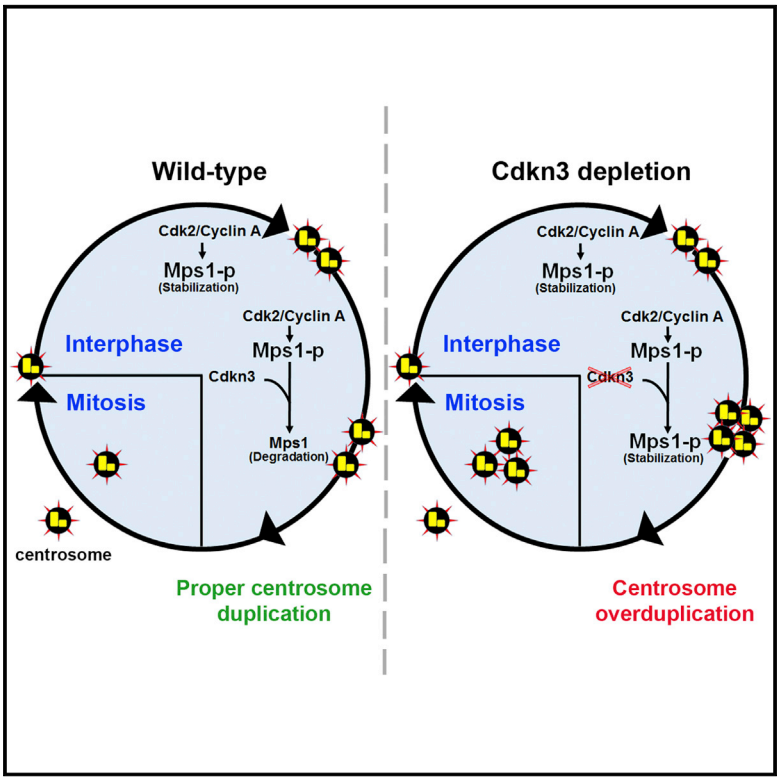
Cell Reports

The Tumor Suppressor Cdkn3 Is Required for Maintaining the Proper Number of Centrosomes by Regulating the Centrosomal Stability of Mps1

Graphical Abstract



Highlights

- Cdkn3 is a binding partner of Mps1, which recruits Cdkn3 to centrosomes
- Cdkn3 is required for maintaining the proper number of centrosomes
- Cdkn3 controls Mps1 stability via a self-regulated feedback loop at centrosomes
- This feedback loop prevents Mps1-mediated centrosome overduplication

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In Brief

Srinivas et al. identify Cdkn3, a Cdk-associated phosphatase, as a direct binding partner of Mps1 kinase. Mps1 recruits Cdkn3 to centrosomes and forms a self-regulatory feedback loop that tightly controls the centrosomal levels of Mps1 to maintain the proper number of centrosomes.

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The Tumor Suppressor Cdkn3 Is Required for Maintaining the Proper Number of Centrosomes by Regulating the Centrosomal Stability of Mps1

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SUMMARY

Supernumerary centrosomes promote the assembly of abnormal spindles in many human cancers. The observation that modest changes in the centrosomal levels of Mps1 kinase can cause centrosome overduplication in human cells suggests the existence of a regulatory system that may tightly control its centrosomal stability. Here, we show that Cdkn3, a Cdk-associated phosphatase, prevents Mps1-mediated centrosome overduplication. We identify Cdkn3 as a direct binding partner of Mps1. The interaction between Mps1 and Cdkn3 is required for Mps1 to recruit Cdkn3 to centrosomes. Subsequently, Mps1-bound Cdkn3 forms a regulatory system that controls the centrosomal levels of Mps1 through proteasome-mediated degradation and thereby prevents Mps1-mediated centrosome overduplication. Conversely, knockdown of Cdkn3 stabilizes Mps1 at centrosomes, which promotes centrosome overduplication. We suggest that Mps1 and Cdkn3 form a self-regulated feedback loop at centrosomes to tightly control the centrosomal levels of Mps1, which prevents centrosome overduplication in human cells.

INTRODUCTION

During the cell cycle, a single centrosome must be duplicated only once during S phase to generate the two centrosomes necessary to form a proper bipolar spindle. A causal association between centrosome overduplication and human cancer development has long been hypothesized (Boveri, 2008). Indeed, supernumerary centrosomes are frequently detected in human cancers, and centrosome abnormalities are considered a common feature of all major classes of human cancers (Godinho and Pellman, 2014).

Mps1 (monopolar spindle 1), first identified in the yeast *S. cerevisiae* (Winey et al., 1991), is an evolutionary conserved dual-specificity kinase that plays multiple roles during the cell cycle (Liu and Winey, 2012). Phenotypic analysis of the original yeast Mps1 mutants identified key functions of the Mps1 kinase in spindle pole body (SPB) duplication (Winey et al., 1991) and the spindle assembly checkpoint (SAC) (Weiss and Winey, 1996). Mps1 also regulates multiple steps during the SPB duplication process (Araki et al., 2010; Elserafy et al., 2014; Holinger et al., 2009; Schutz and Winey, 1998).

In mammalian cells, Mps1 has been most widely studied for its involvement in establishing bipolar spindle attachment and SAC at the kinetochores (Funabiki and Wynne, 2013; Liu and Winey, 2012). However, a requirement for Mps1 in centrosome duplication has been controversial, particularly in human cells depleted of Mps1 (Fisk et al., 2003; Pike and Fisk, 2011; Stucke et al., 2002). In contrast to a lack of Mps1, excess Mps1 levels at centrosomes influence centrosome duplication in mouse and human cells (Fisk et al., 2003; Fisk and Winey, 2001; Kasbek et al., 2007). First, Mps1 is localized not only to SPBs in yeast but also to centrosomes in mammalian cells (Dou et al., 2003; Fisk et al., 2003; Fisk and Winey, 2001; Tyler et al., 2009). Overexpression of mammalian Mps1 leads to centrosome overduplication, whereas overexpression of kinase-inactive Mps1 blocks centrosome duplication and maintenance (Dou et al., 2004; Fisk et al., 2003; Fisk and Winey, 2001; Kasbek et al., 2007). The centrosomal levels of Mps1 are exquisitely controlled by the Cdk2 phosphorylation of Mps1, which is required to prevent the proteasome-mediated degradation of Mps1 and is sufficient to promote centrosome duplication (Fisk and Winey, 2001; Kasbek et al., 2007). Collectively, these studies suggest the existence of a regulatory system that tightly controls the centrosomal levels of Mps1 to prevent centrosome overduplication in human cells.

