Astrin regulates Aurora-A localization

Jian Du[1],[†], **Sandra Jablonski**[2], **Tim J. Yen**[2], and **Gregory J. Hannon**[1],*

[1]Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, Tel: (516) 367 8386, Fax: (516) 367 8874, hannon@cshl.edu

[2]Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111

Abstract

Alterations in the expression and activity of the centrosomal kinase, Aurora-A/STK15, affect genomic stability, disrupt the fidelity of centrosome duplication, and induce cellular transformation. A mitotic spindle-associated protein, astrin/DEEPEST, was identified as an Aurora-A interacting protein by a two-hybrid screen. Astrin and Aurora-A co-express at mitosis and co-localize to mitotic spindles. RNAi-mediated depletion of astrin abolishes the localization of Aurora-A on mitotic spindles and leads to a moderate mitotic cell cycle delay, which resembles the mitotic arrest phenotypes in siAurora-A treated cells. However, depletion of Aurora-A does not affect astrin localization, and co-depletion of both astrin and Aurora-A causes a mitotic arrest phenotype similar to depletion of siAurora-A alone. These results suggest that astrin acts upstream of Aurora-A to regulate its mitotic spindle localization.

Introduction

In mammalian cells, the Aurora kinase family has three members: Aurora-A, -B, and -C. Alterations in the expression and activity of Aurora-A/STK15 affect genomic stability, disrupt the fidelity of centrosome duplication, and induce cellular transformation [1-5]. Aurora-A expression up-regulates at mitosis and it localizes at centrosome and mitotic spindles. Aurora-A T288 phosphorylation activates its kinase activity in late G2 phase at centrosomes, which is prior to and required for the recruitment of CDK1-cyclin B1 to the centrosome. The activated CDK1-cyclin B1 then commits the cell to mitosis, and is in turn required for full activation of Aurora-A [6]. These results explain the mitotic arrest in Aurora-A depleted Hela cells and suggest Aurora-A activation is a crucial early event in mitosis initiation.

Among Aurora-A interacting proteins, TPX2 is identified as an upstream regulator of Aurora-A kinase activity and it localizes on spindles [7-10]. TPX2 was first isolated by co-immunoprecipitation and other biochemical purification in *Xenopus laevis* egg extracts, characterized to be required for the recruitment of XKLP2 (Xenopus kinesin-like protein 2) to

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microtubules to form compact mitotic spindles in Xenopus [11]. In mammalian cells, depletion of TPX2 by RNA interference (RNAi) caused formation of multipolar spindles, similar to the depletion of Aurora-A [7,8,12].

In order to understand Aurora-A's involvement in molecular pathways, we searched for physical Aurora-A partners using yeast two-hybrid screens. Human astrin/DEEPEST is a 134 kDa protein with two coiled-coil domains at the C-terminus, and was originally identified as a mitotic spindle-associated non-motor protein that localized to spindle microtubules from prophase through anaphase [13,14]. Silencing of astrin in Hela cells by RNA interference resulted in a growth arrest and highly disordered multipolar spindles [15]. In the absence of astrin, condensed chromosomes could not align to the spindle equator, ultimately leading to apoptosis [15]. More recently, Astrin was found to interact with p53, kinetochore, and glycogen synthase kinase 3 beta [16-18]. Here we show that astrin interacts with Aurora-A and regulates its localization at mitotic spindles. Epistatic knockdown experiments showed that, like TPX2, astrin is upstream to Aurora-A.

Materials and Methods

Two-hybrid screen

We screened a HeLa cell cDNA library using full length Aurora-A as bait for Aurora-A/STK15 interacting proteins [19]. Sequence analysis revealed two positive clones containing *astrin* coding sequences from nucleotides 10-717 (amino acids 4-239).

Mammalian cell culture

Human primary fibroblast IMR90 and early passage Hela cells were purchased from ATCC (CCL-186 and CCL-2). HeLa cell synchronization was performed by double S-phase arrest/ release. Mitotic extracts were obtained from cells 11 hr after the final release.

Plasmids

Astrin cDNA clones were constructed by three-way cloning from EST clones 1367671 and 567106 (Research Genetics, Inc, Huntsville, AL) into pET23a, pGADGH and pGEX-KG.

