Adducin-1 is essential for mitotic spindle assembly through its interaction with myosin-X

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Mitotic spindles are microtubule-based structures, but increasing evidence indicates that filamentous actin (F-actin) and F-actin–based motors are components of these structures. ADD1 (adducin-1) is an actin-binding protein that has been shown to play important roles in the stabilization of the membrane cortical cytoskeleton and cell–cell adhesions. In this study, we show that ADD1 associates with mitotic spindles and is crucial for proper spindle assembly and mitotic progression. Phosphorylation of ADD1 at Ser12 and Ser355 by cyclin-dependent kinase 1 enables ADD1 to bind to myosin-X (Myo10) and therefore to associate with mitotic spindles. ADD1 depletion resulted in distorted, elongated, and multipolar spindles, accompanied by aberrant chromosomal alignment. Remarkably, the mitotic defects caused by ADD1 depletion were rescued by reexpression of ADD1 but not of an ADD1 mutant defective in Myo10 binding. Together, our findings unveil a novel function for ADD1 in mitotic spindle assembly through its interaction with Myo10.

Introduction

The simple overview of cell division in animal cells is that chromosome segregation is driven by microtubule dynamics in the mitotic spindle, whereas cytokinesis is driven by actin–myosin II dynamics in the contractile furrow. However, increasing evidence indicates that coordinated interactions between the two cytoskeletal systems are necessary to ensure the proper progression of cell division. For example, actin is involved in positioning the spindle via an interaction with astral microtubules (Gundersen and Bretscher, 2003). In contrast, aster and midzone microtubules position the cleavage furrow during anaphase (D’Avino et al., 2005). Remarkably, actin and myosin have been implicated in the organization and function of the spindle under certain conditions (Silverman-Gavrilova and Forer, 2000; Rosenblatt et al., 2004).

Myosin-X (Myo10) belongs to a subclass of unconventional myosins (Berg et al., 2000). Its heavy chain consists of an NH2-terminal motor domain that binds to actin filaments (F-actin) and generates force, a neck domain with three IQ motif binding sites for calmodulin–like light chain, and a long tail domain at the C terminus, which contains three pleckstrin homology (PH) domains, a myosin tail homology 4 (MyTH4) domain, and a FERM (band 4.1, ezrin, radixin, moesin) domain (Kerber and Cheney, 2011). The cluster of three PH domains allows Myo10 to target the plasma membrane via binding to phosphatidylinositol, which contributes to Myo10 distribution to filopodia (Umeki et al., 2011). The MyTH4 domain of Myo10 is sufficient to bind to microtubules (Weber et al., 2004), and this property makes Myo10 an unusual link between the microtubule and actin cytoskeletons. Remarkably, Myo10 has been implicated in the positioning of the meiotic spindles at the cortex in unfertilized Xenopus laevis eggs (Weber et al., 2004; Woolner and Papalopulu, 2012) and in positioning the mitotic spindle parallel to the substratum in cultured cells (Toyoshima and Nishida, 2007). In addition to positioning the spindle, Myo10 is required for mitotic spindle assembly and spindle length control (Woolner et al., 2008). Nevertheless, more studies are required to understand the mechanism of Myo10 regulation of the assembly and function of mitotic spindles.

Adducin (ADD) is an actin-binding protein that is important for the stabilization of the membrane cortical cytoskeleton (Gardner and Bennett, 1987; Hughes and Bennett, 1995) and cell–cell adhesion (Abdi and Bennett, 2008; Naydenov and Ivanov, 2010). The ADD family consists of three closely related genes:
**Results and discussion**

