FEBS Letters 581 (2007) 1661–1672

Cyclin B1/Cdk1 binds and phosphorylates Filamin A and regulates its ability to cross-link actin☆

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Received 19 January 2007; revised 19 February 2007; accepted 14 March 2007

Edited by Francesc Posas

Abstract Substantial actin remodelling occurs prior to mitosis as cells alter their shape in preparation for cytokinesis. In mammalian cells, mitosis is initiated by a heterodimer of cyclin B1 and the cyclin dependent kinase 1 (Cdk1) serine/threonine kinase. In this report. we show that human cyclin B1 binds the actin cross-linking protein Filamin-A (FLNa). The proteins co-immunoprecipitate and co-localize in mitotic human cells. We find that cyclin B1/Cdk1 can phosphorylate FLNa in vitro and reduce its ability to gelate actin. We have also identified serine 1436 as one FLNa residue phosphorylated by cyclin B1/Cdk1 in vitro. Our results suggest a role for cyclin B1/Cdk1 in FLNa-dependent actin remodelling.

Keywords: Cyclin B1; Filamin A; Mitosis; Phosphorylation; Cytoskeleton; Actin; Gelation; Cyclin dependent kinase; Cdk1; Cell cycle

1. Introduction

Cell cycle progression is controlled by cyclin proteins and their binding partners, the cyclin dependent kinases (cdks) [1]. In mammalian cells, mitosis is initiated by the heterodimer of cyclin B1 and cyclin dependent kinase 1 (Cdk1) [2–4]. Cyclin B1/Cdk1 activation initiates mitosis through the phosphorylation of proteins controlling chromatin condensation and cytoskeletal structure [5,6]. A threshold level of cyclin B1 protein and Cdk1 kinase activity is required for G_2/M transition [7] and inhibition of cyclin B1 transcription is one mechanism by which the p53 tumour suppressor inhibits mitosis [8].

As adherent mammalian cells prepare for mitosis, they assume a rounded shape and the total contact area between the cell and its growth substrate decreases [9]. Substantial changes in the actin cytoskeleton occur during this process [9]. In the fission yeast *Schizosaccharomyces pombe,* integrity of the actin cytoskeleton is necessary for cells to undergo mitosis, and inhibition of actin polymerization by lantrunculin B delays the completion of nuclear division [10]. Because of its key role in mitotic initiation, cyclin B1/Cdk1 has long been thought to be involved in mitotic actin remodelling in mammalian cells [11]. Cyclin B1/Cdk1 can control actin remodelling during mitosis, at least in part, by phosphorylating the actin cross-linking protein caldesmon [12]. In this report, we show that cyclin B1/Cdk1 interacts with and can partially regulate the function of the actin cross-linking protein Filamin-A (FLNa, ABP280, FLN1).

Filamins are large (~280 kDa) cytoplasmic proteins initially identified by their ability to bind filamentous actin from non-muscle cells [13]. There are three mammalian Filamins (FLNa, FLNb and FLNc). Each protein contains an amino-terminal actin-binding domain (ABD), a long rod-like domain of 24 repeats and two flexible hinge-like structures (Fig. 1A). Repeats are believed to be docking sites for filamin-binding proteins [15]. Filamins homodimerize via repeat 24 and form a V-shaped structure [13]. The hinges, one between repeats 15 and 16 and the other between 23 and 24, are predicted to allow for conformational flexibility of the FLNa molecule. Of the three human filamin isoforms, FLNa is the most abundant and widely expressed [14]. FLNb also has a broad tissue distribution, but is less abundant than FLNa. FLNc is predominantly expressed in cardiac and skeletal muscle [14]. Filamins are involved in cross-linking cortical actin and can organize filamentous actin into stress fibres and three-dimensional gel-like networks [14]. Filamins also act as scaffolding proteins that link cytoplasmic signalling proteins to the actin cytoskeleton. FLNa is known to bind the GTPase RalA [15] and Trio, a guanine nucleotide exchange factor that is able to induce actin-based ruffling in cells [16]. In addition, granzyme B and the androgen receptor interact with FLNa between hinge 1 and 2 in the FLNa carboxyl-terminus [14,15,17,18].

In this report, we show that cyclin B1 binds to human FLNa and the two proteins co-immunoprecipitate in human cells. Cyclin B1 and FLNa also co-localize in mitotic cells. We find that cyclin B1/Cdk1 phosphorylate FLNa and decrease its

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☆ This work was supported by the National Cancer Institute of Canada, with funds from the Canadian Cancer Society.