Antibodies

Affinity purified anti-Aurora-A, anti-GST-Aurora-A sera [19], mouse monoclonal anti-α-tubulin, -β-tubulin (Sigma, St Louis, MO), -cyclin B1 (BD Sciences, San Diego, CA), -BrdU (Amersham Int. UK), rabbit polyclonal anti-γ-tubulin, -phospho-H3 (Ser-10, Upstate Biotech, Lake Placid, NY), -phospho-Aurora-A (T288) (Cell Signaling Tech., Beverly, MA), -astrin sera (a kind gift from Y.C. Yang, Mackey Memorial Hospital, Taiwan, and D.A. Compton, Dartmouth Medical School, NH) were used. Another rabbit polyclonal anti-astrin antibody against the N-terminal 9 amino acids (MWRVKKLSL) of astrin was affinity purified as previously [19] and used in immunoprecipitation.

Immunoassays and live cell imaging

Immunoassays were as described in [20]. Live cell imaging was done with stable GFP-tubulin-expressing Hela cells that were transfected with various siRNAs and seeded onto cover slips. Cells were mounted in the FCS2 Closed System Live-Cell chamber (Bioptechs, Inc. Butler, PA) for imaging. The images were collected using 100× objective lens on LSM510 confocal microscope (Zeiss, German) at 100 msec/frame.

RNAi

To design target-specific siRNA duplexes, AA(N19)dTdT type sequences were selected from the open reading frames of the astrin (AAGGAGTCTGAAACAGAAGAT and AAGGCAGCAACAACTCATCTC, both worked equally well to knockdown astrin expression levels as judged by western blot). The selected sequences were submitted to BLAST searches against the non-redundant protein database in NCBI to make sure that only the selected genes were targeted. These and siGFP (control), siAurora-A [20] 21nt RNAs were purchased from Dharmacon Res. Inc. (Lafayette, CO). Cells were assayed 24 hr after the last transfection.

Results and Discussion

Astrin interacts with Aurora-A

To search for Aurora-A interacting proteins, a two-hybrid screen was conducted using full length Aurora-A as a bait [19]. Plasmids carrying *astrin* coding sequences (nucleotides 10-717) were isolated as two independent positive clones, suggesting that the N-terminus of astrin (amino acids 4-239) is responsible for the interaction with Aurora-A. A full-length astrin also interacted specifically with Aurora-A in a two-hybrid assay (Fig.1A). Protein complexes precipitated from Hela cell lysates with anti-astrin, or -normal rabbit IgG antibody showed that anti-astrin specifically pulled down astrin, as well as Aurora-A, but not Aurora-B (Fig. 1B), providing confirmation that Aurora-A and astrin specifically associate *in vivo*.

Aurora-A expression is cell-cycle regulated, with peak at mitosis [21]. To check for astrin protein level, Hela cells were synchronized at early S-phase by thymidine and aphidicolin double block. Cells entered the cell cycle and reached prophase at proximately 9 hours and finished mitosis at 13 hours post release (Fig. 1C). Astrin expression increased significantly from prophase to cytokinesis (9-12 hours, Fig. 1C) and returned to basal levels at the next G1 phase, which resembles the expression pattern observed for Aurora-A.

Anti-astrin immunofluorescence showed that astrin dramatically accumulated to centrosomes in prophase, and mitotic spindles, as well as centrosomes, from prometaphase to cytokinesis. This spatial pattern of colocalization coincides precisely with Aurora-A enrichment at mitotic spindles and centrosomes at mitosis (Fig. 1D). In vitro experiments suggest that astrin may be a substrate of Aurora-A. Affinity-purified GST-Aurora-A and -Aurora-A T288D mutant (constitutively active) specifically phosphorylated GST-astrin, while Aurora-A K161M (kinase-dead mutant) did not (data not shown). The two-hybrid binding, co-immunoprecipitation, and colocalization of Aurora-A with astrin all suggest they interact functionally.

Depletion of Astrin delocalizes Aurora-A from multipolar spindles

To assess the functional relevance of the interaction between astrin and Aurora-A, siRNAs were used to effectively deplete each target protein to undetectable levels, as judged by western blotting (Fig. 2A). Immunofluorescence showed that siRNA treatment also abolished protein staining in cells (data not shown). Deprivation of astrin induced the formation of multipolar mitotic spindles in Hela cells (in 55% mitotic cells, compared to 2.3% in control siGFP treated mitotic cells) (Fig. 2B and [15]).