**Association of ADD1 with mitotic spindles through its head domain**

Although ADD1 mainly localized at the cell–cell junctions of MDCK epithelial cells, ADD1 apparently associated with mitotic spindles when the cells entered mitosis (Fig. 1 A). The association of ADD1 with mitotic spindles was also observed in other types of cells, such as HeLa cells (Fig. 1 B). The specificity of the ADD1 staining at mitotic spindles was verified by ADD1 knockdown (Fig. S1 A). In addition, ADD1 no longer associated with mitotic spindles upon the disruption of spindle microtubules by nocodazole (Fig. S1 B). The withdrawal of nocodazole restored the assembly of mitotic spindles and the association of ADD1 (Fig. S1 B). In contrast, the disruption of the actin cytoskeleton by latrunculin did not affect the association of ADD1 with the spindle (Fig. S1 C). As early as prophase, ADD1 was found to associate with mitotic asters before the breakdown of the nuclear envelope (Fig. 1 C). In metaphase and anaphase, ADD1 more visibly associated with spindle fibers and poles (Fig. 1 C). During cytokinesis, ADD1 retained its association with midzone microtubules (Fig. 1 C). Like endogenous ADD1, FLAG epitope–tagged ADD1 (FLAG-ADD1) associated with mitotic spindles throughout mitosis (Fig. S1, D and E).

To determine which domain of ADD1 is responsible for its association with mitotic spindles, several deleted mutants of FLAG-ADD1 were constructed (Fig. 1 D). The ADD1 mutant without the tail domain retained its ability to associate with spindles, indicating that the tail domain is not required for ADD1 to localize at mitotic spindles (Fig. 1 E). Moreover, the tail domain by itself was diffusively distributed in the cytoplasm and failed to associate with mitotic spindles, whereas the head domain by itself was sufficient for the association (Fig. 1 E). These results together indicate that the head domain of ADD1 is responsible for its association with mitotic spindles.

**Phosphorylation of ADD1 at S12 and S355 by CDK1 is crucial for its association with mitotic spindles**

Because the molecular mass of ADD1 increased during the G2/M phase of the cell cycle (Fig. S2 A), we speculated that ADD1 might undergo certain posttranslational modifications in the G2/M phase. Indeed, several phosphorylation sites on ADD1 from the cells arrested in the G2/M phase were identified by mass spectrometry (MS; Fig. S2 B). Remarkably, S12A and S355A mutants became diffusively distributed in the cytoplasm of mitotic cells (Fig. 2 A), whereas S353A, S358A, S465A, and S726A mutants retained their association with mitotic spindles (Figs. 2 B and S2 C). The phosphorylation mimetic mutants S12E and S355E were able to associate with mitotic spindles (Fig. 2 A). Of note, S12D and S355D behaved like S12A and S355A (Figs. 2 B and S2 C), indicating that aspartate does not mimic phosphorylated serine in these two cases.

To facilitate the detection of ADD1 phosphorylation at S12 and S355, antibodies specific to phosphorylated S12 and S355 of ADD1 were generated (Fig. 2 C). The phosphorylation of ADD1 at S12 and S355 was indeed increased in the G2/M phase (Fig. 2, C and D), which was inhibited by RO-3306, a specific inhibitor for the mitotic kinase CDK1 (Fig. 2 E). In vitro, CDK1 was able to directly phosphorylate ADD1 at S12 and S355 (Fig. 2, F and G). These results together suggest that the phosphorylation of ADD1 at S12 and S355 by CDK1 may facilitate the association of ADD1 with mitotic spindles.

**ADD1 associates with mitotic spindles through Myo10**

Because purified FLAG-ADD1 does not bind to polymerized microtubules in vitro (Fig. S3), ADD1 may indirectly associate with mitotic spindles through other spindle-associated proteins. In this study, we demonstrated that FLAG-ADD1 interacts with endogenous Myo10 (Fig. 3 A). To characterize the interaction between ADD1 and Myo10, GFP-fused Myo10 and its mutants were constructed (Fig. 3 B). The NH2-terminal half of Myo10 contains a highly conserved motor domain, three IQ domains, and a coiled-coil domain. The MyTH4 domain and FERM domain in the C terminus of Myo10 are known to mediate the association of Myo10 with microtubules (Weber et al., 2004). We found that FLAG-ADD1 specifically bound to the NH2-terminus (aa 1–439) of the Myo10 motor domain (Fig. 3, C and D). In addition, we demonstrated that the head and neck domains of ADD1 mediated the interaction with Myo10 (Fig. 3 E) and that the mutation of ADD1 at S12 and S355 abrogated its capability to bind to Myo10 (Fig. 3 F). Myo10 depletion significantly (~50%) prevented the association of ADD1 with mitotic spindles, accompanied by spindle distortion, spindle length elongation, and chromosome misalignment (Fig. 3, G and H). These data together suggest that ADD1 may associate with mitotic spindles through its interaction with Myo10.
ADD1 depletion causes disorganized mitotic spindles