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Abbreviations: AA, antibiotic/antimycotic; ABD, actin-binding domain; BSA, bovine serum albumen; β-arr, β-arrestin; CAM KII, calcium/calmodulin-dependent protein kinase II; Cdk1, cyclin dependent kinase 1; DAPI, 4′,6-diamidino-2-phenylindole; D Box, destruction box; ECM, extracellular matrix; ERK, MAPK extracellular signal-regulated kinase; FBS, fetal bovine serum; FLNa, b, c, filamin A, B, C; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HS-P84/90β, heat shock protein 84/90β; IP, immunoprecipitation; MAPK, mitogen activated protein kinase; PBS, phosphate buffered saline; P-KCα, protein kinase Cα; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; WB, Western blot; WCL, whole cell lysate

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doi:10.1016/j.febslet.2007.03.041

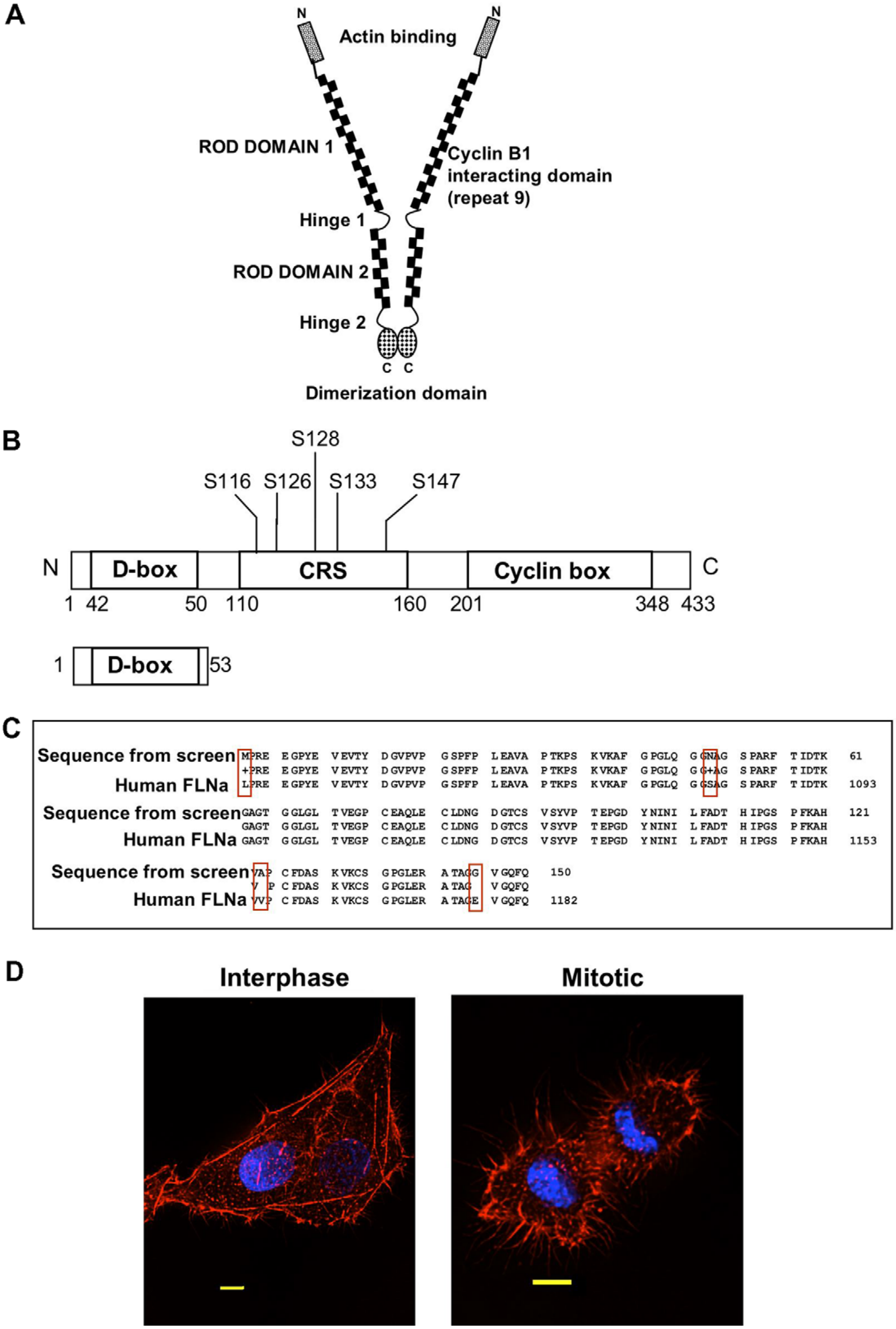

Fig. 1. Interaction between cyclin B1 and Filamin A. (A) Schematic diagram of a FLNa dimer. The protein contains an amino-terminal actin-binding domain (ABD), 24 repeats, 2 hinges and a carboxyl-terminal dimerization domain. Adapted from Stossel et al. [14]. (B) Domain structure of cyclin B1 indicating the D-box, the CRS (cytoplasmic retention sequence) and the Cyclin box. Phosphorylation sites within the CRS are also indicated. The fragment of cyclin B1 used in our two-hybrid screen includes the D-box. (C) Comparison of the interactor sequence from the screen indicates near complete identity with human FLNa. Mouse and human FLNa have high sequence identity. This region of human FLNa spans part of repeats 8 and 10 and all of repeat 9. (D) Actin structure in human HeLa cells differs between interphase and mitotic cells. The yellow scale bar is 10 μM.

ability to cross-link actin in vitro. Our data suggest that cyclin B1/Cdk1 can elicit changes in the actin cytoskeleton by phosphorylating FLNa and reducing its ability to cross-link actin.