More importantly, depletion of astrin totally prevented the normal Aurora-A mitotic spindle localization (Fig. 2C, pointed arrows), although centrosome-associated Aurora-A was still obvious (Fig. 2C). Significantly, Aurora-A was still phosphorylated at the centrosomes of mitotic cells, as shown by anti-phospho-Aurora-A staining (Fig. 2D), suggesting Aurora-A phosphorylation does not require astrin. Neither astrin depletion affects Aurora-B mitotic localization, nor Aurora-A depletion affects astrin spindle localization (data not shown).

The multipolar spindle phenotypes in astrin depleted cells closely resemble those resulting from deprivation of TPX2, another Aurora-A regulating protein, supporting the notion that astrin may also be an Aurora-A regulator. To understand the epistatic relationships among TPX2, astrin, and Aurora-A, we further tested if depletion of TPX2 affects astrin or Aurora-A cellular localization. TPX2 normally localizes to mitotic spindles (Fig. 2E). siTPX2 effectively depleted TPX2 expression in Hela cells as well as its localization at mitotic spindles (Fig. 2E and data not shown). Indeed the knockdown of TPX2 depleted the mitotic spindle localization of both astrin and Aurora-A (Fig. 2F). However, knockdown of either astrin or Aurora-A did not affect TPX2 spindle association (data not shown), suggesting that TPX2 spindle association occurs independent of astrin and Aurora-A, and epistaticly it is upstream of both astrin and Aurora-A. The result that TPX2 associates with centrosomes in the astrin depleted cells also correlates with Aurora-A T288 phosphorylation in these cells (Fig. 2D), because TPX2 is the major stimulator of Aurora-A autophosphorylation.

Significantly, siAstrin leads to a reduction in the density and length of spindle microtubules, judging from the intensity and length of anti-α-tubulin spindle staining (Fig. 2B, 2C). Multipolar spindle arrays were less focused on, but still initiated from disorganized centrosomes with abnormal centrosome numbers, identified by the multiple anti-γ-tubulin staining spots (Fig. 3A). We tested if astrin interacts directly with microtubules, which are made of heterodimers of α and β-tubulins. Immunoprecipitation with anti-astrin antisera specifically co-precipitated both α and β-tubulin (Fig. 3B and data not shown). The amount of astrin-associated α–tubulin increased in mitotic cell extract, consistent with the notion that astrin localizes to mitotic spindles (Fig. 3B).

The kinesin Eg5 is required for the assembly of bipolar spindles in vertebrate cells. It was shown to be one of Aurora-A substrates and involved in the multipolar spindle formation induced by deprivation of TPX2, another Aurora-A regulator. To distinguish the TPX2-Eg5 from astrin-Eg5 relationships, we inhibited Eg5 activity by monastrol, a specific small-molecular inhibitor of Eg5, in siAstrin treated Hela cells (100 µM for 5 hours. Fig. 3C). Both DMSO-treated (control) and monastrol-treated astrin-depleted cells had similar mitotic indexes. However, only 14% of monastrol-treated cells contained multipolar spindles, compared to 54% in the DMSO-treated cells (mock experiment) (Fig. 3C). We then reversed the monastrol inhibition of Eg5 by washing off the small molecular inhibitor and allowing cells to incubate with active Eg5 for one hour. These cells showed significant recovery of multipolar spindles (53%), comparable to that of astrin-depleted cells without Eg5 inhibition (54%. Fig. 3C). These results indicate that in the absence of astrin, which is similar to TPX2 inactivated cells, the Eg5 is required for the formation of multipolar spindles.

Among the multiple centrosomes in astrin knockdown cells, anti-centrin-2 staining showed that only two of those centrosomes associated with centrin-2, indicating that these are the authentic duplicated centrosomes (Fig. 2D). To determine the temporal multiple centrosomes formed in siAstrin-treated cells, we combined cell synchronization with RNAi. HeLa cells were transfected with siRNAs in the interval between the thymidine and aphidicolin blocks. Centrosomes were examined by γ-tubulin and centrin-2 staining 6 to 14 hours after the cells were released into the cell cycle (Mitosis. Fig. 3D). The numbers of cells showing multiple γ-tubulin spots did not increase in either siGFP transfected cells or in siAstrin transfected interphase cells (Fig. 3D). However, in siAstrin treated mitotic cells, multiple γ-tubulin foci increased dramatically from prometaphase to post-metaphase, indicating that the multiple functional microtubule organizing centers are synthesized *de novo*, resulting in the formation of multipolar mitotic spindles within one cell cycle after depletion of astrin (Fig. 3D). Live cell imaging using HeLa cells stably expressing GFP-α-tubulin confirmed the phenotype (data not shown). However, multiple centrin-2 foci (>2) cell populations were very low and did not change dramatically in either siGFP (control) or siAstrin transfected cells, suggesting these

centrosomes do not recruit centrin-2 in pericentriolar material. This result is in sharp contrast to the siTPX2 phenotype in which multipolar spindles form from multiple split centrosomes, each containing centrin proteins [7]. The result further suggests that, although both astrin and TPX2 are upstream of Aurora-A, they regulate Aurora-A and mitotic spindle assembly differently.

