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Mitotic kinases regulate MT-polymerizing/MT-bundling activity of DDA3

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Abstract

Mitotic kinases orchestrate cell cycle processes by phosphorylation of cell cycle regulators. DDA3, a spindle-associated phosphor-protein, is a substrate of mitotic kinases that control chromosome movement and spindle microtubule (MT) dynamics. Through a mass spectrometry analysis, we identified phosphorylation sites on the endogenous mitotic DDA3, which include Ser22, Ser65, Ser70, and Ser223. Phosphorylation of these residues converts interphase form of DDA3 to mitotic form by changing its biochemical activity, as unphosphorylated DDA3 processed both the MT polymerizing and bundling activities, whereas phosphor-mimic mutants lost both activities, only retaining the MT-binding activity. We found that mitotic kinases, such as Cdk1, Aurora A, and Plk1, phosphorylate DDA3 *in vitro*. Whereas Cdk1 and Aurora A negatively regulate MT-polymerizing and MT-bundling activities, Plk1 does not affect these activities. Interestingly, the phosphorylation of DDA3 by Aurora A and Plk1 inhibits the phosphorylation by other kinases, indicating that sequential phosphorylation is important for the regulation of DDA3 function. We conclude that kinases control the function of DDA3 in the cell cycle by regulating its MT-polymerizing/bundling activities through sequential phosphorylation.

Keywords

DDA3; Mitotic kinase; phosphorylation; MT-polymerizing activity; MT-bundling activity; Mitosis

1. INTRODUCTION

Microtubules (MTs) constitute cytoskeleton structure that maintains cell shape and mediates cell migration in interphase. MTs assemble into a spindle structure in mitosis, which generates force for chromosome congression and segregation through polymerization/

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depolymerization of MTs [1]. The dynamic turnover of the MTs in mitosis is controlled by MT-associated proteins, such as DDA3 [2], and by MT depolymerases, such as Kif2a [3,4,5,6,7]. The biochemical activity and cellular localization of MT-associated proteins and MT depolymerases are regulated by mitotic kinases, such as cyclin-dependent kinase 1 (Cdk1), Aurora A kinase, and Polo-like kinase 1 (Plk1) [8,9,10]. We previously reported that Plk1 positively regulates, and Aurora A negatively regulates the function of Kif2a in mitosis [11]. Similarly, we showed that DDA3 directly interacts with spindle MTs and regulates the spindle dynamics by recruiting Kif2a to the mitotic spindle [2]. DDA3 is also hyperphosphorylated in mitosis, although the function of its phosphorylation remains unknown.

Cell cycle kinases control mitotic progression by regulating the biochemical activity and/or cellular location of their substrates through direct phosphorylation [9,10,11]. Thus, temporal activation of kinases controls the timing of substrate phosphorylation and cell cycle progression. Given that DDA3 is hyperphosphorylated in mitosis, we analyzed the functional consequence of DDA3 phosphorylation and showed here that its MT-polymerizing activity and MT-bundling activity are negatively regulated by mitotic kinases.

2. Materials and Methods

2.1 Plasmids and Antibodies

Full-length DDA3 WT and mutants were subcloned into the pET28a vector (Invitrogen) with a N-terminal His tag, and purified. DDA3 WT and mutants were subcloned into pCS2+ containing a N-terminal GFP tag. Point mutations in DDA3 were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagen).

Rabbit antibodies against Mad2, DDA3, cyclin B and GFP were described previously [2,12]. Anti-β-tubulin E7 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank.

2.2 Cell culture, siRNAs and Transfection

HeLa S3 and HeLa cells were cultured in Dulbecco's modified Eagles Medium containing 10% fetal bovine serum (Invitrogen) and antibiotics. HeLa S3 cells were synchronized at prometaphase by a thymidine-nocodazole treatment (an 18-h thymidine arrest and then a 6-h release, followed by a 14-h nocodazole arrest [13,14].

siRNAs were synthesized by Dharmacon, Inc. using previously characterized sequences [2,15]. Sequence targeting DDA3 was 5'-AAGCAAGACTTCAGTAGCATT-3'. The control siRNA (siGL2) was 5'-CGTACGCGGAATACTTCGATT-3'. siRNAs were transfected into HeLa cells using DharmaFect 1 (Dharmacon, Inc.).

DNA transfection was performed using Effectene (Qiagen) or Lipofectamine 2000 (Invitrogen) as instructed by the manufacturers.

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2.3 Mapping the phosphorylation sites in DDA3

Purification of the DDA3 protein—The DDA3 gene was fused to GFP and S-peptide tags in the pBabe-Puro vector and subsequently transfected into Phoenix-Ampho packaging cells. The viral supernatant was used to infect HeLa S3 cells to isolate stable clones after selection with 0.4 μg/ml puromycin for 2 weeks. A clone stably expressing DDA3-S-GFP gene at a level comparable to that of endogenous DDA3 was used to purify the DDA3 protein by two-step affinity chromatography as described previously [2,10,16,17].