2. Materials and methods

2.1. Yeast two-hybrid

The yeast two hybrid was performed as previously described [19]. The pLexA pBTM116 cyclin B1 bait plasmid (pLex-B1) was constructed using the amino-terminal 53 amino acids of cyclin B1 using the primers 5′GACCCGGGGGCGCTCCGAGTCACCAGG3′ (sense) and 5′CAGTCGACGTTAACTGACTTTGTTACCAAT3′(anti-sense). The PCR fragment was digested with SmaI and SalI and inserted into the SmaI/SalI site of pBTM116. A mouse 10.5 day old embryonic library cloned into the VP16 plasmid was used as prey. Histidine-positive colonies of L40 yeast were lysed in liquid nitrogen and assayed for β-galactosidase activity on filters. Positive colonies were further analyzed after loss of the pLex-B1 plasmid and if no self-activation occurred, inserts from β-galactosidase positive colonies were sequenced. Sequences were analyzed using the National Center for Biotechnology Information (NCBI) nucleotide BLAST database.

2.2. Cell culture

HeLa cells and 293 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). M2 and A7 were gifts from Dr. Thomas Stossel (Brigham and Women's Hospital, Harvard Medical School). HeLa cells were maintained in Minimal Essential Medium alpha (Invitrogen Life Technologies, Burlington, Ont., Canada) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (AA) (Invitrogen). 293 cells were maintained in Dulbecco Modified Eagle medium (Invitrogen) supplemented with 10% FBS and 1% AA. M2 and A7 cells were maintained in Minimal Essential Media supplemented with 10% FBS, 1% AA, 1% sodium pyruvate, 1% L-glutamine, 10 mM HEPES (Invitrogen) and the A7 cells were also supplemented with 0.5 mg/ml of G418 (Invitrogen). All cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Plasmid DNA and transfections

GFP-tagged cyclin B1 was a gift of Dr. Anja Hagting (Wellcome/CRC Institute) and FLNa was a gift of Dr. Fumihiko Nakamura (Brigham and Women's Hospital, Harvard Medical School). For transfection, M2, A7 and HeLa cells were seeded at 3.0×10^5 cells in 100 mm plates and transfected the following day with ExGen 500 (Fermentas Life Sciences, Burlington, Ont., Canada) according to manufacturers protocol.

2.4. Co-immunoprecipitation and immunoblotting

HeLa and 293 cells were treated with 100 ng/ml nocodazole (Sigma–Aldrich, Oakville, Ont., Canada) overnight (14–16 h). The next day, cells were collected, lysed in a buffer of 1% NP-40, 50 mM Tris pH 7.4, 5 mM EDTA, 150 mM NaCl containing a 1× protease inhibitor cocktail (Sigma), left on ice for a minimum of 30 min and centrifuged at 4 °C for 10 min. at 14000 rpm. The supernatant was collected and protein concentration was determined using the Bradford method (BioRad, Mississauga, Ont., Canada). Antibodies used were filamin-A (Neomarkers, Montreal, Que., Canada; Chemicon International, Temecula, CA and Santa Cruz, Santa Cruz, CA), cyclin B1 (Neomarkers and Santa Cruz), myt1 (Santa Cruz), normal mouse (Santa Cruz), actin (MP Biomedicals, Aurora, OH) and MPM2 (Upstate Biotechnology, Lake Placid, NY). For co-precipitation, 250–750 μg of cell lysate was incubated with 0.5–1 μg of cyclin B1, FLNa, normal mouse, GFP (Santa Cruz) or FLAG (Sigma) antibody. The mixture was incubated from one hour to overnight at 4 °C with gentle rocking. Lysates were then incubated with protein-A or G beads (Sigma and Santa Cruz) from 1 h to overnight at 4 °C. Beads were washed 3× with lysis buffer and the beads were eluted with 2× SDS sample buffer by boiling for 8 min. Samples were resolved by 7.5–12% gradient SDS–PAGE and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Blots were then blocked with 5% milk in TBS-T (20 mM Tris pH 7.5, 150 mM NaCl and 0.1% Tween 20) for between 1 hr to overnight at 4 °C, washed in TBS-T 3× totalling 15 min. (this wash step was done between each treatment), probed with the specific primary antibody indicated in each figure overnight at 4 °C, washed, incubated with a horseradish peroxidase secondary antibody (BioRad) for 45 min., washed, treated with ECL (Amersham Biosciences, Piscataway, NJ) and exposed to X-ray film.

2.5. Glutathione S-transferase (GST) protein construction

Human FLNa fragments were cloned into pGEX-4T1 vector (Pharmacia). The FLNa fragments are FLNa-1, 33 kDa representing the actin-binding domain; FLNa-2, 110 kDa, repeats 1–10; FLNa-3, 60 kDa, repeats 11–16 and FLNa-4, 80 kDa, repeats 17–24. GST-FLNa fusion proteins were expressed in *Escherichia coli* BL-21 cells (Invitrogen) according to standard protocols, and affinity purified by glutathione-sepharose-4B beads (Amersham Biosciences) and Thrombin (Amersham Biosciences) cleaved to remove the GST moiety. Three mutants were created in repeats 12–15 using PCR mutagenesis and cloned into pGEX-4T1. The mutated sites are serine 1436 (**S**PFK → **G**PFK), serine 1533 (R**S**PFK → R**G**PFK) and serine 1630 (**S**PYR → **A**PYR). Cyclin B1 fragments corresponding to amino-terminal amino acids 1–40 of cyclin B1; amino-terminal amino acids 40–53 of cyclin B1; amino-terminal amino acids 1–53 of cyclin B1; a deletion mutant lacking the first 53 amino acids; and a full length cyclin B1 construct were cloned into the pGEX-4T1 vector. All constructs were sequenced before use.