Astrin is epistatic to Aurora-A

To understand further the Aurora-A and astrin interactions, we checked their knockdown phenotypes. Depletion of Aurora-A induced mitotic arrest (Fig. 4A). Flow cytometry analysis of cells depleted of astrin indicated a delay in mitotic progression, judging from the increase in cell populations carrying 4N DNA content (24% G2/M cell population in siAstrin transfected cells, compared to 8% in siGFP, 60% in siAurora-A, or 62% in double transfected cells) (Fig. 4A). This mitotic delay was also evident by immunostaining of Phospho-H3 positive cells, which increased significantly following siAstrin treatment (Fig. 4B).

Astrin regulates G2/M progression by affecting cyclinB1 localization. When cells approach mitosis, cyclin B1 localizes to the centrosomes and mitotic spindles at prophase to metaphase (Fig. 4C and [6]). Additionally CDK1 activity increases, providing a key initiator for mitotic progression. In Aurora-A depleted cells, cyclinB1 associated CDK1 kinase activity is significantly down-regulated [6]. Astrin deprivation diminished localization of cyclin B1 at centrosomes and mitotic spindles (Fig. 4C). Consequently, cyclin B1-associated CDK1 kinase activity in these cells was significantly inhibited, as judged from immunoprecipitated kinase assay (Fig. 4D). However, there is still an obvious remaining CDK1 activity in the astrin-depleted cells, which may explain the mitotic delay, but not the mitotic arrest in these cells (Fig. 4D). These results indicate that, similar to Aurora-A [6], astrin is required for localization of cyclin B1 at the centrosome and mitotic spindle, as well as for CDK1 kinase activation.

Depletion of astrin and Aurora-A also affected cell proliferation shown here by a growth curve (Fig. 4E). Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assays confirmed the increase in the ratio of apoptotic cells in these cells: 9% in siAstrin transfected cells, compared to 1.6% in siGFP cells, 19% in siAurora-A cells, and 20% in siAurora-A+siAstrin cells. So Aurora-A plus astrin or Aurora-A alone depleted cells showed the same degree of cell cycle arrest, a similar decrease in cell proliferation and an increase of apoptosis (Fig. 4). Considering together with the above results that astrin knockdown affected Aurora-A spindle localization, but silencing of Aurora-A did not delocalize astrin, these data indicates that astrin acts as an upstream regulator of Aurora-A to organize its spatial distribution, which may direct Aurora-A access to key substrates.