Cyclin-dependent kinase inhibitor 3 (Cdkn3) is a dual-specificity phosphatase that dephosphorylates monomeric Cdk2 at Thr160 (Gyuris et al., 1993; Hannon et al., 1994). As the phosphorylation of Thr160 is necessary for full Cdk2 activity and cell-cycle progression, overexpressed Cdkn3 delays the G_1-S phase transition (Poon and Hunter, 1995). Notably, Cdkn3 has been found to be aberrantly expressed in many human carcinomas, and variant Cdkn3 mRNA transcripts that code for proteins lacking Cdk2-dephosphorylating activity have been identified (Yeh et al., 2000; Yu et al., 2007), suggesting a potential role for Cdnk3 in suppressing tumor progression. Recently, Cdkn3 was also found to be involved in SAC signaling and centrosome maintenance (Nalepa et al., 2013), although it is unclear whether the increased numbers of centrosomes in Cdkn3-depleted cells are a consequence of failed mitosis because of defects in SAC signaling or centrosome overduplication.

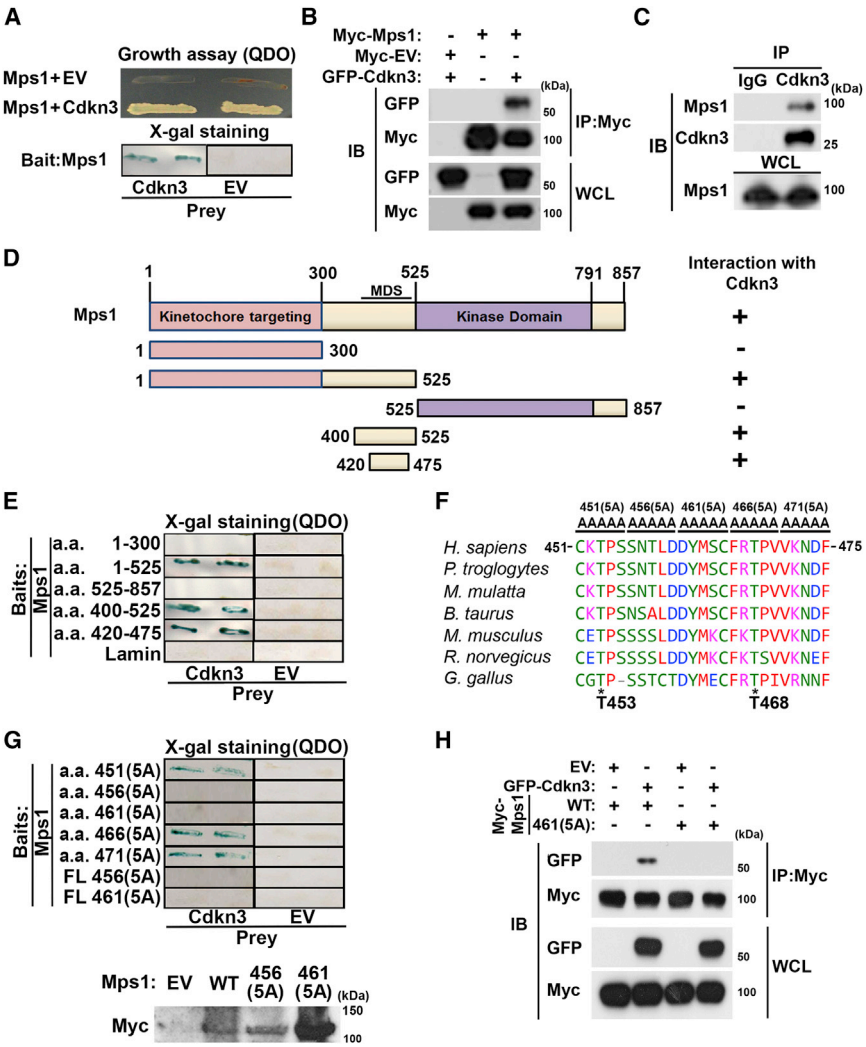

Figure 1. Cdkn3 Is a Direct Binding Partner of Mps1

(A) Isolation of Cdkn3 as an interacting partner of Mps1. Yeast two-hybrid assay is shown. The interaction between Cdkn3 and Mps1 was evaluated by colony growth (top) and X-gal staining (bottom) assays in quadruple-dropout media (QDO) (SD/-Leu/-Trp/-His/-Ura). Each yeast colony grown under permissive conditions on double-dropout plates (DDO) (SD/-Leu/-Trp) was streaked on the QDO plates. EV, empty vector.

(B) 293T cells transiently expressing Myc-Mps1 and GFP-Cdkn3 were subjected to immunoprecipitation (IP) with antibodies against Myc-epitope and immunoblot (IB) analysis. WCL, whole-cell lysates.

(C) Asynchronously growing MDA-MB-231 cells were subjected to immunoprecipitation analysis with antibodies against Cdkn3.

(D) Structural motifs of Mps1 with a summary of the Cdkn3:Mps1 interactions are shown.

(D and E) Yeast two-hybrid assay. The interaction between Cdkn3 and Mps1 was evaluated by colony growth and β-galactosidase assay. Each yeast colony grown on the DDO plates was streaked on the QDO plates.

(F) Alignment of the putative Cdkn3-binding sequence of Mps1 between different species with the changes to generate point mutants of Mps1 defective in binding Cdkn3 that were used for yeast two-hybrid assay.

(G) Each yeast colony grown on the QDO plates was re-streaked on the QDO plates for colony growth and β-galactosidase assay (top panels). The expression levels of the indicated Myc-tagged Mps1 baits in yeast grown in DDO were determined by immunoblot analysis (bottom panels).

(H) Lysates from 293T cells transiently expressing Myc-Mps1 and GFP-Cdkn3 were subjected to immunoprecipitation analysis with antibody against the Myc-epitope.

Here, we show that Cdkn3 is essential for preventing Mps1-mediated centrosome overduplication in human cells. We have isolated Cdkn3 as a direct binding partner of Mps1 using yeast two-hybrid screening and identified critical amino acid residues in Mps1 that are responsible for the Cdkn3:Mps1 interaction. Using siRNA-mediated depletion-reconstitution, we demonstrate that the Cdkn3:Mps1 interaction is required for maintaining the proper number of centrosomes, as failure to recruit Cdkn3 to Mps1 at centrosomes contributes to centrosome overduplication. Importantly, we show that Mps1 recruits Cdkn3 to centrosomes and forms a regulatory system that controls its own centrosomal levels in a proteasome-dependent manner. This self-regulatory feedback loop tightly controls the centrosomal levels of Mps1 to maintain the proper number of centrosomes.