2.6. In vitro cyclin B1/FLNa interaction

For experiments with whole cell lysates (WCL) and baculovirus purified FLNa, GST linked cyclin B1 proteins were loaded onto 30 μl of glutathione sepharose-4B beads, left at room temperature rotating for 30 min to 2 h and washed 3× with phosphate buffered saline (PBS). The beads containing the GST-cyclin constructs were then incubated with 1 mg of total 293 or HeLa lysates in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% NP-40, 0.5% deoxycholate and 0.1% SDS) or 357 ng of FLNa for 30 min to overnight at 4 °C. Samples were then washed 3× in PBS. The binding of recombinant FLNa constructs to recombinant GST-cyclin B1 constructs was carried out by combining equal amounts of the GST cyclin B1 and FLNa (GST free) with 30 μl of glutathione sepharose-4B beads as above. Precipitated proteins were detected by a mixture of antibodies that detect distinct FLNa regions: Neomarkers FLN Ab-1, Santa Cruz FLNa E-3 and FLNa H-300 as well as Chemicon FLNa mab1680 according to manufacturer's instructions.

2.7. In vitro cyclin B1/Cdk1 phosphorylation

Human FLNa was purified from HeLa cells according to a previously published protocol [20]. Human FLNa expressed in baculovirus was purified according to Nakamura et al. [21]. Cyclin B1/Cdk1 was either purchased as an active enzyme derived from recombinant proteins (Upstate) or immunopurified from HeLa cells. For immunopurification, HeLa were treated with 100 ng/ml nocodazole (Sigma) overnight and active kinase immunoprecipitated with a cyclin B1 antibody and protein-G beads as above. Beads were washed 2× in lysis buffer (same as above) they were then washed 2× in E1A buffer (50 mM Tris pH 7.4, 1 mM EDTA, 0.5% NP-40, 250 mM NaCl), then washed 3× in kinase buffer (50 mM HEPES pH 7.0, 5 mM $MnCl_2$, 10 mM $MgCl_2$, 0.8 mM EGTA). The beads were left in kinase buffer and 10 μCi of ^{32}P-ATP and 0.05 mM cold ATP was added for 30 min. The reaction was stopped by the addition of 2× SDS sample buffer, samples were boiled for 8 min, loaded onto 10% PAGE, dried and exposed to a phosphorimager screen and/or Storm 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA) or autoradiography. Quantitation was performed using ImageQuant TL software (Molecular Dynamics). In the case of recombinant cyclin B1/Cdk1, the kinase assay was performed according to manufacturers instructions using 10 μCi $[\gamma - {}^{32}P]$-ATP and 1 mM ATP. Kinase reactions were electophoresed and quantitated as above or blotted onto P30 filter paper as per the manufacturer's instructions, added to BetaMax scintillation fluid (ICN Biomedicals, Irvine, CA, USA) and counted with a Wallac 1414 Liquid Scintillation Counter (Fisher Scientific Limited, Ottawa, Ont., Canada). Chicken gizzard FLN (RDI, NJ) and thrombin cleaved GST purified human FLNa constructs were also used as substrates for immunopurified cyclin B1/Cdk1 using the kinase protocols above.

2.8. Actin gelation assays

The ability of FLNa to gelate actin was measured by an actin sedimentation assay (Cytoskeleton, Denver, CO) or by falling ball viscometry [21]. For falling ball viscometry, FLNa was incubated with either cyclin B1/Cdk1 or bovine serum albumen (BSA) at 30 °C for the times incubated in the figure legends and then added to an actin polymerization solution containing 23 μM G-actin according to manufacturer's instructions (Cytoskeleton) in a final volume of 100 μl. Samples were then loaded into a capillary tube and incubated for 1-h at room temperature and viscosity measured as described [21], except that Blue Tack (Home Depot, Ont., Canada) was used to seal the capillary. For actin sedimentation, FLNa was added to protein-G beads containing immunopurified cyclin B1/Cdk1 and the kinase reaction was carried out as above, followed by removal of the supernatant containing FLNa. Purified polymerized actin (Cytoskeleton) was added to the samples at room temperature for 1 h, followed by 14000 rpm spinning at 4 °C for 30 min. The supernatant was then removed and the pellet resuspended in 2× SDS sample buffer, heated at 80 °C for 8 min and electrophoresed on a 10% PAGE gel. The gel was then stained in Coomassie brilliant blue solution overnight at room temperature, de-stained and dried. Gelled actin was quantitated using an Alpha Innotech video documentation device and quantitated using Alpha Imager software. Roscovitine (1.3 μM final concentration) and olomoucine (14 μM final concentration) (Calbiochem, San Diego, CA) were added to the immunopurified cyclin B1/Cdk1 complexes in kinase buffer (50 mM HEPES pH 7.5, 1 mM Dithithreitol, 0.02% (w/v) Tween 20, 100 mM NaCl) and incubated at RT for 30 min, after which the beads were washed in kinase buffer (50 mM HEPES pH 7.0, 5 mM $MnCl_2$, 10 mM $MgCl_2$, 0.8 mM EGTA) and incubated as above in the presence of the inhibitors.