Liquid chromatography-tandem mass spectrometry—An 100-μm i.d. capillary with a 5-μm pulled tip was packed with 10 cm of 5-μm Aqua C18 material (Phenomenex). The desalting column was then equilibrated for 30 min with Buffer A (5% acetonitrile/0.1% formic acid) and the protein digest was pressure loaded onto it. The column was placed inline with a quaternary HPLC (Agilent Technologies) and analyzed by an one-step separation that consisted of a 120-min gradient from 0 to 100% Buffer B (80% acetonitrile/ 0.1% formic acid). As peptides eluted from the microcapillary column, they were electrosprayed directly onto an LTQ mass spectrometer (Thermo Fisher Scientific) with the application of a distal 2.4-kV spray voltage. A cycle of one full-scan mass spectrum (400 – 1,400 m/z) was followed by three data-dependent tandem mass spectrometry spectra at a 35% normalized collision energy.

Analysis of tandem mass spectrometry—Tandem mass spectrometry spectra for phosphor-peptides was analyzed by using the following software analysis protocol. Poor-quality spectra were removed from the dataset by using an automated spectral quality assessment algorithm [18]. Tandem mass spectrometry spectra remaining after filtering were searched with the SEQUEST algorithm against a human database concatenated to a decoy database in which the sequence for each entry in the original database was reversed [19]. No enzyme specificity was considered for any search. SEQUEST results were assembled and filtered by using the DTASelect program (version 2.0) [20]. DTASelect 2.0 uses a linear discriminant analysis to dynamically set XCorr and DeltaCN thresholds for the entire dataset to achieve a user-specified false-positive rate (5% in this analysis).

2.4 Immunofluoresence

HeLa cells on coverglasses were fixed with −20°C methanol for 5 min. Subsequently, cells were permeabilized and blocked with PBS-BT (1× PBS, 3% BSA, and 0.1% Triton X-100) for 30 min at room temperature. Coverslips were then incubated in primary and secondary antibodies diluted in PBS-BT. Images were acquired with Openlab 5.2 (Improvision) under a Zeiss Axiovert 200M microscope using a 1.4NA Plan-Apo 100x oil immersion lens.

2.5 MT Binding and Bundling Assays

Assembly-competent α/β tubulin was isolated as described [21]. Recombinant His-DDA3 was incubated with 2 mM GTP, 10 μg/ml each of leupeptin, pepstatin, and chymostatin, 20 μM taxol, and 3.6 μM taxol-stabilized MTs in BRB80 buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) at room temperature for 30 min and then pelleted through a 150 μl 40% glycerol cushion containing 20 μM taxol and protease inhibitors in the BRB80 buffer at 100,000× *g* for 20 min at 30°C. Pellets were washed three times with the BRB80 buffer and

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analyzed by SDS-PAGE, followed by silver staining and Western blotting. For the MT-stabilization assay, recombinant proteins were incubated with α/β tubulin (final concentration 2 μM) in BRB80 containing 33% glycerol for 30 min at 30°C. Samples were then pelleted through a glycerol cushion and analyzed by silver staining.

MT-bundling assay was performed with modifications of a published protocol [22]. Recombinant proteins were incubated with taxol-stabilized MTs (final concentration 2 μM) in BRB80 plus 1 mM DTT, 2 mM ATP, and 150 mM KCl for 15 min at room temperature. Samples were then fixed with 10 volume of BRB80 containing 1% glutaraldehyde at room temperature for 5 min, overlaid onto a 5 ml of cushion (BRB80 plus 25% glycerol) and spun onto coverslips at 16,000 rpm for 70 min in a Beckman SW41Ti rotor at 22°C. Coverslips were then fixed with −20°C methanol for 10 min, rehydrated, and stained with the antibodies indicated.

2.6 Kinase Assay

To analyze phosphorylation of DDA3 (Figs. 1D and 4), 100 nM recombinant Aurora A, Plx1, and Cdk1/cyc B1 (purified from asynchronous *Sf9* cells) were incubated with varying amounts of recombinant His-DDA3 in the presence of 100 μM unlabeled ATP, 2 μCi [γ $-^{32}$P] ATP, in 10 μl kinase reaction buffer (20 mM Hepes, pH 7.8, 15 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mg/ml BSA) for 30 min at room temperature. Histone H3 and casein are substrates commonly used to assay Aurora A and Plk1 activity, respectively. The reactions were stopped by addition of SDS and analyzed by SDS-PAGE and PhosphoImager.

3. RESULTS and DISSCUSSION

3.1 DDA3 is phosphorylated by mitotic kinases

It has been previously reported that DDA3 has MT-polymerizing activity in interphase cells [23] and regulates MT spindle-dynamics in mitosis [2]. Interestingly, DDA3 also has MT-bundling activity in interphase cells but lose it in mitotic cells (Fig 1A). Consistent with this observation, the formation of the mitotic spindle is abnormal in DDA3-depleted cells [2]. DDA3 binds to MTs *in vitro* (Fig. 1B). *In vivo*, DDA3 is associated with MTs in interphase (Fig. 1A), and with spindle in mitosis [11].