The effect of ADD1 depletion on mitotic spindles was analyzed (Fig. 4 A). We found that ADD1 depletion led to disorganized mitotic spindles (Fig. 4 B), which were characterized by distorted spindles (Fig. 4 C), elongated spindle length (Fig. 4 D and E), and multipolar spindles (Fig. 4 F). In addition, ADD1 depletion caused aberrant chromosome alignment, including misalignment and nonalignment (Fig. 4 G). Of note, most of the cells with distorted spindles also exhibited elongated spindle length, accompanied by chromosome nonalignment (Fig. 4 B). Importantly, the mitotic defects induced by ADD1 depletion were restored by the reexpression of FLAG-ADD1 but not by the S12A/S355A mutant (Fig. 4 C–G). Because the S12A/S355A mutant failed to interact with Myo10 (Fig. 3 D), our results together suggest that the interaction of ADD1 with Myo10 may be important for the assembly and function of mitotic spindles. Like FLAG-ADD1, the mutant lacking the tail domain (Δtail) was able to rescue the defects in ADD1-depleted cells (Fig. 4).

ADD1 depletion causes aberrant congression and segregation of chromosomes in mitotic cells

ADD1 depletion significantly increased the mitotic index in HeLa cells (Fig. 5, A and B). Next, the effect of ADD1 depletion on mitosis was monitored in living HeLa cells that stably expressed mCherry–histone H2B (Fig. 5 C). ADD1 depletion apparently caused aberrant congression and segregation of chromosomes in mitotic cells (Fig. 5 C and Videos 1–5). This aberrance can be classified into three types (Fig. 5 D). In type I, chromosomes undergo incomplete congression and then proceed to segregation, which leads to micronuclei in daughter cells (Video 3). In type II, chromosomes proceed to segregation without congression, which often leads to multiple nuclei in daughter cells (Video 4). In type III, chromosomes undergo several rounds of incomplete congression but do not proceed to segregation, which eventually leads to cell apoptosis (Video 5). The aberrance in chromosomal congression/segregation by ADD1 depletion led to prolonged mitosis (Fig. 5 E).

ADD1 is well known for its function in the stabilization of the membrane cortical cytoskeleton and cell–cell junctions, which relies on its interaction with F-actin and spectrin (Hughes and Bennett, 1995; Abdi and Bennett, 2008). ADD1 binds to F-actin via its tail domain (Mische et al., 1987; Kuhlman et al., 1996; Li et al., 1998); however, we show here that the head domain of ADD1 is responsible for its association with mitotic spindles and its interaction with Myo10. This observation indicates that the function of ADD1 is not always dependent on F-actin and reveals a novel function of ADD1 through its head domain. In addition, we demonstrate that the phosphorylation of ADD1 at Ser12 (in the head domain) and Ser355 (in the neck domain) by CDK1 is essential for the interaction of ADD1 with Myo10,
which diminishes the interactions of ADD1 with F-actin, and is subsequently phosphorylated by CDK1, which leads to ADD1’s interaction with Myo10 and its association with mitotic spindles.