2.9. Immunofluorescence

Cells were treated as stated previously [22]. Briefly, cells were plated on coverslips, washed twice with PBS and fixed with 4% paraformaldehyde at 4 C for $\sim$1 h, and washed 3× with PBS over 20 min (same for all wash steps). Cells were then washed and permeabolized (0.02% triton-X 100 in PBS) for 5 min, followed by washing and blocking in 5% normal goat serum for between 1 h and overnight. The blocker was removed and cyclin B1 Ab-3 or Ab-4 (Neomarkers) 1:500 was added to the cells for between 1 h to overnight at 4 C. Following PBS wash, 1:500 Alexa-conjugated 546 nm goat anti-mouse secondary (Molecular Probes, Eugene, OR) was added for 30 min, washed, and 1:1000 FLNa Ab-1 (Neomarkers) was added for 1 h to overnight at 4 C. Following PBS wash, 1:500 Alexa-conjugated 488 nm goat anti-mouse secondary was added for 30 min, washed, and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) was added for 10 min followed by a wash. Cells were stained with DAPI (Sigma) to visualize DNA and actin was visualized using AlexaFluor 546 phalloidin (Molecular Probes). Slides were imaged using a Leica DMIRB inverted stage microscope and deconvoluted using Open Lab software (Improvision, Lexington, MA).

3. Results

3.1. Two hybrid analysis identifies FLNa as a cyclin B1 interacting protein

To identify proteins that interact with cyclin B1, we used a yeast two-hybrid screen utilizing the amino-terminal 53 amino acids of human cyclin B1 as bait (Fig. 1B). This portion of cyclin B1 includes the destruction box (D-box), a region of the protein that regulates proteolytic destruction of cyclin B1 [23]. We used a mouse embryonic 10.5 day old cDNA library as prey and screened $\sim 1 \times 10^7$ colonies. A list of interactors with identifiable homology is shown in Table 1. The most commonly identified interactor was mouse HSP84/HSP90β, likely due to its capacity to bind unfolded proteins. Other interactors include the Pax3 transcription factor, ribosomal proteins, PCNA and peroxiredoxin I. Both PCNA and peroxiredoxin I have previously been shown to interact with cyclin B1/Cdk1 [24,25], suggesting that our screen is identifying bona fide cyclin B1 interacting proteins. We decided to pursue FLNa as a cyclin B1 interactor because of the marked differences in actin structure between interphase and mitotic cells (Fig. 1D). Mitotic cells have fewer and shorter stress fibres and ectopic expression of cyclin B1/Cdk1 has previously been reported to alter the actin cytoskeleton [11]. We reasoned that regulation of FLNa activity could be one possible pathway through which cyclin B1/Cdk1 might affect actin remodelling at mitosis. The FLNa domain that interacts with cyclin B1 includes all of FLNa repeat 9 and part of repeats 8 and 10 (Fig. 1A).

Table 1
Cyclin B1 interactors

Gene	Hits	GI accession
Filamin A (FLNa)	1	13752160
Mouse HSP84 (HSP90β)	6	194026
Mouse BMS1-like ribosome assembly protein	1	21410150
Mouse Pax3 transcription factor	1	6679210
Mouse ribosomal protein L4 (Rpl4)	1	31340596
Mouse mitochondrial carrier triplet repeat 1 (Mcart1)	1	57977298
Mouse up-stream binding factor (UBF) transcription factor	1	20913288
Mouse nicalin homolog (zebrafish) (Ncln)	1	33469042
Mouse U1 small nuclear ribonuclearprotein 70 kDa	2	12852074
Mouse Peroxiredoxin I	1	54035545
Mouse PCNA	1	26353219
Mouse signal peptide, CUB domain, EGF-like 1 (Scube1)	1	12738839

3.2. Interaction of cyclin B1 and FLNa in mammalian cells

In order to confirm a physical interaction between human FLNa and cyclin B1, we next determined whether cyclin B1 and FLNa could co-precipitate in human cells. The abundance of cyclin B1 is very low in normally cycling cells because of the small number of cells entering into mitosis. We therefore increased cyclin B1 protein levels in human HeLa and 293 cells by treating them with nocodazole, a microtubule destabilizing agent that arrests cells in G2/M, increasing cyclin B1 protein levels and cyclin B1/Cdk1 kinase activity [26]. Nocodazole increases cyclin B1 protein levels but has no effect on FLNa (Fig. 2A). As shown in Fig. 2B, a cyclin B1 antibody immunoprecipitates a detectable amount of FLNa in HeLa and 293 cells treated with nocodazole (top panels). FLNa was not precipitated by control normal mouse serum. There is no detectable actin in the immunoprecipitates (lower panels), indicating that the FLNa/cyclin B1 co-precipitation is not due to indirect association of either protein with actin. While co-precipitation is detectable, only a small fraction of FLNa is associated with cyclin B1, likely due to the large intracellular excess of FLNa relative to cyclin B1. To determine whether FLNa and cyclin B1 interact in untreated cells and to determine if there is a reciprocal interaction between the two proteins, we next transfected M2 cells, a human melanoma cell line lacking endogenous FLNa, with cyclin B1 and FLNa. We used a GFP-cyclin B1 allele for transfection so that the molecular weight of the transfected protein ($\sim$90 kDa) was distinguishable from the heavy chain of the precipitating anti-