A major change in the mitotic DDA3 is its hyper-phosphorylation during mitosis, as indicated by its mobility shift in SDS-PAGE, which can be reversed by a treatment with lambda phosphatase (Fig. 1C). Consistent with the mitotic phosphorylation of DDA3, we found that recombinant DDA3 was phosphorylated by several mitotic kinases *in vitro,* including Aurora A, Plk1 and cyclin B/Cdk1 (Fig 1D).

To investigate whether the phosphorylation of DDA3 regulates MT-bundling activity in mitosis, we first analyzed phosphorylation sites of DDA3 in mitosis. Hyperphosphorylated DDA3 was purified from prometaphase HeLa S3 cells stably expressing DDA3 tagged with the S-peptide and GFP [2]. Mass spectrometry analysis revealed that DDA3 is phosphorylated at several residues, including Ser22, Ser65, Ser70, Ser223, and Ser225. Identification of these phosphor-peptides was statistically significant, as indicated by their high scores of XCorr and DeltaCN (Fig 1E).

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We have previously reported that the phosphorylation of Ser225 during mitosis is essential for chromosome congression [24]. To investigate the function of the DDA3 phosphorylation of the other sites, we mutated Ser residues to Ala (DDA3-S22/65/70A, DDA3-S223A, DDA3-S225A; non-phosphorable mutants) or to Asp or Glu (DDA3-S22/65/70E, DDA3-S223D, DDA3-S225D; phosphor-mimicking mutants). Next, we analyzed the effect of phosphorylation on the mitotic localization of DDA3. All these mutants were correctly localized to the spindle in mitosis (data not shown), suggesting that phosphorylation of these residues is not required for their association with the spindle in mitosis. We then investigated whether the phosphorylation of these Ser residues is required for the mitotic function of DDA3 in chromosome congression. Depletion of DDA3 generated unaligned chromosomes in metaphase cells (Fig. 2A) [2] and this knock-down phenotype is rescued by GFP-DDA3, and to a slight lesser extent, by GFP-DDA3-S22/65/70A, and GFP-DDA3S223A (Fig. 2B), suggesting that the phosphorylation of Ser22, Ser65, Ser70, and Ser233 residues is not essential for chromosome congression, in contrast to the phosphorylation on Ser225. We have also reported previously that N-terminal region of DDA3 shows dominant-negative effect against endogenous DDA3 by blocking the association of the full-length DDA3 with the spindle [25]. As shown in Figure 2C, transfection of GFP-DDA3-N-S22/65/70A and GFP-DDA3-N-S22/65/70E mutants into HeLa cells also generated unaligned chromosome, indicating that the phosphorylation of Ser 22/65/70 does not affect dominant-negative function of N-terminal region.

3.2 Phosphorylation of DDA3 regulates MT-polymerizing/MT-bundling activities

Next, we examined the effect of phosphorylation on the ability of the full-length DDA3 to polymerize α/β tubulin in an *in vitro* reconstituted system. Recombinant DDA3 or its phosphor-mimicking mutants, DDA3-S65E, DDA3-S22/70E, DDA3-S223D, and DDA3-S225D were incubated with α/β tubulin. The amounts of polymerized MTs were pelleted through a glycerol cushion and assayed by silver-staining (Fig 3A). Whereas wild-type DDA3 showed MT polymerizing activity, all phosphor-mimic mutants have no MT-polymerizing activity (Fig 3A), suggesting that phosphorylation of DDA3 regulates DDA3 function in MT polymerization.

To test whether the phosphorylation affect MT binding ability of DDA3, recombinant DDA3 or its phosphor-mimicking mutants were incubated with pre-polymerized and taxol-stabilized MTs. DDA3 and MTs were pelleted through a glycerol cushion and the amounts of proteins pelleted were assayed by silver-staining (Fig 3B). Both phosphor-mimicking mutants and WT DDA3 bound to Taxol-stabilized MTs, indicating that the phosphorylation does not affect the binding of DDA3 to MTs.

Lastly, we analyzed the effect of DDA3 on MT bundling *in vitro* and found that phosphorylation of DDA3 negatively regulates its MT-bundling activity, as phosphor-mimicking mutants showed little or no MT-bundling activity (Fig. 3C).

3.3 Cdk1 and Aurora A regulate MT-polymerizing/MT-bundling activity of DDA3

We investigated which mitotic kinases control MT-polymerization/MT-bundling activity of DDA3 in the cell cycle. Recombinant DDA3 was phosphorylated by cyclin B/Cdk1, Aurora

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A, or Plx1 and incubated with α/β-tubulin. Polymerized MTs were then assayed by silver staining (Fig. 4A). MT-polymerizing activity was decreased from samples with DDA3 phosphorylated by Cdk1 (Fig. 4A, lanes 4 vs 6) and Aurora A (Fig. 4A, lanes 14 vs 16). In contrast, phosphorylation of DDA3 by Plx1 did not affect its MT-polymerizing activity.