Acknowledgments

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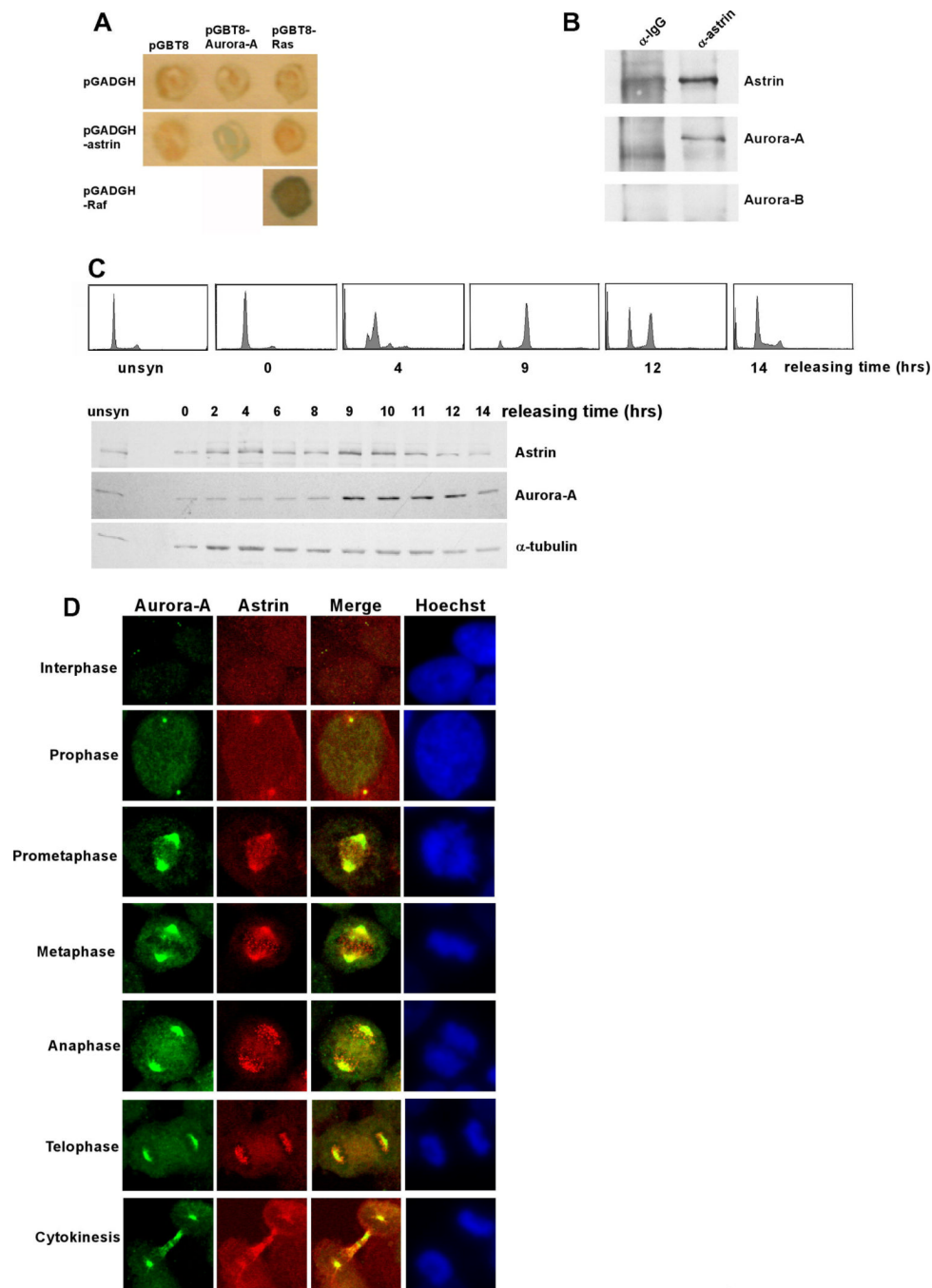
Figure 1. Astrin and Aurora-A interact
(A) Transformants of *S. cerevisiae* pJ64-4a were patched on synthetic complete plates and transferred to Whatman No.1 paper for β-galactosidase assays with X-gal as substrate at 37° C for 1 hour. **(B)** Hela cell lysates were immunoprecipitated with anti-IgG (negative control), or -astrin, respectively. The immunoprecipitates were analyzed by Western blotting. Proteins are labeled at right. **(C)** Hela cells were synchronized at S-phase by double thymidine and aphidicolin arrest, and then released into the cell cycle. Cells at various time-points were collected and analyzed by flow cytometry or by immunoblot. "Unsys" indicates the unsynchronized cells. Numbers indicate the hours from the release point. **(D)** Hela cells grown