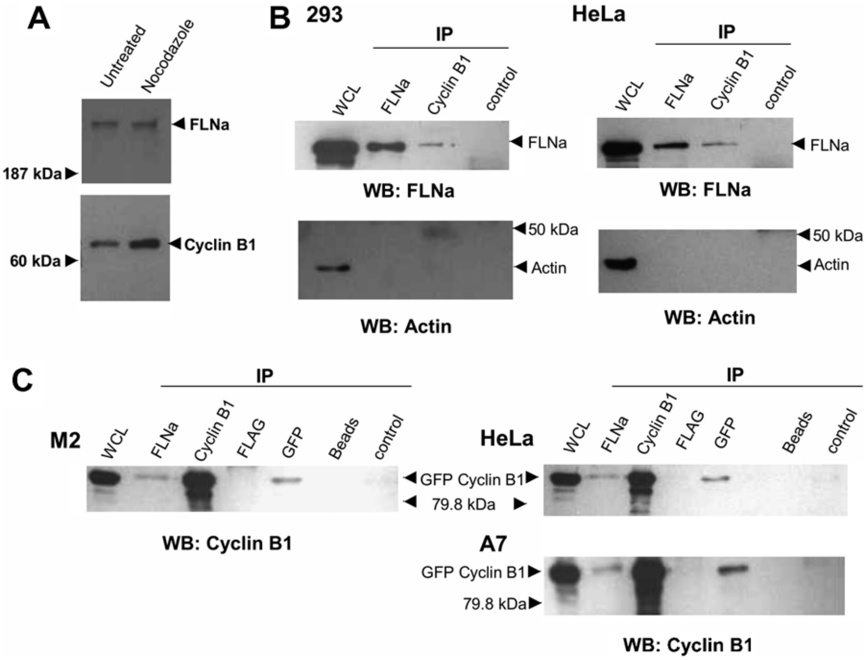

Fig. 2. Cyclin B1 binds to human FLNa. (A) Nocodazole (100 ng/ml) treatment of HeLa cells increases cyclin B1 protein levels but not FLNa. (B) Co-immunoprecipitation of cyclin B1 and FLNa in human 293 and HeLa cells treated with nocodazole (100 ng/ml). The upper panel is a Western blot for FLNa from whole cell lysate (WCL), or FLNa, cyclin B1 or normal mouse IgG immunoprecipitation. FLNa is approximately 280 kDa. The lower panel is the same immunoprecipitation blotted for actin. (C) Cyclin B1 co-immunoprecipitates with FLNa in FLNa-deficient M2 cells transfected with FLNa and a GFP-cyclin B1 allele (left panel). Antibodies used for immunoprecipitation are indicated. Normal mouse serum and FLAG serve as controls. GFP-cyclin B1 was detected by blotting with a cyclin B1 antibody. (Right panel) Cyclin B1 co-immunoprecipitates with FLNa in filamin-containing HeLa and A7 cells transfected with a GFP-cyclin B1 allele.

body. As shown in Fig. 2C, a FLN antibody immunoprecipitates cyclin B1 in M2 cells (left panel). Cyclin B1 is also precipitated by a GFP antibody but not by control normal mouse serum or a FLAG antibody control. Transfection of GFP-tagged cyclin B1 into the FLNa-expressing human cell lines HeLa and A7 similarly shows cyclin B1 co-immunoprecipitation with FLNa (Fig. 2C, right panel). A7 is an M2 variant that has been engineered to express FLNa at normal levels. We have performed co-immunoprecipitation experiments with multiple cyclin B1 and FLNa antibodies from different commercial sources with similar results (not shown), indicating that FLNa and cyclin B1 physically interact.

3.3. *Interaction of purified cyclin B1 with FLNa*

To further examine the cyclin B1-FLNa interaction, we created recombinant GST proteins containing our initial bait and smaller fragments thereof. These fragments correspond to the initial bait (amino acids 1–53), the D-box (amino acids 42–50) and non-D-box fragment (amino acids 1–40). These GST-proteins were incubated with whole cell lysates from HeLa and 293 cells to determine whether they could precipitate FLNa. As shown in Fig. 3A, amino acids 1–40 and 1–53 both bind FLNa in the cell lysates, whereas the construct with amino acids 40–53 and the GST protein did not. We then determined whether baculovirus purified human FLNa could interact with these proteins. As shown in Fig. 3B (top panel), cyclin B1 amino acids 1–40, full length cyclin B1, and to a lesser extent amino acids 1–53 bound to FLNa. A small background FLNa band is detectable in 40–53, 54–433 and GST alone lanes. This background binding may be due to a small amount of FLNa binding the glutathione sepharose beads since the BL-21 lysate alone also shows some binding (Fig. 3B). A Coomassie stain of the baculovirus FLNa is shown in the lower panel of Fig. 3B. Taken together, these results map the FLNa interaction domain to cyclin B1 amino acids 1–40.