Next, we explored the mitotic kinases responsible for regulating MT-bundling activity of DDA3. *In vitro* phosphorylated DDA3 was incubated with Taxol-stabilized MTs after the kinase reaction. Whereas Cdk1 and Aurora A negatively regulate MT-bundling activity of DDA3, phosphorylation by Plx1 did not alter the degree of MT bundling by DDA3 (Fig. 4B). To eliminate the possibility that these phenotypes derives from phosphorylation of α/β-tubulin by kinases *in vitro*, we treated cells with the inhibitors against Cdk1 or Aurora A after the kinase reaction with DDA3, but prior to incubation with α/β-tubulin, and observed the same phenotypes (Fig. 4B, lower panels). Taken together, the mitotic Cdk1 and Aurora A kinases inhibit MT-polymerization activities and MT-bundling activities of DDA3.

To explore the biochemical and cellular mechanism of the DDA3 phosphorylation in mitosis, we analyzed the relationship between mitotic kinases and the sequential phosphorylation on DDA3. Recombinant DDA3 was first posphorylated by a kinase with unlabeled ATP at room temperature and then a specific inhibitor against this kinase was added to the kinase reaction. Subsequently, a second kinase was incubated with pre-phosphorylated DDA3 in the presence labeled ATP. As shown in Figure 4C, Cdk1 directly phosphorylates DDA3, leading to an up-shift of DDA3. Furthermore, DDA3 phosphorylated by Cdk1 is a substrate for other mitotic kinases, Aurora A and Plk1. However, DDA3 phosphorylated by Aurora A or Plk1 cannot be further phosphorylated for other mitotic kinases. Given that Cdk1, Aurora A, and Plk1 recognize different substrate motifs and that DDA3 pre-phosphorylated by Cdk1 can be phosphorylated by Aurora A and Plk1, but not vice versa, it is unlikely that these three mitotic kinases compete for the same set of Ser and Thr residues for phosphorylation. We conclude that the order of DDA3 phosphorylation is important for the regulation of its function.

DDA3 associates with, bundles, and polymerizes MTs *in vitro*, even though no bundling activity of endogenous DDA3 itself is observed *in vivo* [2,15]. Ectopically expressed DDA3 also has MT-bundling activity in interphase but not in mitotic cells (Fig 1A). In interphase cells, endogenous DDA3 is not sufficient to bundle MTs because its expression is downregulated [2,15]. In mitotic cells, high levels of endogenous DDA3 may be sufficient to bundle spindle MTs, which would interfere the structure and function of the mitotic spindle. How does cell negatively regulate the bundling activity of DDA3 in mitosis to maintain MT dynamics? To understand the biochemical mechanism of this regulation, we developed an *in vitro* reconstitution system using recombinant DDA3 and mitotic kinases. We found that phosphorylation of DDA3 by mitotic kinases disrupted its MT-bundling activity, suggesting that kinases active in mitosis blocks the MT-bundling activity of DDA3, thereby maintaining proper dynamics of mitotic spindle.

In summary, we characterized a biochemical mechanism for regulation of DDA3 activity by mitotic kinases in the cell cycle. This mechanism is critical for mitotic function of DDA3 and for cellular processes in mitosis such as spindle dynamics and chromosome movement.

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Highlights

- DDA3 is hyper-phosphorylated mitotic protein.
- DDA3 is phosphorylated by Plk1, Aurora A, and Cdk1 *in vitro*.
- Phosphorylation down-regulates MT-polymerizing and MT-bundling activities of DDA3.
- Aurora A and Cdk1 negatively regulate MT-polymerizing and MT-bundling activities of DDA3.