RESULTS

Identification of Cdkn3 as a Binding Partner of Mps1

To better understand Mps1 function in mitotic progression and centrosome maintenance, we searched for protein partners that directly bind Mps1 using the yeast two-hybrid method with full-length Mps1 as bait. Screening of a HeLa cDNA library identified multiple colonies containing cDNA of full-length Cdkn3 (aas 1–212). The interaction between Mps1 and Cdkn3 was verified in a directed two hybrid for growth on medium lacking histidine as well as β-galactosidase assay (Figure 1A). Consistent with this finding, transiently expressed Myc-tagged Mps1 co-immunoprecipitated with GFP-tagged Cdkn3 (Figure 1B). Furthermore, endogenous Mps1 also co-immunoprecipitated with endogenous Cdkn3 (Figure 1C), indicating that Cdkn3 is a binding partner of Mps1. Using the two-hybrid interaction, we performed a fine mapping of the Cdkn3-binding site on Mps1. Notably, the Mps1 domain (aas 400–525) containing a Cdk2 phosphorylation-dependent Mps1 degradation signal (MDS), which controls the stability of Mps1 at centrosomes (Kasbek et al., 2007), was both necessary and sufficient for Cdkn3 binding (Figures 1D and 1E). Further deletion analysis of Mps1 revealed that a small motif of Mps1 (aas 420–475) was sufficient for the interaction with Cdkn3 (Figures 1D and 1E), suggesting that this small amino acid stretch is essential for Cdkn3 binding. Next, based

on sequence conservation among species (Figure 1F), a series of alanine-scanning point mutations were introduced in this minimal motif (aas 420–475). Mps1 point mutants in which residues 456–460 or 461–465 (Figure 1F) were replaced with alanine (SNTLD/AAAAA, 456(5A) or DYMSC/AAAAA, 461(5A)), but not surrounding amino acid residues, failed to bind Cdkn3 (Figure 1G, top panels). Similarly, full-length Mps1 mutants containing these alanine substitutions also failed to bind Cdkn3 (Figure 1G, top panels), although these mutants were properly expressed in yeast (Figure 1G, bottom panel). Furthermore, when Myc-Mps1 and GFP-Cdkn3 were transiently co-expressed in 293T cells and subjected to immunoprecipitation analysis using anti-Myc antibodies, wild-type Myc-Mps1 ($Mps1^{WT}$), but not 461(5A) mutant ($Mps1^{461(5A)}$), was co-immunoprecipitated together with GFP-Cdkn3 (Figure 1H), suggesting that amino acid residues 456–465 in Mps1 are essential for binding Cdkn3.

Cdkn3 Is Required for Maintaining the Proper Number of Centrosomes, but Not SAC Signaling

Cdkn3 is a dual-specificity phosphatase that functions as an interphase Cdk inhibitor (Gyuris et al., 1993; Hannon et al., 1994). Interestingly, recent evidence indicates the requirement for Cdkn3 in promoting the SAC and centrosome maintenance, as siRNA-mediated depletion of Cdkn3 causes SAC silencing and supernumerary centrosome formation in multinucleated HeLa cells (Nalepa et al., 2013), which could be attributed to the recognized function of Mps1. Thus, we speculated that Mps1 and Cdkn3 may function as a complex in promoting the SAC. To test this, we first determined the mRNA abundance of Cdkn3 in various cell lines by real-time PCR (qPCR) analysis (Figure S1A). Cdkn3 was highly expressed in asynchronously growing MDA-MB-231, DU145, and AGS cells. MDA-MB-231 cells were transfected with four different siRNAs to deplete endogenous Cdkn3 (Figure S1B). As determined by qPCR analysis and immunoblot analysis (Figures S1B and S1C), each siRNA transfection efficiently caused a similar decrease in Cdkn3 mRNA and protein levels. Notably, when challenged with the microtubule depolymerizer nocodazole for 20 hr, MDA-MB-231 cells depleted of Cdkn3 were arrested in mitosis with a similar mitotic index to that seen in MDA-MB-231 cells transfected with non-silencing control siRNA (Figure S1D). Under this condition, MDA-MB-231 cells depleted of an essential SAC component, Mps1 or Mad2, markedly decreased the mitotic index, as expected (Figure S1D). Similarly, DU-145 and HeLa cells depleted of Cdkn3 showed an increased mitotic index and mitotic rounded-up cells comparable to controls after treatment with nocodazole (Figures S1D and S1E). Together, these results suggest that Cdkn3 is unlikely to be required to promote SAC signaling, although we cannot rule out the possibility that even a small amount of Cdkn3 might be sufficient for SAC.

In contrast, knockdown of Cdkn3 significantly increased the number of cells with greater than or equal to three centrosomes in the MDA-MD-231, DU-145, and HeLa cell lines, as determined by immunofluorescence analysis using anti-γ-tubulin staining (Figure 2A). Notably, the centrosomes in these Cdkn3-depleted cells were present in close proximity without any measurable induction of multinucleated cells (Figures S2A and S2B) similar to that seen in Plk4-overexpressed cells (Figures S2C and S2D; Habedanck et al., 2005), suggesting a potential role for Cdkn3 in maintaining the proper number of centrosomes rather than in the SAC. To further rule out contributions from errors in mitosis and/or cytokinesis, Cdkn3-depleted HeLa and DU-145 cells were arrested in S phase using hydroxyurea (HU), which permits ongoing centrosome duplication in the absence of DNA replication or cytokinesis (Meraldi et al., 1999). Importantly, Cdkn3 depletion caused a significant increase in the number of cells with supernumerary centrosomes and centrioles, as determined by anti-γ-tubulin and anti-CP110 (a centriole marker) staining (Figures 2B and 2C). The effect of Cdkn3 siRNA on centrosome overduplication was specific to the depletion of Cdkn3 because transiently expressed GFP-Cdkn3 significantly reduced the number of cells with greater than or equal to three centrosomes (Figure 2D). Furthermore, as determined by immunofluorescence analysis using antibodies against Cdkn3 and the centrosome marker γ-tubulin, endogenous Cdkn3 localized at centrosomes, whereas siRNA-mediated depletion of Cdkn3 abolished the centrosome localization of Cdkn3 and increased centrosome number (Figure 2E). Similarly, transiently expressed mCherry-tagged Cdkn3 also localized to centrosomes (Figure 2F), excluding the possibility of non-specific immunofluorescence staining. Together, our data suggest that Cdkn3 is a centrosomal protein that plays an essential role in maintaining the proper number of centrosomes. Therefore, we hypothesized that the Cdkn3:Mps1 interaction may occur at centrosomes and play a key role in maintaining the proper number of centrosomes.