on cover slips were fixed and stained with Hoechst 33324, and antibodies directly against astrin and Aurora-A.
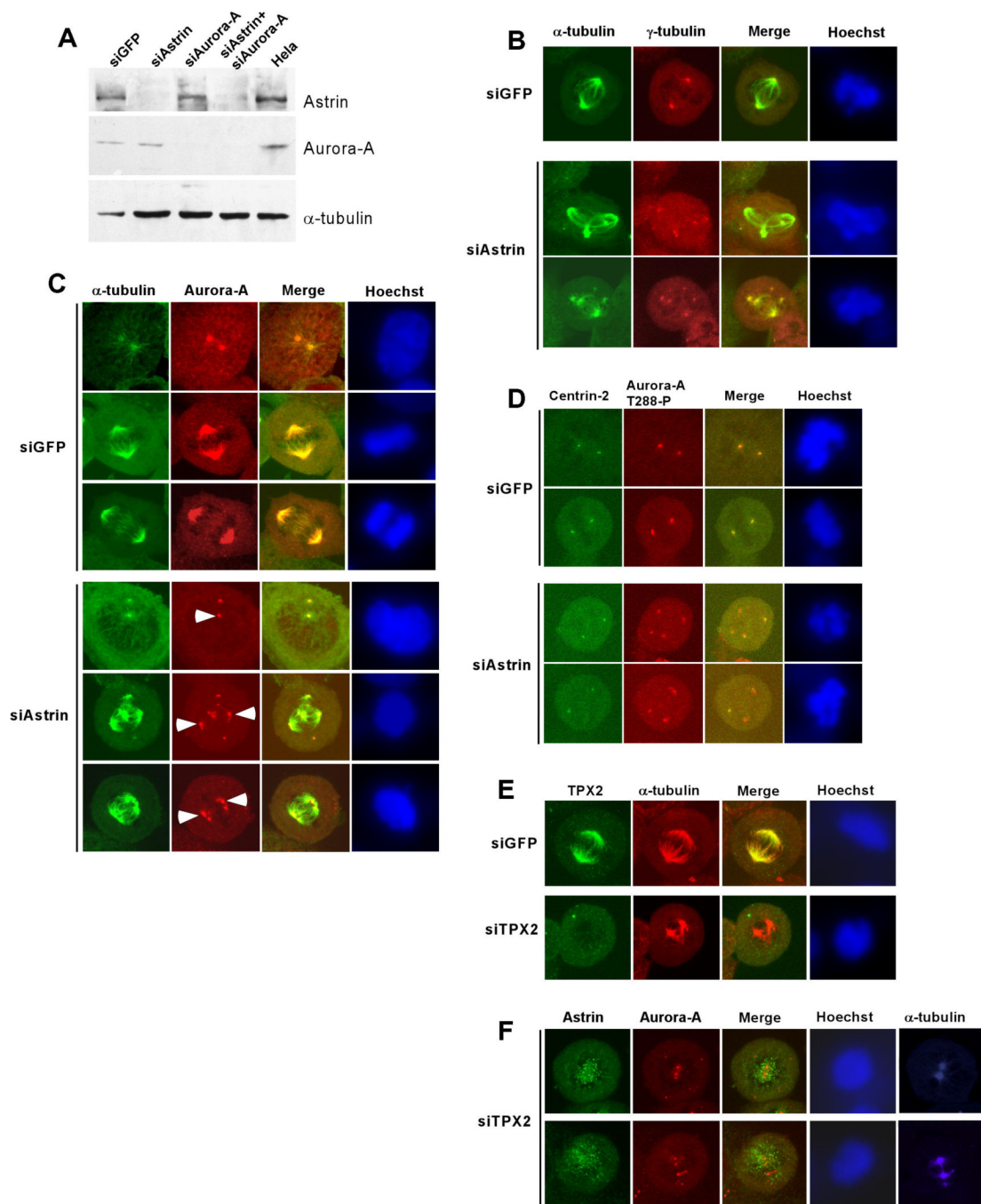
Figure 2. Depletion of astrin delocalizes Aurora-A from multipolar spindles
(**A**) Hela cells transfected with siGFP (control), siAstrin, siAurora-A, or both were lysed and subjected to immunoblot analysis. Proteins are indicated on the right. **(B), (C), (D), (E)** Hela cells seeded on cover slips were transfected twice with indicated siRNAs at a 24-hour interval. Then the cells were fixed for immunofluorescence with Hoechst 33342 and indicated antibodies, respectively.
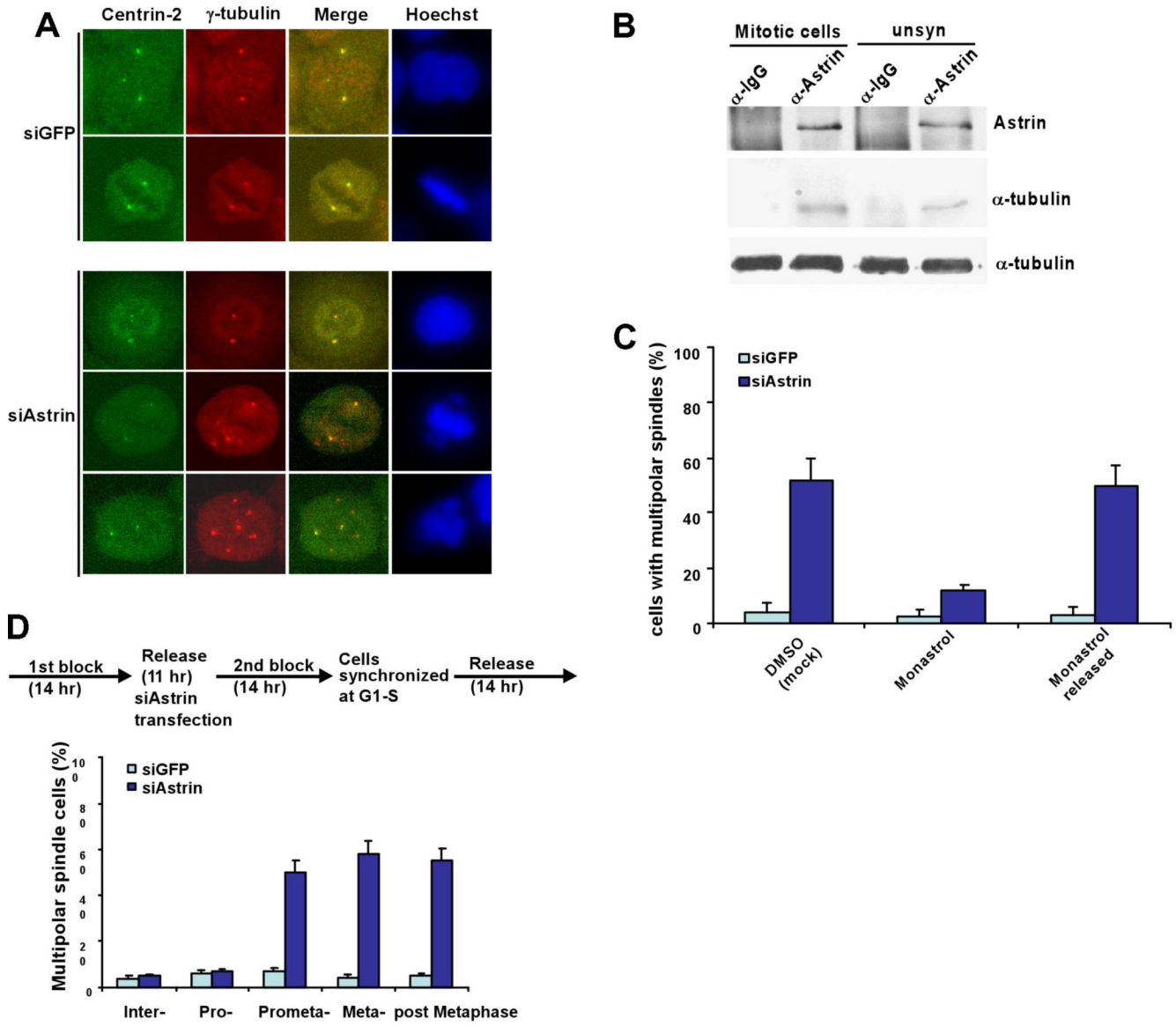
Figure 3. Like Aurora-A, astrin regulates mitotic spindles and centrosome formation
(A) Hela cells transfected with siGFP (control) or siAstrin were fixed for immunofluorescence with Hoechst 33342 and anti-centrin 2 and γ-tubulin antibodies, respectively. **(B)** Hela cell lysates from unsynchronized or mitotic