The myosin motor domain has relatively few known interacting proteins other than F-actin. For example, the unusually suggesting that the phosphorylation of ADD1 by CDK1 may induce conformational changes, leading to the exposure of the head domain to Myo10. Therefore, we propose a possible scenario in which ADD1 is first phosphorylated at the C-terminal MARCKS-related domain by a kinase that is activated in the G2/M phase, which diminishes the interactions of ADD1 with F-actin, and is subsequently phosphorylated by CDK1, which leads to ADD1’s interaction with Myo10 and its association with mitotic spindles.

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large loop 2 region in the motor domain of myosin-IX is reported to bind to calmodulin (Liao et al., 2010), and the myosin chaperone UNC-45 is likely to bind to the myosin motor domain (Gazda et al., 2013). In this study, we show that ADD1 binds to the motor domain of Myo10. This finding affords the possibility that the motor activity of Myo10 may be modulated by ADD1 binding. It has been shown that the binding of the lissencephaly protein Lis1 to the motor domain of dynein (a microtubule-based motor protein) affects the dynein mechanical behavior, leading to a prolonged interaction of dynein with microtubules and thereby to slower microtubule sliding (McKenney et al., 2010; Huang et al., 2012).

Figure 3.  ADD1 phosphorylation at S12 and S355 is required for its interaction with the motor domain of Myo10. (A) The cell lysates prepared from HeLa cells or those stably expressing FLAG-ADD1 were incubated with anti-FLAG M2 affinity resins. The bound proteins were eluted from the resins with FLAG peptides and analyzed by immunoblotting (IB) with anti-Myo10 or anti-FLAG. WCL, whole-cell lysates. (B) GFP-fused Myo10 (GFP-Myo10) and mutants were transiently expressed in HEK293 cells and were analyzed by immunoblotting with anti-GFP. The scheme shows the domain structures of GFP-Myo10. CC, coiled-coil domain; PEST, polypeptide enriched in proline, glutamic acid, serine, and threonine residues. (C and D) FLAG-ADD1 was transiently coexpressed with GFP-Myo10 or its mutants in HEK293 cells. GFP-Myo10 was immunoprecipitated (IP) by anti-GFP, and the immunocomplexes were analyzed by immunoblotting with anti-FLAG or anti-GFP. (E) GFP-Myo10-N was transiently coexpressed with [+ ] or without (–) the FLAG-ADD1 Δtail mutant in HEK293 cells. The cell lysates were incubated with anti-FLAG, and the immunocomplexes were analyzed by immunoblotting with anti-GFP or anti-FLAG. (F) GFP-Myo10-N was transiently coexpressed with FLAG-ADD1 or its mutants in HEK293 cells. FLAG-ADD1 was immunoprecipitated by anti-FLAG, and the immunocomplexes were analyzed by immunoblotting with anti-GFP or anti-FLAG. (G) HeLa cells were infected with lentiviruses expressing shRNAs to Myo10 (sh-Myo10 #1 and sh-Myo10 #2) or to luciferase (sh-Luc.) as a control. The expression levels of Myo10, ADD1, and β-tubulin (as a loading control) were analyzed by immunoblotting with the indicated antibodies. (H) The cells, as in G, were stained for ADD1, α-tubulin, and DNA. Arrows indicate misaligned chromosomes. The percentage of ADD1 association with mitotic spindles in the total number of mitotic cells counted was measured (n > 270). Values (means ± SD) are from three independent experiments. **, P < 0.01. Bars, 5 µm.
Figure 4. Depletion of ADD1 results in distorted, elongated, and multipolar spindles. (A) HeLa cells were infected with lentiviruses expressing shRNAs to ADD1 (shADD1 #1), Myo10 (shMyo10 #1), or luciferase (sh-Luc). FLAG-ADD1 or mutants (S12A/S355A and Δtail) were reexpressed in the cells whose endogenous ADD1 had been depleted. An equal amount of whole-cell lysates was analyzed by immunoblotting (IB) with the indicated antibodies. (B) The cells were stained for ADD1, α-tubulin, and DNA. (C) The percentage of distorted spindles in the total number of mitotic cells counted was measured (n > 700). (D) The cells were stained for ADD1 (gray), α-tubulin (red), and DNA (blue). The ratio of spindle length to cell diameter was measured (n > 90). (E) The spindle length of the mitotic cells was measured (n > 90). The results were obtained from three independent experiments. The best-fit Gaussian distribution is shown in the black curves. (F) The percentage of multipolar spindles in the total number of mitotic cells counted was measured (n > 700). (G) The percentage of aberrant chromosome alignment in the total number of mitotic cells counted was measured (n > 700). Values (means ± SD) are from three independent experiments. *, P < 0.05; **, P < 0.01. Bars, 5 µm.
nucleate branched networks of short actin filaments were found to be involved in the formation of the spindle F-actin (Mitsushima et al., 2010; Field et al., 2011), suggesting that the spindle F-actin might be branched, short F-actin structures. Myo10 was initially identified as a motor protein localized to the filopodia (Berg et al., 2000), which are a type of membrane protrusion that contains a core of parallel F-actin. These studies together suggest that Myo10 may bind to parallel and branched F-actin under different circumstances. It is not known whether this property of Myo10 is affected by ADD1 binding. In summary, this work not only unveils a novel role for ADD1 in the spindle assembly but also highlights the significance of ADD1–Myo10 interactions in mitosis.