The Cdkn3:Mps1 Interaction Is Required for Recruiting Cdkn3 to Centrosomes

Centrosome reduplication requires Cdk2/cyclin A (Balczon, 2001; Meraldi et al., 1999). As Cdkn3 directly binds and inhibits Cdk2 activity (Gyuris et al., 1993; Hannon et al., 1994), it was difficult to evaluate the importance of the Cdkn3:Mps1 interaction in centrosome maintenance by siRNA-mediated depletion of Cdkn3 alone. Therefore, using the Cdkn3-binding-deficient Mps1 point mutants, we determined the importance of the Cdkn3:Mps1 interaction in the centrosomal localization of Cdkn3. To address this, endogenous Mps1 in MDA-MB-231 cells was depleted and replaced with an exclusively centrosomal-localized version of Mps1 by inserting the PACT domain at the C terminus of GFP-Mps1 (GFP-Mps1-PACT) (Kasbek et al., 2007). These cells were subsequently treated with the proteasome inhibitor MG132 to exclude the potential complication of Mps1 degradation at the centrosome (Kasbek et al., 2007). The centrosomal levels of endogenous Cdkn3 relative to the centrosomal marker γ-tubulin in cells expressing either wild-type (GFP-$Mps1^{WT}$-PACT) or mutant (GFP-$Mps1^{461(5A)}$-PACT) defective in Cdkn3 binding were quantified. As expected, GFP-$Mps1^{WT}$-PACT exclusively localized to centrosomes (Figure 3A). Endogenous Cdkn3 also well co-localized with GFP-$Mps1^{WT}$-PACT at centrosomes (Figure 3A). Similar to GFP-$Mps1^{WT}$-PACT, GFP-$Mps1^{461(5A)}$-PACT also exclusively localized at centrosomes (Figure 3A). However, the centrosomal levels of endogenous Cdkn3 were significantly lower than those in cells expressing GFP-$Mps1^{WT}$-PACT, although some residual centrosomal localization of Cdkn3 was still detected (Figures 3A and 3B), which may possibly occur through its interaction with Cdk2

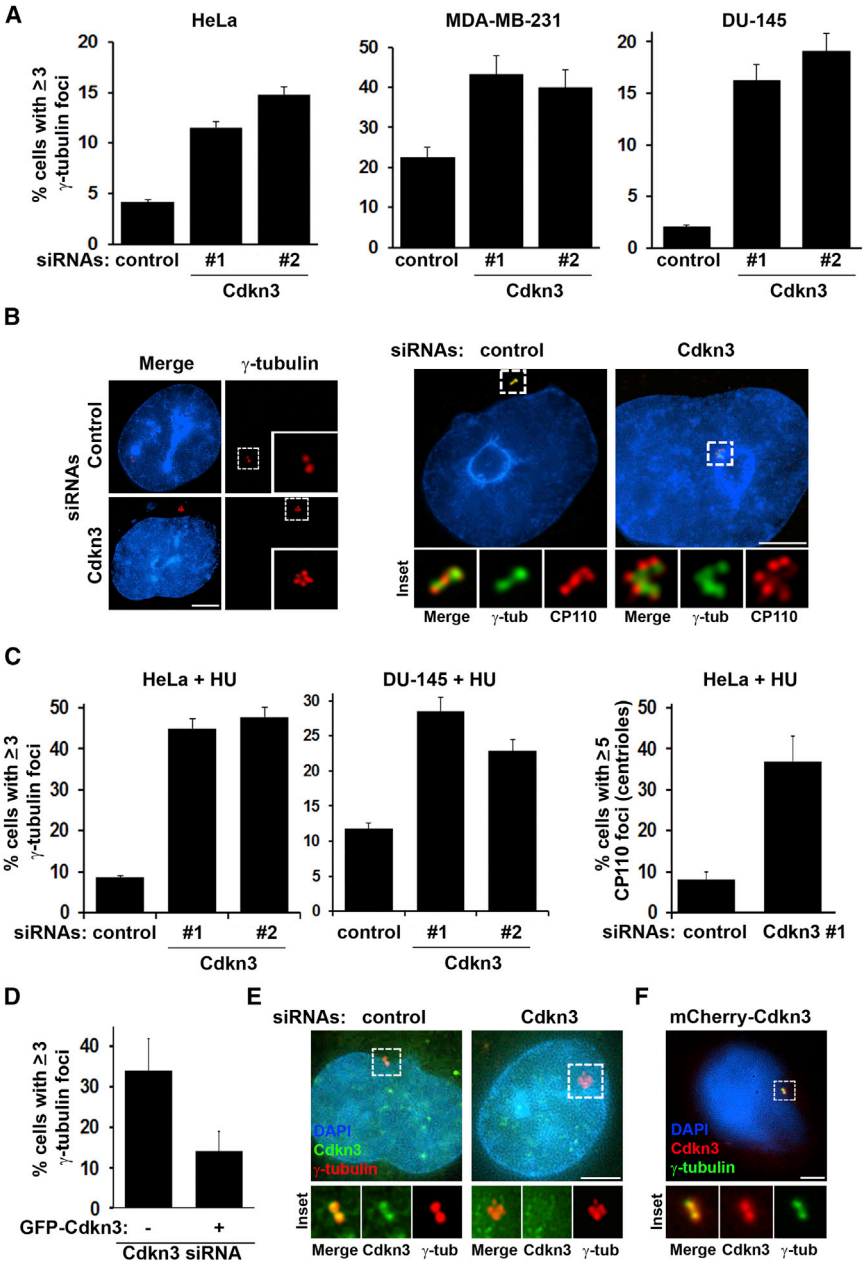

Figure 2. Cdkn3 Is Required for Maintaining the Proper Number of Centrosomes

(A–C) Asynchronously growing cells from the indicated lines were transfected with non-silencing or siRNA against Cdkn3 two times with a 24-hr interval. At 48 hr after transfection, the cells were subjected to immunofluorescence analysis using antibodies against γ-tubulin and DAPI. For (B) and (C), the indicated cell lines were treated with hydroxyurea (HU) for 48 hr before fixation. Quantification results of the numbers of γ-tubulin or CP110-positive foci are shown (n > 40 cells per condition from two independent experiments; ±SD). (B) Representative image of DU-145 cells from (C) is shown.