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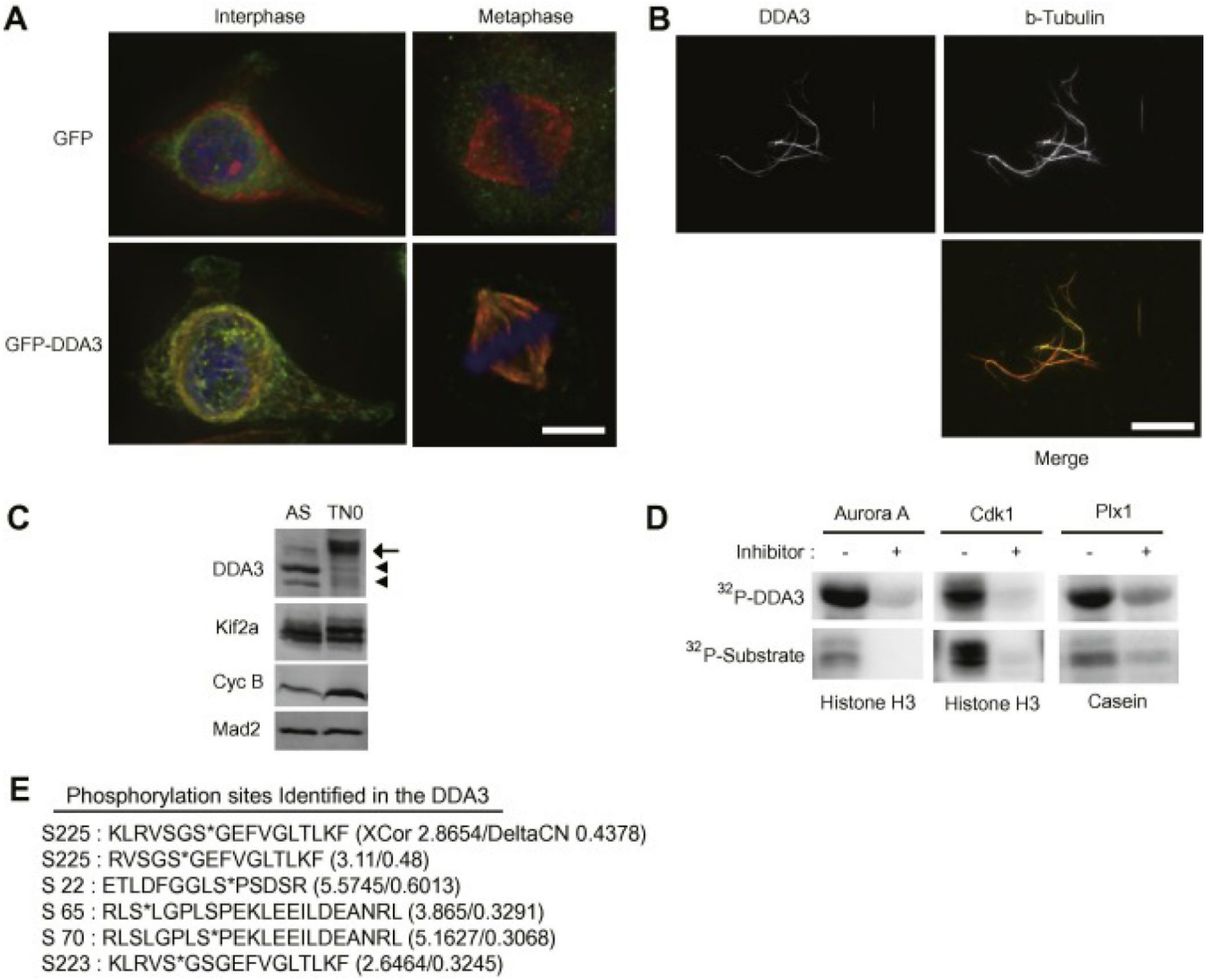

Figure 1. DDA3 is phosphorylated by mitotic kinases.

(A) HeLa cells were transfected with GFP and GFP-DDA3. Images are maximum projections from deconvolved z stacks of representative HeLa cells stained for GFP (green), β-tubulin (red), and DNA (blue). Scale bar, 5 μm. **(B)** Recombinant His-DDA3 (125 nM) was incubated with Taxol-stabilized microtubules for 30 min at room temperature, fixed, and sedimented onto coverslips. Immuno-fluorescence staining of DDA3 (green) and tubulin (red) were shown. Scale bar, 50 μm. **(C)** HeLa cells were synchronized by a thymidine-nocodazole (TN0) block and then harvested. The arrows point to phosphorylated mitotic DDA3 and arrowheads point to two forms of interphase DDA3 [2,15]. AS, unsynchronized cells. **(D)** Recombinant His-DDA3 was phosphorylated by recombinant Aurora A, cyclin B/Cdk1 and Plx1 (the *Xenopus* homolog of human Plk1) with or without respective inhibitor (100 nM VX680 for Aurora A inhibition, 10 μM Purvalenol for Cdk1 inhibition, 100 nM BI 2536 for Plx1 inhibition) in the presence of radioactive ATP. The control substrates are as follow: Histone H3 for Aurora A and cyclin B/Cdk1, and Casein for Plx1. **(E)** DDA3-S-GFP cells were synchronized at prometaphase by a thymidine-nocodazole arrest and the DDA3

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protein was tandem-affinity-purified from the mitotic DDA3-S-GFP cells and analyzed by mass spectrometry. Peptides from phosphorylated DDA3 are listed with XCorr and DeltaCN scores indicated.

