unregulated PP2A activity leads to centriole amplification and chromosomal instability and should therefore be considered as a potential oncogenic factor.

Materials and methods

Cell culture and double-stranded RNAi (dsRNA)

Drosophila S2 cell culture, in vitro dsRNA synthesis, and RNAi treatments were performed as previously described (Rogers and Rogers, 2008). In brief, cells were cultured in Sf900II SFM media (Life Technologies). RNAi was performed in 6-well plates. Cells (50–90% confluency) were treated with 10 µg dsRNA in 1 ml media and replenished with fresh media/dsRNA every other day for 4–7 d. The gene-specific primer sequences used to amplify DNA templates for RNA synthesis are shown in Table S1. Control dsRNA was synthesized from control DNA template amplified from a non-GFP sequence of the pEGFP-N1 vector (Takara Bio Inc.) using the primers 5′-CGCTTTTCTGGATTCATCGAC-3′ and 5′-TGAGTAACCTGAGGCTATGG-3′ (all primers used for dsRNA synthesis in this study begin with the T7 promotes sequence 5′-TAATACGACTCACTATAGGG-3′). For the *Drosophila* kinome screen, 185 dsRNA were purchased from the Harvard Fly RNAi Center (those with amplicon numbers in Table S2). Cell cycle arrest was induced by treating cells for ≥24 h with a final concentration of either

0.5 mM mimosine (for a G1-phase arrest), 1 µM hydroxyurea + 10 µM aphidicolin (S-phase arrest), 1.7 µM 20-hydroxyecdysone (G2-phase arrest), or 12 h of 30 µM colchicine (M-phase arrest; this treatment produces a mitotic index of ~30%) as previously described (Rogers et al., 2009). OA was used at final concentrations ranging from 1 to 100 nM.

Immunofluorescence microscopy

For immunostaining, S2 cells were fixed and processed exactly as previously described (Rogers and Rogers, 2008) by spreading S2 cells on concanavalin A–coated, glass-bottom dishes and fixing with 10% formaldehyde. Primary antibodies were diluted to concentrations ranging from 1 to 20 µg/ml. They included rabbit and guinea pig anti–pericentrin-like protein (PLP; produced in laboratory), anti-GFP antibodies (monoclonal JL8 and polyclonal Living Colors; Takara Bio Inc.), rabbit anti-Plk4 KD (produced in laboratory), anti-V5 (Life Technologies), anti–α-tubulin DM1a and γ-tubulin GTU88 (Sigma-Aldrich), mouse and rabbit antiphospho–histone H3 (Millipore; Cell Signaling Technology), anti-FLAG M2 (Sigma-Aldrich), and FITC-conjugated anti–α-tubulin DM1a (Sigma-Aldrich). Secondary antibodies (conjugated with Cy2, Rhodamine red-X, or Cy5 [Jackson ImmunoResearch Laboratories, Inc.]) were used at manufacturer-recommended dilutions. Hoechst 33342 (Life Technologies) was used at a final dilution of 3.2 µM. Cells were mounted in 0.1 M *n*-propyl galate, 90% (by volume) glycerol, and 10% PBS solution. Specimens were imaged at room temperature using a DeltaVision Core system (Applied Precision) equipped with a microscope (IX71; Olympus), a 100× objective, NA 1.4, and a cooled charge-coupled device camera (CoolSNAP HQ^2; Photometrics). Images were acquired with softWoRx v1.2 software (DeltaVision). Statistical analyses of centriole and mitotic spindle counts were performed using two-tailed two-sample *t* tests and assuming equal variances.

Immunoblotting

S2 cell extracts were produced by lysing cells in PBS and 0.1% Triton X-100. The Bradford protein assay (Bio-Rad Laboratories; per manufacturer's instructions) was used to measure lysate protein concentrations. Laemmli sample buffer was then added, and samples were boiled for 5 min. The efficiency of RNAi was determined by Western blotting of treated cell lysates; equal total protein was loaded for each sample, and the integrated densities of chemiluminescent bands (measured with ImageJ [National Institutes of Health]) were normalized relative to the integrated densities of the loading control. Either endogenous α-tubulin or transfected Nlp-EGFP (a constitutively expressed nuclear protein; Rogers et al., 2009) was used as a loading control.

Antibodies

Escherichia coli–expressed GST- or maltose-binding protein–Slimb (amino acids 1–91) proteins were purified on either glutathione-Sepharose or amylose resin (Rogers et al., 2008, 2009). Guinea pig antisera were raised against GST-tagged purified Slimb protein, and the corresponding maltose-binding protein fusion was used for antibody affinity purification by precoupling to Affi-Gel 10/15 resin (Bio-Rad Laboratories). Rabbit anti-Plk4 polyclonal antibodies were raised against a peptide (KLPHERITLEAVLC) corresponding to the carboxy-terminus of the Plk4 KD. Antibodies were affinity purified from antisera using resin with coupled peptide. Additional antibodies used for Western blotting include polyclonal anti-Tws and anti-Mts (gift from T. Uemura, Kyoto University, Kyoto, Japan; Shiomi et al., 1994), monoclonal anti-Mts (Millipore), monoclonal anti-GFP JL8, and polyclonal anti-GFP Living Colors, and anti-FLAG used at 1:1,000 dilutions. HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) were prepared according to the manufacturer's instructions and used at 1:1,500 dilutions.