(D) Quantification of the numbers of γ-tubulin foci in GFP-positive HeLa cells (n > 40 cells per condition). The Cdkn3 siRNA was transfected as above, except 10 ng of plasmid encoding GFP-tagged Cdkn3 was included in the second transfection.

(E) Cdkn3 localizes to centrosomes. DU-145 cells were prepared as in (B) and subjected to immunofluorescence analysis using antibodies against Cdkn3 and γ-tubulin.

(F) MDA-MB-231 cells transiently expressing mCherry-Cdkn3 were subjected to immunofluorescence analysis. Inset represents the boxed area.

White bars represent 5 μm. See also Figures S1 and S2.

or residual endogenous Mps1. Nevertheless, these results suggest that the Cdkn3:Mps1 interaction promotes Cdkn3 recruitment to the centrosome.

Cdkn3 Destabilizes Mps1 at Centrosomes

Notably, siRNA-mediated depletion of Cdkn3 significantly increased the centrosomal levels of endogenous Mps1 in MDA-MB-231 cells, as determined by immunofluorescence analysis (Figures 3C and 3D). The centrosomal levels of Mps1 are regulated by proteasome-mediated degradation (Kasbek et al., 2007). Indeed, MG132 treatment increased the centrosomal levels of Mps1 in MDA-MB-231 cells (Figure 3D). In contrast, the increased centrosomal levels of endogenous Mps1 in MDA-MB-231 cells depleted of Cdkn3 were not significantly further augmented by MG132 treatment (Figure 3D), which suggests that Cdkn3 controls the centrosomal levels of Mps1. Conversely, enforcing the centrosomal localization of Cdkn3 by inserting a PACT domain (Gillingham and Munro, 2000) at the C terminus of GFP-tagged Cdkn3 (GFP-Cdkn3-PACT) significantly decreased the centrosomal levels of endogenous Mps1 in both MDA-MB-231 and HeLa cells (Figures 3E and 3F). This decrease was due to the proteasome-mediated degradation of Mps1 at centrosomes, as MG132 treatment restored the centrosomal levels of Mps1 in MDA-MB-231 cells expressing GFP-Cdkn3-PACT (Figure 3E). Together, our data suggest that Cdkn3 negatively controls the centrosomal levels of Mps1 by either directly or indirectly promoting the proteasome-mediated degradation of Mps1 at centrosomes.

The Cdkn3:Mps1 Interaction Is Required for Destabilizing Mps1 at Centrosomes

The MDS motif within Mps1 (aa 420–507) is responsible for destabilizing Mps1 at the centrosome, whereas Cdk2 phosphorylation within this MDS motif, especially at Thr468 residue (Figure 1F), promotes the centrosomal stability of Mps1 (Kasbek et al., 2007). Given that the MDS motif nearly overlaps with the

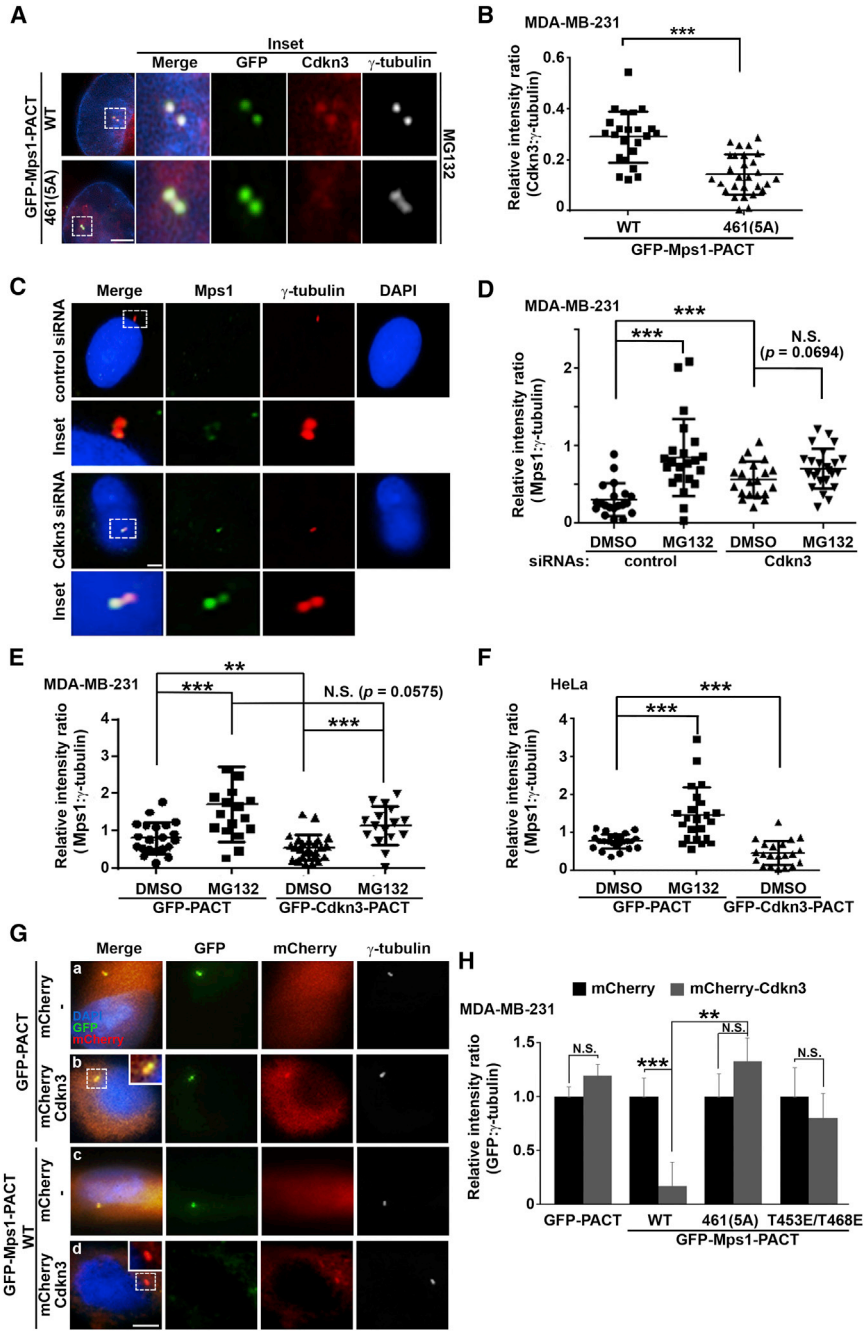

Figure 3. The Cdkn3:Mps1 Interaction Is Required for Controlling the Centrosomal Stability of Msp1

(A and B) MDA-MB-231 cells were transiently transfected with an expression vector encoding the indicated siRNA-resistant GFP-Mps1-PACT together with siRNA against endogenous Mps1. At 48 hr after transfection, cells were treated with MG-132 (20 μM) for 4 hr and subjected to immunofluorescence analysis using antibodies against Cdkn3 and γ-tubulin. Inset represents the boxed area. (B) Quantification results of the centrosomal levels of Cdkn3 relative to γ-tubulin are shown (n > 30 cells per condition; ±SD).