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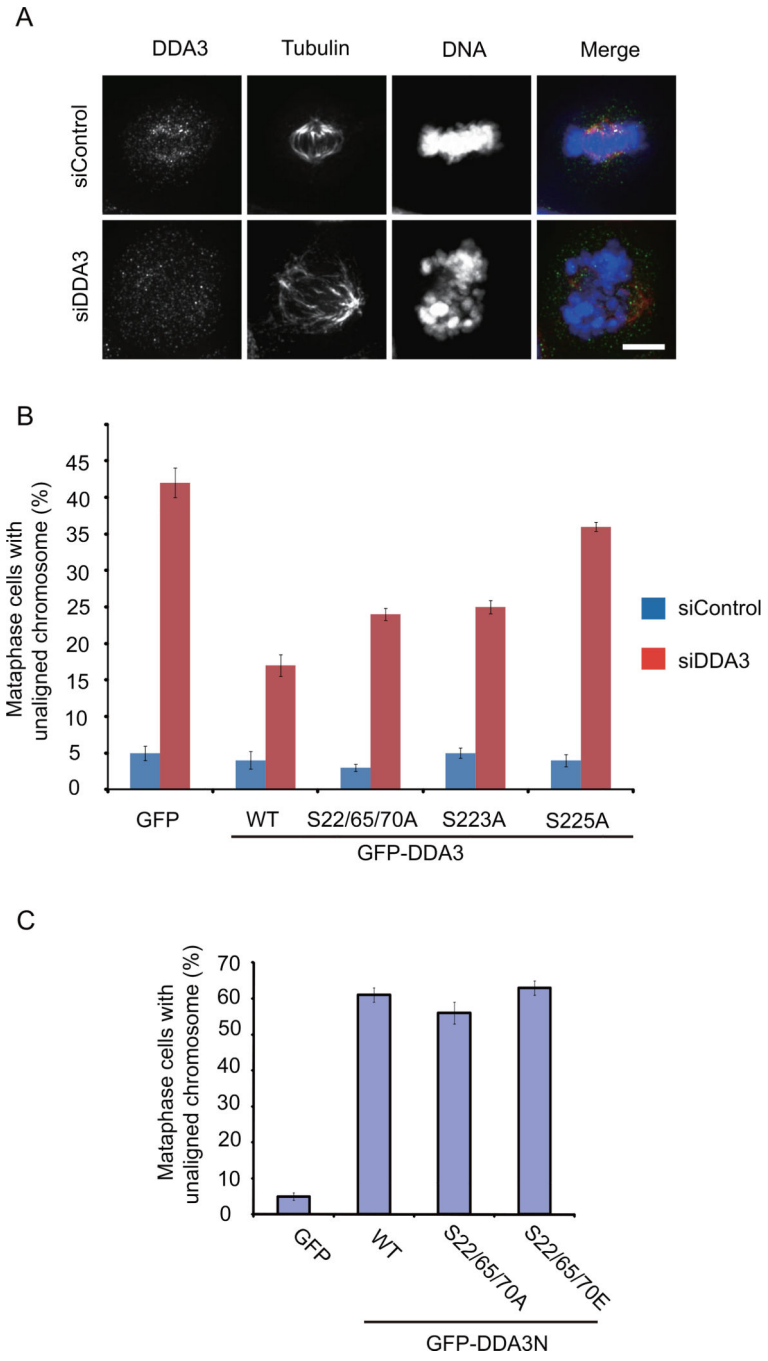

Figure 2. Phosphorylation of N-terminus is not essential for chromosome congression.

(A) HeLa cells were transfected with control siRNA (siControl) or DDA3 siRNA (siDDA3). Images are maximum projections from deconvolved z stacks of representative HeLa cells stained for DDA3 (green), β-tubulin (red), and DNA (blue). Scale bar, 5 μm. **(B)** HeLa cells were transfected with a control siRNA or a previously characterized siRNA targeting DDA3 [2,15]. Twenty-four hours later, cells were transfected again with GFP, GFP-DDA3, GFP-DDA3-S225A, GFP-DDA3-S223A, and GFP-DDA3-S22/65/70A. Cells were fixed at 36 hr post-transfection and stained. The percentage of metaphase cells with unaligned

chromosomes over total metaphase cells were quantified and plotted (n=100). **(C)** HeLa cells were transfected or co-transfected with indicated DNA constructs. The percentage of metaphase cells with unaligned chromosomes over total metaphase cells were quantified and plotted (n=100).