Materials and methods

Materials

The rabbit polyclonal antibodies specific to ADD1 pS12 and pS355 were generated using synthesized peptides C-SRAAVVTpSP and C-KSRpSPG-SPVGE, respectively, as antigens (GeneTex, Inc.). The rabbit anti-ADD1 (H-100), mouse anti-β-tubulin (D-10), mouse anti-cyclin B1, and mouse anti-Myo10 (C-1) antibodies were purchased from Santa Cruz Biotechnology, Inc. The mouse anti-FLAG (M2), rabbit anti-FLAG, mouse anti-α-tubulin (DM1A), mouse anti-GFP (B-2), mouse anti-β-actin antibodies, and nocodazole were purchased from Sigma-Aldrich. The mouse anti-T7 antibody was purchased from EMD Millipore. The mouse anti-GFP antibody and X-tremeGENE HP were purchased from Roche. The HRP-conjugated goat anti–rabbit or goat anti–mouse antibodies and rabbit anti–mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. Alexa Fluor 488- and Alexa Fluor 546–conjugated secondary antibodies and Lipofectamine were purchased from Invitrogen. The CDK1 inhibitor RO-3306 was purchased from Enzo Life Sciences. Purified CDK1/cyclin B1 was purchased from EMD Millipore.

Plasmids and mutagenesis

For FLAG-ADD1, the human ADD1 cDNA from pEGFP-C1-ADD (Chen et al., 2007) was cloned into pCMV-3Tag-3A vector (Agilent Technologies) by SacI and BamHI sites. For deleted mutants of FLAG-ADD1, the corresponding cDNA fragments were amplified by PCR using pCMV-3Tag-3A-ADD1 as the template with the following primers and were subcloned into pCMV-3Tag-3A vector. For the tail mutant (aa 1–430), the forward primer was 5’-CGGGATCCCGATGAATGGTGATTCTCGTGCTGCG-3’ and the reverse primer was 5’-GAATTCGCAAGTGCCCGAATCACCGTCACT-3’. For the head domain (aa 1–350), the forward primer was 5’-CGGGATCCCGATGAATGGTGATTCTCGTGCTGCG-3’ and the reverse primer was 5’-GGAATTCTTTGTACTTCTCAGGATTCAGCAGGAC-3’. For the tail domain (aa 431–737), the forward primer was 5’-CGGGATCCATGTGCTCCCCACTCAGACACAGTTTT-3’ and the reverse primer was 5’-GGAGAATTCGAGTCACTCTTCTTCTTGCT-3’. All point mutations in pCMV-3Tag-3A-ADD1 were generated by the site-directed mutagenesis kit (QuickChange; Agilent Technologies) and were confirmed by dyeoxy-DNA sequencing. The mutagenic primers for making mutations