We determined whether amino acids 1–40 could interact with regions of FLNa other than those identified from the two hybrid screen. To this end, we created truncated versions of FLNa: FLNa-1 (FLNa actin-binding domain; MW: 33 kDa), FLNa-2 (FLNa repeats 1–10; MW: 110 kDa), FLNa-3 (FLNa repeats 11–16; MW: 60 kDa) and FLNa-4 (repeats 17–23 and dimerization domain; MW: 80 kDa). These proteins are schematically depicted in Fig. 3C. FLNa-2 binds detectably to cyclin B1 1–40 and no interaction was observed with either FLNa-3 or FLNa-4. (Fig. 3D, left panel) There is some interaction between FLNa-1 to cyclin B1 depicted in Fig. 3D (left panel), but this interaction is not consistently observed. FLNa-2 interacts with cyclin B1 residues 1–40 and does not appreciably bind cyclin B1 residues 54–433 or GST (Fig. 3E). Taken together, our data suggest a model for cyclin B1/FLNa interaction where cyclin B1 amino acids 1-40 bind the FLNa amino-terminal region in repeat 9.

3.4. *Cyclin B1 and FLNa co-localize in human cells*

To establish that cyclin B1 and FLNa interact in intact cells, we used deconvolution immunofluorescence to determine the intracellular localization of both proteins as a function of the cell cycle. Cyclin B1 and FLNa proteins co-localize strongly in mitotic cells in telophase and metaphase (Fig. 4). In interphase cells, cyclin B1 and FLNa have little super-imposable distribution suggesting that the two proteins interact primarily in mitotic cells. To confirm the specificity of mitotic cyclin B1/