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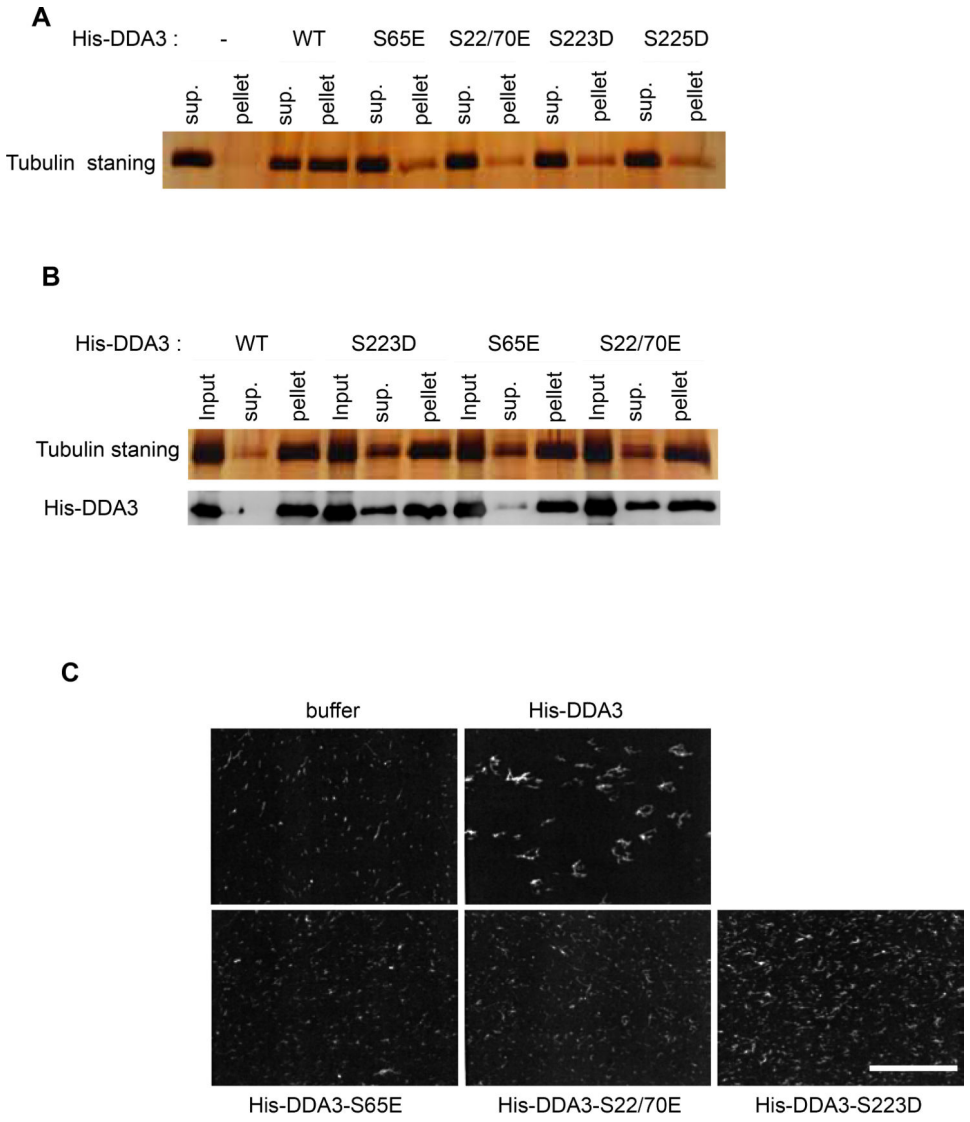

Figure 3. Phosphorylation of DDA3 regulates MT polymerizing and bundling activities.

(A) Recombinant His-DDA, His-DDA3-S65E, His-DDA3-S25/70E, His-DDA3-S223D and His-DDA3-S225D (at 125 nM each) were incubated with α/β tubulin (final concentration 2 μM) in BRB80 containing 33% glycerol for 30 min at 30°C, but without Taxol, as indicated. Samples were then pelleted through a glycerol cushion and analyzed by silver staining. Sup, supernatant. **(B)** Recombinant His-DDA3, His-DDA3-S65E, His-DDA3-S25/70E, and His-DDA3-S223D (at 260 nM each) were incubated with Taxol-stabilized MTs, and MT-associated DDA3 was analyzed in a co-pelleting assay. Equivalent amount of each sample was analyzed by Silver staining and Western blotting. **(C)** Recombinant His-DDA3, His-DDA3-S65E, His-DDA3-S25/70E, and His-DDA3-S223D (at 260 nM each) were incubated with Taxol-stabilized microtubules for 30 min at room temperature, fixed, and sedimented onto coverslips. Immuno-fluorescence staining of tubulin was shown. Scale bar, 300 μm.