(C and D) MDA-MB-231 cells were transfected with either control or Cdkn3 siRNAs for 48 hr and subjected to immunofluorescence analysis using antibodies against Mps1 and γ-tubulin.

(E and F) MDA-MB-231 (E) or HeLa (F) cells were transiently transfected with an expression vector encoding GFP-PACT or GFP-Cdkn3-PACT. At 48 hr after transfection, cells were subjected to immunofluorescence analysis using antibodies against Mps1 and γ-tubulin. (D–F) Quantification results of the centrosomal levels of Mps1 relative to γ-tubulin are shown (n > 20 cells per condition; ±SD).

(G) MDA-MB-231 cells were transiently transfected with expression vectors encoding GFP-PACT or the indicated GFP-Mps1-PACT together with mCherry or mCherry-Cdkn3. At 24 hr after transfection, cells were subjected to immunofluorescence analysis using antibodies against γ-tubulin to visualize centrosome.

(H) Quantification results of the centrosomal levels of GFP relative to γ-tubulin are shown (n > 30 cells per condition; ±SD).

Student's t test was performed to determine the statistical significance (**p < 0.01; ***p < 0.001; N.S., not significant). Where indicated, cells were treated with MG-132 (20 μM) for 4 hr before fixation. Inset represents the boxed area. White bars represent 5 μm. See also Figure S3.

Mps1 binding site of Cdkn3 (Figures 1D–1G), we wished to determine whether the Cdkn3:Mps1 interaction controls the stability of Mps1 at the centrosome using the point mutants of Mps1 defective in binding Cdkn3 (Figures 1G and 1H). To address this, GFP-Mps1-PACT or GFP-PACT was transiently expressed in MDA-MB-231 cells together with either mCherry-tagged Cdkn3 or mCherry alone. The centrosomal levels of GFP-Mps1-PACT or GFP-PACT relative to the centrosomal marker γ-tubulin were then quantified. As expected, GFP-PACT and GFP-Mps1-PACT exclusively localized to centrosomes in cells co-expressing mCherry (Figure 3G). Overexpressed mCherry-Cdkn3 was also found in centrosomes, but the centrosomal levels of GFP-PACT relative to γ-tubulin were not changed (Figures 3Ga, 3Gb, and 3H). In contrast, the relative centrosomal levels of GFP-Mps1^{WT}-PACT were markedly decreased in cells co-expressing mCherry-Cdkn3 compared with mCherry (Figures 3Gc, 3Gd, and 3H). These results are consistent with the decrease in the centrosomal levels of endogenous Mps1 caused by targeting Cdkn3 to centrosomes (Figures 3E and 3F). Importantly, the relative centrosomal levels of the GFP-Mps1$^{461(5A)}$-PACT mutant defective in binding Cdkn3 were not measurably affected by co-expressing mCherry-Cdkn3 (Figure 3H), indicating the requirement of the Cdkn3:Mps1 interaction to inhibit the centrosomal stability of Mps1. Furthermore, the Cdkn3:Mps1 interaction may destabilize Mps1 at centrosomes by altering

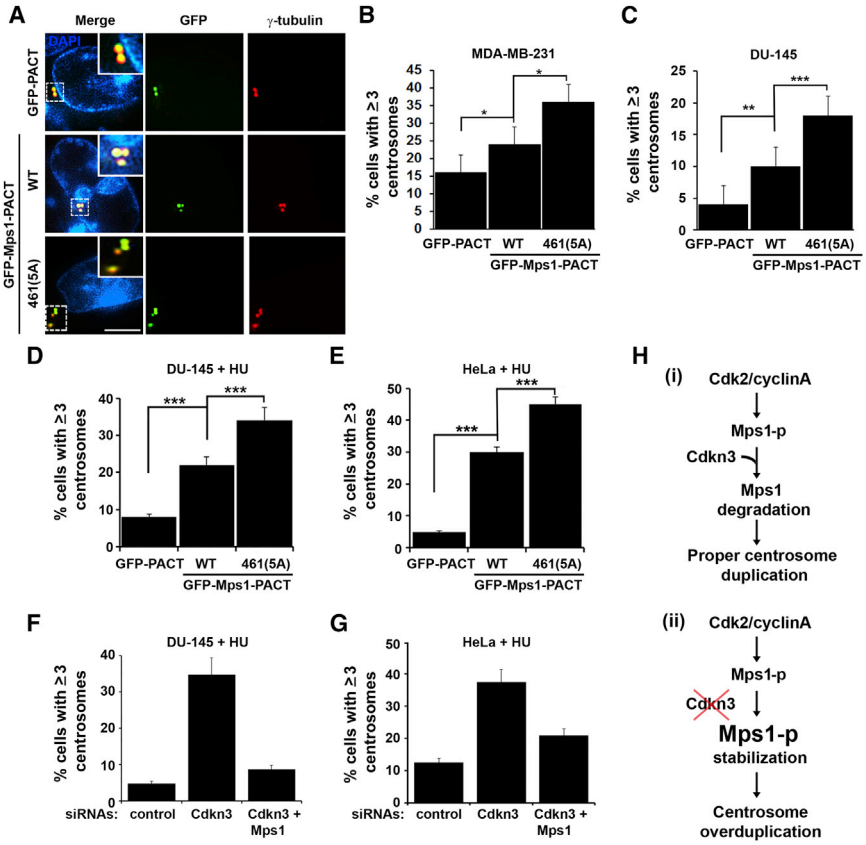

Figure 4. The Cdkn3:Mps1 Interaction Is Required for Maintaining the Proper Number of Centrosomes

(A–E) The indicated cell lines were transiently transfected with an expression vector encoding GFP-PACT fused with either wild-type or mutant Mps1 defective in binding Cdkn3. At 48 hr after transfection, cells were subjected to immunofluorescence analysis using antibodies against γ-tubulin. Inset represents the boxed area, and the white bar represents 5 μm (A). (B–E) Quantification of centrosome number is shown (n > 30 cells expressing GFP-Mps1-PACT per condition; ±SD). For (D) and (E), cells were further treated with HU for 24 hr before fixation. Student's t test was performed to determine the statistical significance (*p < 0.05; **p < 0.01; ***p < 0.001).