Figure 5. ADD1 depletion causes aberrant congression and segregation of chromosomes. (A) HeLa cells expressing mCherry–histone H2B were infected with lentiviruses expressing shRNAs to ADD1 (sh-ADD1 #1 and #2) or luciferase (sh-Luc). The expression level of ADD1 or actin (as a loading control) was analyzed by immunoblotting (IB) with the indicated antibodies. (B) The cells were stained for DNA, and the percentage of mitotic cells in the total number of cells was measured (n > 2,044). Values (means ± SD) are from three independent experiments. **, P < 0.01. (C) Images of HeLa cells expressing mCherry–histone H2B were acquired by time-lapse epifluorescent microscopy (Axio Observer.D1). Frames were taken every 3 min for 24 h. Arrows indicate misaligned chromosomes. Bar, 5 µm. (D) The defect in chromosome movement caused by ADD1 depletion was classified into three types (see the seventh paragraph in Results and discussion for details). The percentage of each type of defect in the total chromosomal aberrance was measured (n > 25). (E) The duration from chromosome condensation to anaphase onset was measured (n > 25). The results are expressed as box and whisker plots. The horizontal line in each box is the median. The boxes represent 50% of all measurements from the 25th to 75th percentile, and whiskers represent the 10th and 90th percentiles. Values (means ± SD) are from three independent experiments.
at S12, S355, S358, and G656, and S726 were shown in Table S1. To construct the plasmid encoding the T7-tagged ADD1 head domain (aa 1–350) or Δtail (aa 1–430), cDNAs from pCMV-3Tag-3A-ADD1-head or pCMV-3Tag-3A-ADD1-tail were subcloned into the pET21d vector (EMD Millipore).

The pEGFP-Myo10 plasmid was a gift from B.S. Lee (The Ohio State University, Columbus, OH) and was described previously (Berg and Cheney, 2002). The full-length bovine Myo10 cDNA (aa 1–2,052) was amplified by PCR and cloned into pEGFP-N1 (Clontech, Takara Bio Inc.). To construct the pEGFP plasmids encoding GFP-Myo10-N (aa 1–946) or GFP-Myo10-C (aa 946–2,052), the corresponding cDNA fragments were PCR amplified using pEGFP-Myo10 as the template with the following primers and were subcloned into the pEGFP-C3 vector (Takara Bio Inc.). For GFP-Myo10-N, the forward primer was 5′-CCAGAATTTGAGAACAATCTCTCCCGACGAG-3′ and the reverse primer was 5′-CCAGAACCTTCCTCAACTGCAAGATGCAGACG-3′. For GFP-Myo10 aa 1–748 (the motor domain), the forward primer was 5′-CCAAAGGTCATGAGAACAATCTCTCCCGACGAG-3′ and the reverse primer was 5′-AAAAGAATCTTTCGAGGACTCGAAGCTTCCGAG-3′.

To express the FLAG-ADD1 wild-type (WT) or S12A/S355A mutant by lentivirus infection, the corresponding cDNAs were PCR amplified using pEGFP-C1 vector (Takara Bio Inc.). For GFP-Myo10 aa 1–439 or aa 440–748, the corresponding cDNA fragments were PCR amplified using pEGFP-Myo10 as the template with the following primers and were subcloned into the pEGFP-C1 vector (Takara Bio Inc.). For GFP-Myo10 aa 1–439, the forward primer was 5′-CAAGATCTGATGAGAACAATCTCTCCCGACGAG-3′, and the reverse primer was 5′-CAGAATTTGAGAACAATCTCTCCCGACGAG-3′. For GFP-Myo10 aa 440–748, the forward primer was 5′-CCAGAATTTGAGAACAATCTCTCCCGACGAG-3′, and the reverse primer was 5′-CAGAATTTGAGAACAATCTCTCCCGACGAG-3′.
condensation) to anaphase onset was measured for ≥25 cells from three independent experiments.

**References**


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