cells were immunoprecipitated with anti-IgG (negative control), or -astrin antibodies, then subjected to immunoblot analysis (upper and middle panels). The whole lysates were immunoblotted with anti-tubulin to insure equal loading (lower panel). Proteins are indicated on the right. **(C)** Hela cells transfected with siGFP (negative control) or siAstrin were treated with DMSO (mock lanes) or monastrol (Monastrol lanes) for 5 hours, or cells released from monastrol inhibition for another 1 hour (Monastrol released lanes). Cells were fixed for anti-γ-tubulin immunofluorescence to assess the centrosome numbers in the cells. **(D)** siAstrin transfection was done after the first thymidine arrest during the double thymidine and aphidicolin synchronization. Released cells after aphidicolin treatment were collected at the indicated time-points and subjected to anti-centrin-2 and -γ-tubulin immunostaining. Cells carrying multiple (>2) γ-tubulin spots were counted and plotted. More than 300 interphase and 300 mitotic cells were counted.
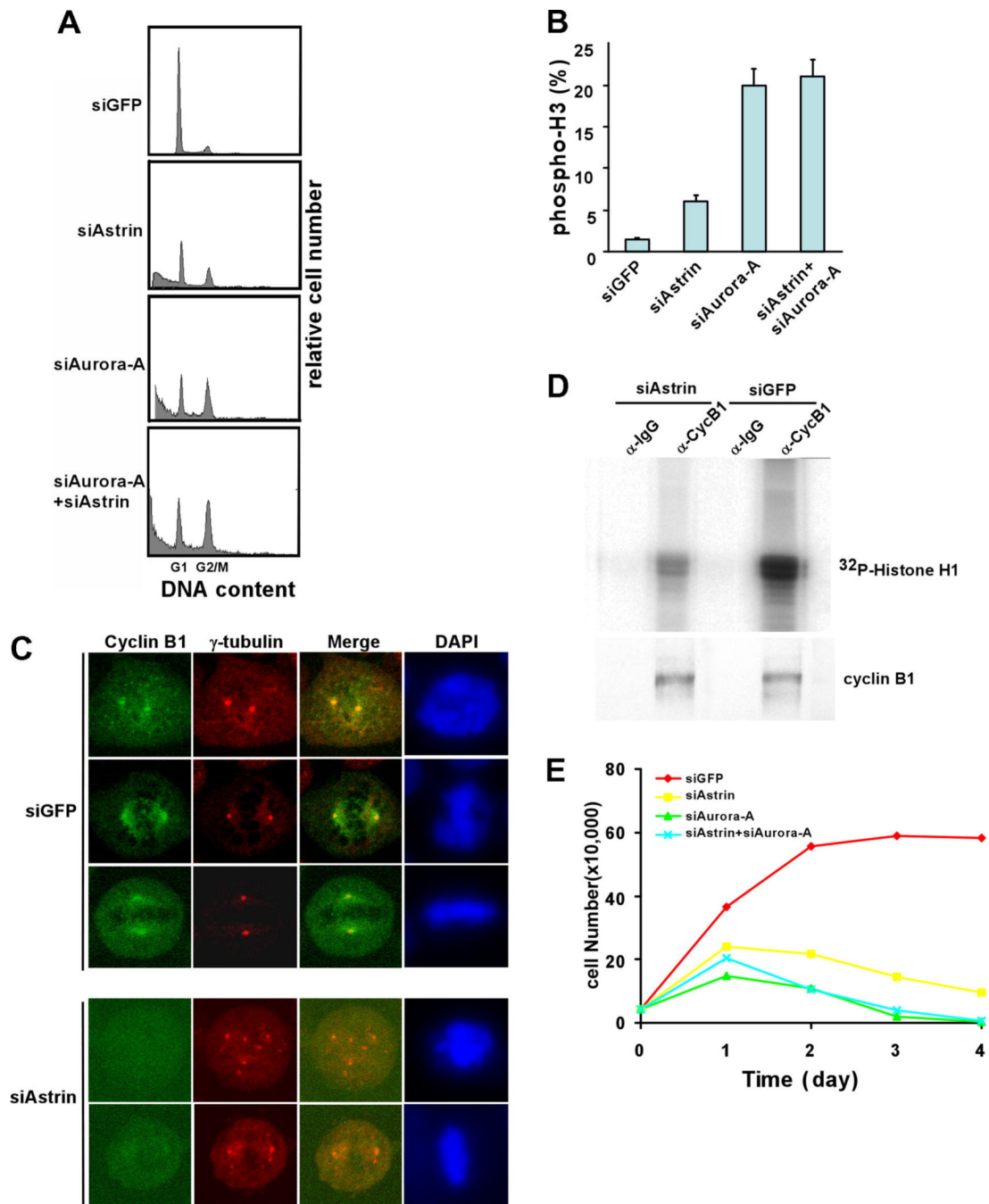
Figure 4. Astrin is epistatic to Aurora-A
(A) Hela cells transfected with various siRNAs were collected and fixed for flow cytometry.
(B) cells in **(A)** were stained with anti-phospho-H3 immunofluorescence. **(C)** Cells in **(A)** were immunostained with anti-cyclin B1 and -γ-tubulin antibodies. **(D)** Equal amounts of cell extracts from siGFP (control) and siAstrin treated cells were immunoprecipitated with anti-cyclin B1 antibody. The resulting precipitates were tested both for CDK1 kinase assay using histone H1 as a substrate (upper panel), and by immunoblot with anti-cyclin B1 antibody (lower panel) to ensure equal loading. **(E)** Hela cells were seeded into 35 mm plates and transfected with siRNAs. At the indicated time (days), cells were collected, cell numbers counted and plotted as growth curves. This is a represent of three repeated experiments.