(F and G) Using the indicated siRNAs (100 nM total), asynchronously growing DU-145 or HeLa cells were transfected twice with a 24-hr interval. After the second transfection, cells were treated with HU for 48 hr and subjected to immunofluorescence analysis using antibodies against γ-tubulin and DAPI (n > 40 cells per condition; ±SD).

(H) Schematic model. Cdk2 phosphorylation of Mps1 (Mps1-p) promotes the stabilization of Mps1-p at the centrosome. In turn, Mps1-p recruits Cdkn3 to the centrosome, which promotes the proteasome-mediated degradation of Mps1, possibly via the dephosphorylation of Mps1-p in a direct or indirect manner. This self-regulated feedback loop at centrosomes tightly controls the centrosomal levels of Mps1 for proper centrosome duplication (i). Conversely, in the absence of Cdkn3, Mps1-p accumulates at centrosomes and leads to centrosome overduplication (ii).

See also Figure S4.

the Cdk2-dependent phosphorylation of the MDS motif in Mps1 because the relative centrosomal levels of the GFP-Mps1$^{T453E/T468E}$-PACT mutant that mimics Cdk2 phosphorylation (Kasbek et al., 2007) were not significantly changed by co-expressing mCherry-Cdkn3 (Figures 3H and S3A). Of note, the difference in the centrosomal levels of Mps1 was not due to the transfection efficiency because each of the Mps1 constructs was comparably expressed, as determined by immunoblot analysis (Figure S3B). Furthermore, this inability of Cdkn3 to destabilize Mps1$^{T453E/T468E}$ at centrosomes was not due to defects in the Cdkn3:Mps1 interaction, as determined using quantitative β-galactosidase as well as immunoprecipitation analysis, whereas non-phosphorylatable Mps1$^{T453A/T468A}$ was less able to bind Cdkn3 than Mps1$^{T453E/T468E}$ (Figures S3C and S3D). Moreover, preventing Cdk2 phosphorylation was sufficient to promote Mps1 degradation at centrosomes in the absence of Cdkn3 binding because the relative centrosomal levels of the compound mutant defective for both Cdkn3 binding and Cdk2 phosphorylation (Mps1$^{461(5A)-T453A/T468A}$) were markedly lower than Mps1$^{461(5A)}$ but were restored by MG132 treatment (Figure S3E). Collectively, our data suggest that the Cdkn3:Mps1 interaction is required to destabilize Mps1 at centrosomes and that Cdk2 phosphorylation of Mps1 may create a self-regulated feedback loop that controls the centrosomal levels of phosphorylated Mps1 by recruiting Cdkn3 to centrosomes.

The Cdkn3:Mps1 Interaction Is Required for Maintaining the Proper Number of Centrosomes

Using the Mps1 point mutants defective in binding Cdkn3, we further determined the importance of the Cdkn3:Mps1 interaction in the maintenance of centrosome number in MDA-MD-231 and DU-145 cells. As shown previously, overexpressed GFP-Mps1^{WT}-PACT marginally but measurably increased the number of cells possessing greater than or equal to three centrosomes compared to the negative control, GFP-PACT alone (Figures 4A–4C; Kasbek et al., 2007). Importantly, the Cdkn3:Mps1 interaction was required for the maintenance of centrosome numbers because Mps1 point mutants defective in binding Cdkn3 (GFP-Mps1$^{461(5A)}$-PACT) further increased the number of cells possessing greater than or equal to three centrosomes (Figures 4B and 4C). To further confirm this, GFP-Mps1-PACT was transiently expressed in DU-145 and HeLa cells, which were subsequently arrested in S phase using HU. We noticed that overexpressed GFP-Mps1^{WT}-PACT significantly increased the number of cells possessing greater than or equal to three centrosomes (Figures 4D and 4E). Importantly, under this condition, overexpressed GFP-Mps1$^{461(5A)}$-PACT further increased the number of cells possessing greater than or equal to three centrosomes (Figures 4D and 4E), suggesting that the Cdkn3:Mps1 interaction is required for maintaining the proper number of centrosomes.

Finally, we determined whether stabilized Mps1 at centrosomes in cells depleted of Cdkn3 is required for centrosome overduplication. To test this, endogenous Mps1 and Cdkn3 were simultaneously depleted in either DU-145 or HeLa cells using individual siRNAs. Then, the cells were subsequently arrested in S phase with HU for 48 hr. Whereas Cdkn3 depletion caused a significant increase in the number of cells with supernumerary centrosomes, simultaneous deletion of Mps1 and Cdkn3 significantly suppressed the number of cells with supernumerary centrosomes in both cell lines (Figures 4F and 4G). Of note, this suppression of centrosome overduplication was not simply due to an inefficient depletion of Cdkn3, as determined by qPCR analysis (Figure S4A). Furthermore, transient expression of GFP-tagged wild-type Mps1, but not a kinase-dead mutant, significantly increased the number of cells possessing greater than or equal to three centrosomes only in the absence of Cdkn3 (Figure S4B), indicating the role of Cdkn3 in preventing Mps1-induced centrosome overduplication. Taken together, our results suggest that the Cdkn3:Mps1 interaction is required for maintaining the proper number of centrosomes by regulating the stability of Mps1 at centrosomes (Figure 4H).