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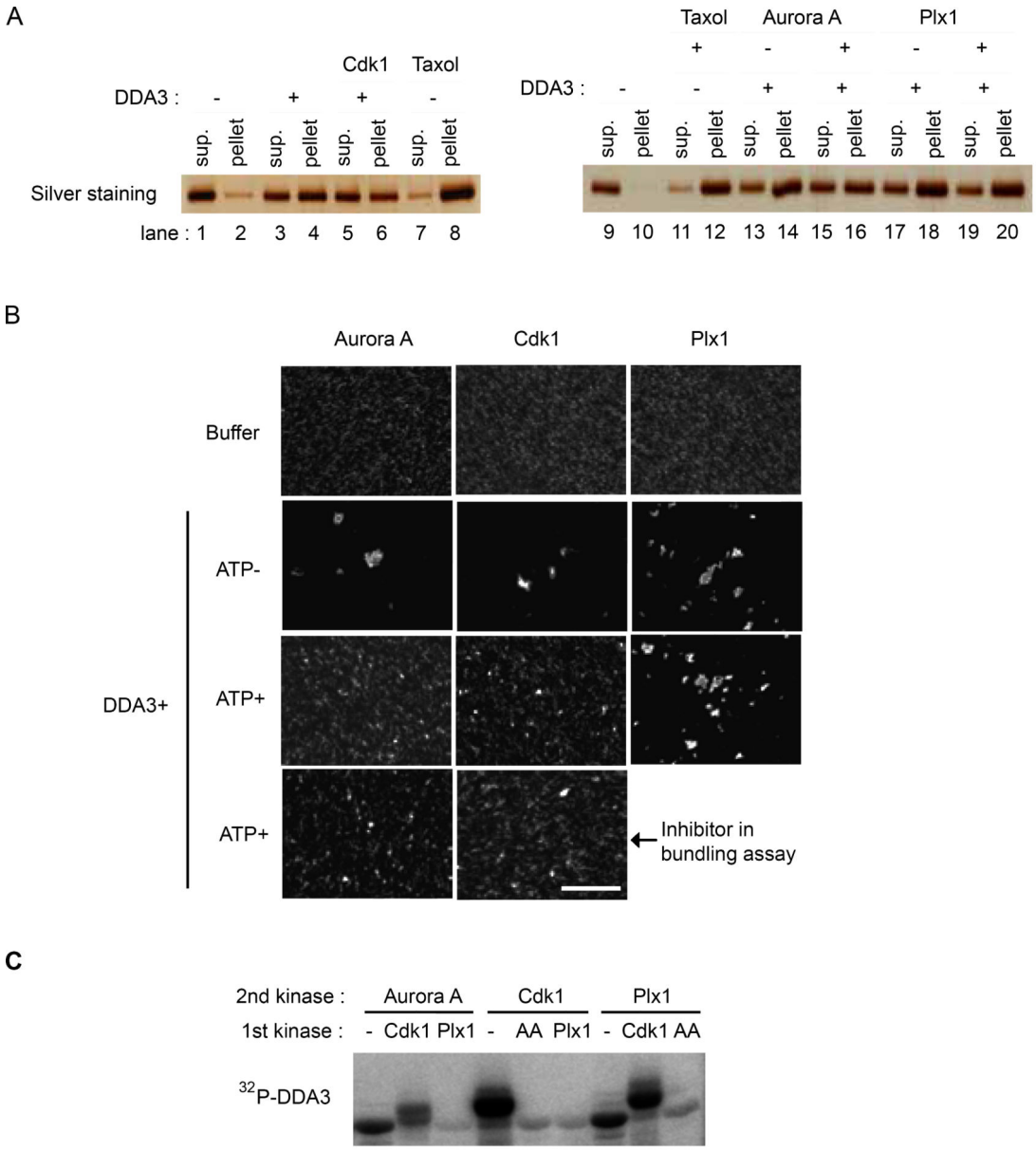

Figure 4. Aurora A and Cdk1 regulate MT polymerizing and bundling activities of DDA3.

(A) Recombinant His-DDA3 was phosphorylated by recombinant Aurora A, cyclin B/Cdk1, or Plx1 for 30 min at room temperature in the presence of unlabeled ATP. Next, phosphorylated His-DDA3 (at 125 nM each) were incubated with α/β tubulin (final concentration 2 μM) in BRB80 containing 33% glycerol for 30 min at 30°C, without Taxol, as indicated. Samples were then pelleted through a glycerol cushion and analyzed by silver staining. **(B)** Recombinant His-DDA3 was phosphorylated by recombinant Aurora A, cyclin B/Cdk1, or Plx1 in the presence of unlabeled ATP. Then, phosphorylated His-DDA3 was incubated with Taxol-stabilized microtubules for 30 min at room temperature, fixed, and sedimented onto coverslips. Kinase inhibitors (100 nM VX680 for Aurora A inhibition, 10 μM Purvalenol for Cdk1 inhibition) were treated after kinase assay to block phosphorylating of MTs. Immuno-fluorescence staining of tubulin was shown. Scale bar, 300 μm.

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(C) Recombinant His-DDA3 was first phosphorylated by recombinant Aurora A, cyclin B/Cdk1, or Plx1 in the presence of unlabeled ATP for 30 min at room temperature. Respective kinase inhibitors (100 nM VX680 for Aurora A inhibition, 10 μM Purvalenol for Cdk1 inhibition, 100 nM BI 2536 for Plx1 inhibition) were then added to kinase reaction mixture on ice for 20 min. Next, phosphorylated His-DDA3 was phosphorylated by a second recombinant kinase as indicated in the presence of radioactive ATP for 30 min at room temperature. Reaction mixtures were assayed by SDS-PAGE and PhosphorImager.