Constructs and transfection

A genomic region of 208 residues immediately upstream of the fly SAS-6 gene (called SAS-6p) was subcloned into the pMT vector (Life Technologies) to serve as a promoter of Plk4-GFP, constitutively promoting low level expression of this transgene, and was used for localization experiments (Rogers et al., 2009). cDNAs encoding SV40 ST (Addgene), Plk4 (Rogers et al., 2009), *Mts/CG7109* (EST database: *Drosophila* LD26077, accession no. AY058571), *Tws/CG6235* (EST database: *Drosophila* LD12394, accession no. AY061152), and human *Tws/PPP2R2B* (Open Biosystems) were subcloned into the inducible metallothionein promoter pMT vector and tagged with either FLAG, GFP, mCherry, or V5. Expression of all constructs was induced by addition of 0.5–2 mM copper sulfate to the media. Transient transfections were performed using the Nucleofector II (Lonza) according to manufacturer's instructions.

In vitro kinase and phosphatase assays

For in vitro kinase assays, *Drosophila* $(His)_6$-tagged Plk4 KD + DRE (amino acids 1–317) was cloned into the pET28a vector, expressed in BL21(DE3) *E. coli*, and purified on TALON resin (Takara Bio Inc.) according to the manufacturer's instructions. Kinase assays were conducted in reaction buffer (40 mM Na Hepes, pH 7.3, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.1 mM dithiothreitol, and 0.1 mM PMSF) and containing 10% (final concentration by volume) glycerol and 85 µM γ-[^{32}P]ATP at 25°C for 1–2 h. Some assays included 6 µg of the generic phosphorylation substrate and purified and dephosphorylated bovine brain myelin basic protein (Millipore). Reactions were terminated by the addition of Laemmli sample buffer and boiling. Reaction samples were resolved on SDS-PAGE, Coomassie stained, and then exposed to x-ray film (or a phosphorimaging screen [Molecular Dynamics]) to detect radiolabeled bands.

For in vitro phosphatase assays, purified *Drosophila* $(His)_6$-tagged Plk4 KD + DRE with or without the corresponding kinase-dead Plk4 mutant (a D156N point mutation; also bacterially expressed and purified on TALON resin) were first incubated with γ-[^{32}P]ATP to generate phosphorylated Plk4 (kinase-dead Plk4 was included in this assay because it incorporated more ^{32}P radiolabel than active Plk4). Specifically, 4.3 µM (final concentration) wt-Plk4, 4.7 µM kinase-dead Plk4, and 85 µM γ-[^{32}P]ATP were incubated in the conditions described in the previous paragraph. After 2 h at 25°C, Plk4 was bound to fresh TALON resin, and the resin was washed with reaction buffer to remove ATP. To assay the dephosphorylation of Plk4 by purified human PP2A (Millipore), resin-bound phosphorylated Plk4 was incubated with various concentrations of PP2A and OA (1 µM final concentration when present) in reaction buffer at 37°C for 1.5 h. Samples were resolved on SDS-PAGE, Coomassie stained, and then exposed to x-ray film to detect radiolabeled bands.

Flow cytometry

S2 cells (10^6) were pelleted at 1,000 *g* for 5 min, resuspended in 0.5 ml PBS, and vortexed while intermittently adding 0.5 ml of cold 100% ethanol. Fixed cells were incubated on ice for 20 min, pelleted (1,000 *g* for 5 min), and resuspended in a 0.5 ml propidium iodide (PI)-RNase solution (50 µg/ml PI + 100 µg/ml RNase Type1 I-A [QIAGEN] in PBS). After 20 min, cells were passed through a 12 × 75–mm flow cytometry tube (Falcon; Thermo Fisher Scientific). Cytometric analysis was performed in the Arizona Cancer Center/Arizona Research Laboratories Division of Biotechnology Cytometry Core Facility using a FACScan flow cytometer (BD) equipped with an air-cooled 15-mW argon ion laser tuned to 488 nm. List mode data files consisting of 10,000 cells gated on forward scatter versus side scatter were acquired and analyzed using CellQuest Pro software (BD). Appropriate electronic compensation was performed by acquiring cell populations stained with PI individually as well as an unstained control.

Centriole purification

Mitotic centrioles were purified as previously described by Mitchison and Kirschner (1986). In brief, a 400-ml S2 cell suspension culture was treated with 25 µM colchicine for 24 h, pelleted, and washed with the following buffers in succession: (a) PBS, (b) 0.1× PBS + 8% sucrose, (c) 8% sucrose, (d) lysate buffer (1 mM Tris-HCl, pH 8.0, and 8 mM 2-mercaptoethanol), and (e) lysate buffer + IGEPAL CA-630. The cell lysate was centrifuged for 3 min at 1,500 *g* at 4°C, and the supernatant was spun through a 2 ml Ficoll cushion (20% [wt/wt] Ficoll 400, 0.1% [wt/vol] IGEPAL CA-630, 1 mM EDTA, and 8 mM 2-mercaptoethanol, pH 8.0) at 26,000 *g* for 15 min at 4°C. 2 ml of the supernatant above the Ficoll cushion was removed, loaded onto a 30-ml 20–70% sucrose cushion (prepared in 0.1% Triton X-100, 1 mM EDTA, and 8 mM 2-mercaptoethanol, pH 8.0), and centrifuged for 1.5 h at 27,000 rpm in a SW28 rotor (Beckman Coulter) at 4°C. Gradients were fractionated into 0.5-ml fractions and boiled in Laemmli sample buffer for 5 min.

Online supplemental material

Fig. S1 is an illustration that shows our current understanding of the cell cycle–dependent regulation of Plk4. Fig. S2 shows that Plk4 protein levels in S2 cells are unaffected in an RNAi screen of the *Drosophila* kinome. Fig. S3 shows that Tws RNAi eliminates mitotic centrosomes in S2 cells but does not affect cell cycle progression. Table S1 lists the primer sequences that were used to generate dsRNA in this study. Table S2 shows a list of *Drosophila* kinases depleted in an RNAi screen and their effects on Plk4 protein levels. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201107086/DC1.

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