DISCUSSION

Excess Mps1 levels at centrosomes or the expression of a nondegradable form of Mps1 promotes centrosome overduplication in mouse and human cell lines (Cui et al., 2010; Fisk and Winey, 2001; Kasbek et al., 2007), suggesting the existence of a regulatory system, whose identity has remained elusive, that tightly controls the centrosomal levels of Mps1. Our current study reveals that Cdkn3 plays a key role in controlling the centrosomal stability of Mps1 and in maintaining the proper number of centrosomes based on the following results: (1) MDA-MB-231, DU-145, and HeLa cells depleted of Cdkn3 exhibited centrosome overduplication; (2) Cdkn3 depletion increased the centrosomal levels of Mps1, whereas Cdkn3 overexpression decreased the Mps1 levels in a proteasome-dependent manner; (3) the centrosomal levels of Mps1 mutants defective in binding Cdkn3 were unaffected by overexpression of Cdkn3; (4) these Mps1 mutants caused centrosome overduplication; and (5) centrosome overduplication in cells depleted of Cdkn3 was suppressed by simultaneously depleting Mps1. As the Cdkn3:Mps1 interaction promotes Cdkn3 recruitment to centrosomes (Figures 3A and 3B), our data suggest that the Cdkn3:Mps1 interaction at centrosomes may create a situation where Mps1 is recognized by the factors (Kasbek et al., 2010; Liu et al., 2013) that drives its own degradation at centrosomes, creating a self-regulatory feedback loop for proper centrosome duplication. Of note, it has been suggested that the centrosomal levels of Mps1 are exquisitely controlled by Cdk2 phosphorylation within the MDS motif of Mps1, separately from other pools of Mps1 (Fisk and Winey, 2001; Kasbek et al., 2007). Similarly, either knockdown or overexpression of Cdkn3 specifically affected the centrosomal (Figure 3), but not the overall protein, levels of Mps1 (Figures S1C and S3D). Furthermore, unlike wild-type Mps1, the centrosomal levels of the phosphomimetic $Mps1^{T453E/T468E}$ mutant were not affected by overexpression of Cdkn3 (Figure 3H) despite its ability to bind Cdkn3 (Figures S3C and S3D), suggesting that reversing the Cdk2 phosphorylation of Mps1 is a prerequisite step for Cdkn3 to promote Mps1 degradation at centrosomes. Future research efforts will be required to test whether Cdkn3 is the phosphatase responsible for directly reversing Cdk2 phosphorylation of the MDS motif in Mps1 at centrosomes.

Cdkn3 binds both monomeric Cdk2 and cyclin-A-bound Cdk2 (Gyuris et al., 1993; Hannon et al., 1994; Poon and Hunter, 1995). However, Cdkn3 only dephosphorylates Thr160 of monomeric Cdk2 and does not block the kinase activity of cyclin-A-bound Cdk2 (Poon and Hunter, 1995). Thus, upon degradation or dissociation of cyclin A from Cdk2 at centrosomes, Cdkn3 may promote the dephosphorylation of Cdk2 as well as Mps1, and Cdkn3 thereby simultaneously counters Cdk2 activity and prolonged Mps1 stabilization at centrosomes to prevent centrosome overduplication. It is also possible that increasing Cdk2 activity at centrosomes in cells depleted of Cdkn3 may promote Cdk2 phosphorylation of Mps1, which in turn further stabilizes Mps1 at centrosomes, as seen in MBA-MD-231 cells (Figure 3C). Nonetheless, an Mps1 mutant defective in binding Cdkn3 was stabilized at centrosomes independent of Cdkn3 (Figure 3H) and caused centrosome overduplication (Figure 4). These results provide direct evidence that the Cdkn3:Mps1 interaction plays a key role in maintaining the proper number of centrosomes by suppressing the stability of Mps1 at centrosomes. Therefore, whereas Cdk2 phosphorylation promotes the stabilization of Mps1 at the centrosome, we propose that Cdk2 phosphorylation of Mps1 recruits Cdkn3 to the centrosome to promote the proteasome-mediated degradation of Mps1 possibly through dephosphorylating Mps1 in a direct or indirect manner. This self-regulated feedback loop at centrosomes tightly controls the centrosomal levels of Mps1, which prevents centrosome overduplication (Figure 4H). Furthermore, by tying Cdkn3 to Mps1, this system may be poised to extinguish the centrosomal Mps1 signal as soon as Cdk2 activity drops (e.g., cyclin A degradation), yet it can be re-engaged upon the reactivation of Cdk2 during S phase in the next cycle.

Cdkn3 has been found to be mutated or aberrantly expressed in many human tumors, and variant Cdkn3 mRNA transcripts that code for proteins lacking Cdk2-dephosphorylating activity have been identified (Yeh et al., 2000; Yu et al., 2007). Thus, in the future, it will be interesting to determine the extent to which the dysregulated centrosomal levels and/or phosphatase activity of Cdkn3 found in human tumors may affect the stability of Mps1 at centrosomes that may contribute to centrosome overduplication, multipolar chromosome missegregation, and tumor progression.

EXPERIMENTAL PROCEDURES

More detailed methods are described in the Supplemental Experimental Procedures.

Yeast Two Hybridization

Full-length and fragments of human Mps1 (Kang et al., 2007) were PCR cloned into the pGBKT7 vector (Clontech) as bait. The two-hybrid screen was performed according to the manufacturer's instructions using human cDNA library in pGADT7 prey vectors (Matchmaker Gold Yeast Two-Hybrid System; Clontech), and the interaction was evaluated by colony growth on

quadruple-dropout plates lacking Trp, Leu, His, and Ura (QDO), as well as via β-galactosidase assay for validation.

Immunoblotting and Immunoprecipitation Analysis

Cells were lysed with either passive lysis buffer (Roche) or 1% NP-40 lysis buffer (25 mM Tris-HCl [pH 7.4] and 150 mM NaCl) supplemented with complete mini (Roche) and phosphatase inhibitor cocktails (Sigma). For immunoprecipitation analysis, total cell lysates were incubated with 1 μg of anti-Myc antibody (Sc-40; Santa Cruz Biotechnology) at 4°C overnight. Protein A/G plus agarose beads (Santa Cruz) were added and incubated at 4°C for 1 hr. After incubation, the beads were washed three times with 1% NP-40 lysis buffer and subjected to immunoblot analysis.

Microscopy Analysis and Quantification

Images were acquired at RT with 3D-SIM using a Super Resolution Microscope (Nikon) equipped with an iXon EM+ 885 EMCCD camera (Andor) mounted on a Nikon Eclipse Ti-E inverted microscope with a CFI Apo TIRF (100 ×/1.40 oil) objective and processed with the NIS-Elements AR software. Centrosome numbers were quantified using 3D projection of the images in NIS-Elements AR software followed by manual counting of centrosomes in individual cells. For centrosomal fluorescence intensity measurements, a background correction method was used.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.039.

AUTHOR CONTRIBUTIONS

V.S., M.K., J.W., and P.L. conducted the experiments. S.H.L. designed the experiments and wrote the paper.

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