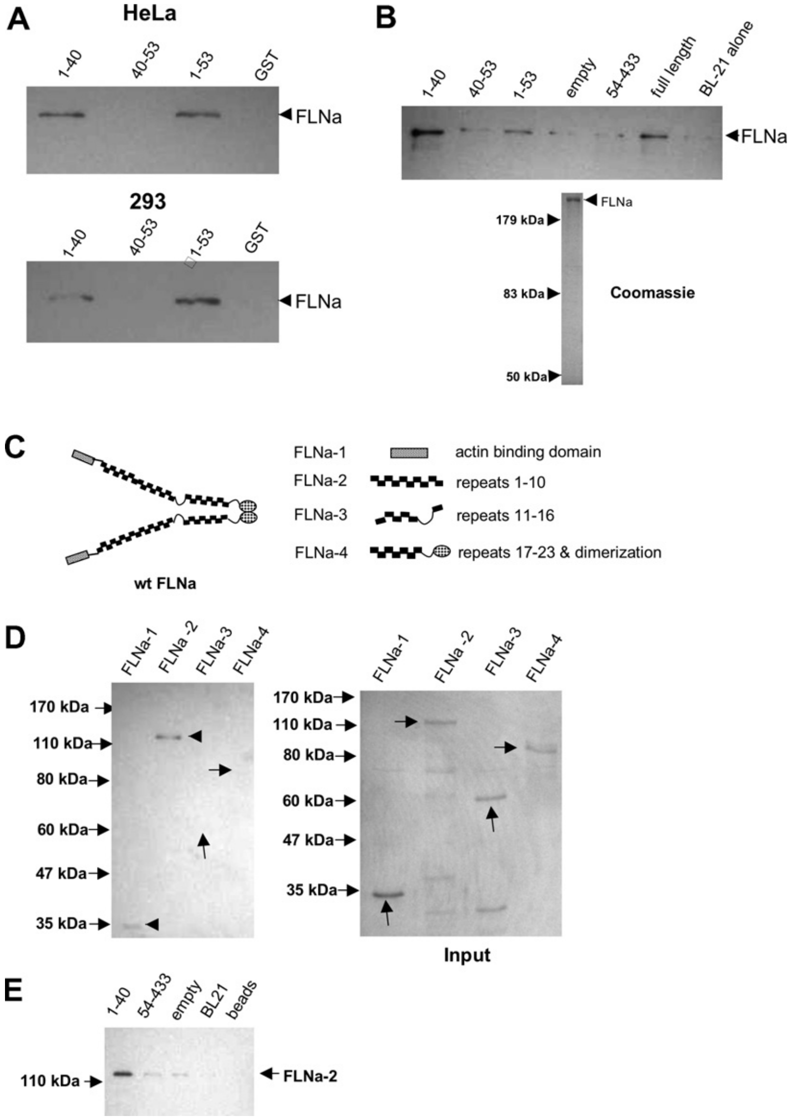

Fig. 3. Mapping cyclin B1/FLNa interaction sites. (A) GST fusion proteins derived from the indicated cyclin B1 amino acids were incubated with either HeLa or 293 total cell lysates. Bound FLNa is detected by Western blotting for FLNa. (B) Cyclin B1 GST constructs bind purified FLNa. GST fusion proteins derived from the indicated cyclin B1 amino acids were incubated with baculovirus purified human FLNa and bound FLNa detected by Western blotting. BL-21 bacterial lysates were used as a control. Coomassie stained baculovirus purified human FLNa is shown in the lower panel. (C) Schematic depiction of FLNa truncations used for cyclin B1 interaction analysis. (D) FLNa-2 is the major site for cyclin B1 interaction. One microgram of cyclin B1 amino acids 1–40 was incubated with 1 μg of the indicated FLNa fragments (FLNa 1–4) and bound FLNa detected by Western blotting for FLNa (left panel). Four different FLNa antibodies were used to blot the membrane. The input FLNa fragments are shown in a Coomassie gel in the right panel. (E) GST-cyclin B1 amino acids 1–40 and 54–433 were incubated with the FLNa-2 fragment and interacting proteins detected by Western blotting for FLNa. GST alone, beads alone and BL-21 cells alone were controls.

FLNa co-localization we next determined whether the Myt1 protein also co-localizes with FLNa. Myt1 is involved in the inhibitory phosphorylation of Cdk1 at threonine 14 during prophase of the cell cycle, and is localized to the endoplasmic reticulum and Golgi complex both during interphase and mitosis [27]. As is shown in Fig. 4C, the Myt1 protein is cytoplasmic at interphase. In mitotic cells, the distribution of Myt1 and FLNa does not show substantial super-imposable distribution. This indicates that the co-localization of cyclin B1 with FLNa in mitotic cells is specific.

3.5. Cyclin B1/Cdk1 phosphorylate FLNa

Cyclin B1/Cdk1 is known to phosphorylate the actin associated proteins actopaxin, paxillin and caldesmon [28–30]. FLNa is a substrate for the kinases p21-activated kinase-1 (Pak1) [31] and protein kinase Cα[32]. We next determined whether FLNa (purified from chicken gizzard, human cells, or baculovirus) could be substrates for cyclin B1/Cdk1 kinase activity. As shown in Fig. 5A, purified recombinant cyclin B1/Cdk1 (Upstate) phosphorylates human FLNa in a time- and dose-dependent manner (upper and lower panels, respectively). Phosphorylation of FLNa is maximal at ~1 h. Recombinant cyclin B1/Cdk1 (Upstate) phosphorylates baculovirus purified human FLNa and immunopurified cyclin B1/Cdk1 phosphorylates chicken gizzard FLN in a dose-dependent fashion (Fig. 5B, bottom and top, respectively).

To further explore FLNa phosphorylation by cyclin B1/Cdk1, we determined whether full length FLNa contained an

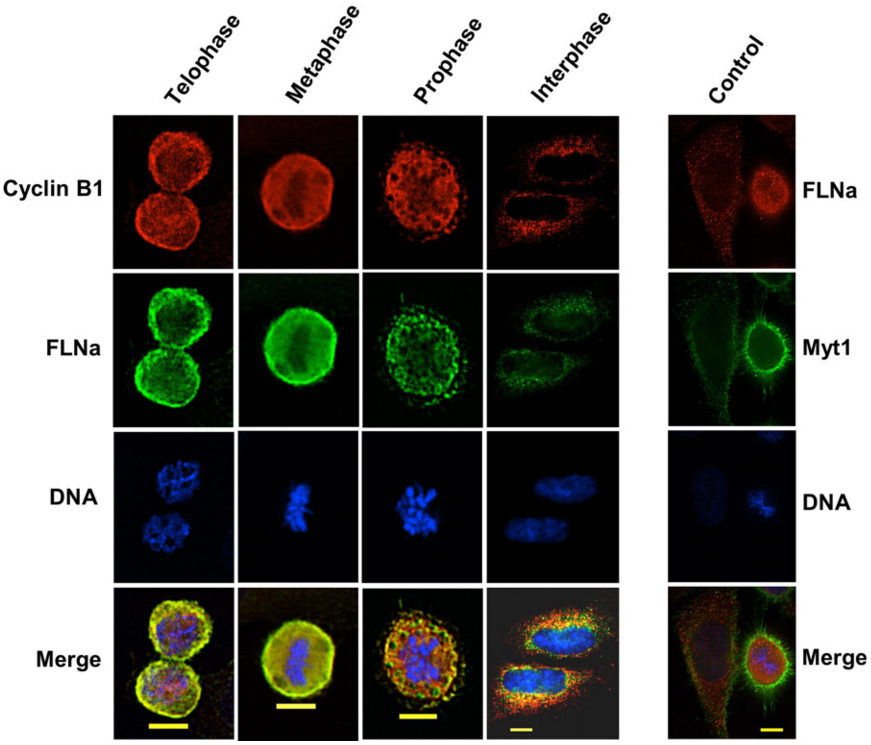

Fig. 4. Cyclin B1 and FLNa co-localize in mitotic and interphase cells. (Left panels) HeLa cells at the labelled cell cycle phases were stained for cyclin B1 (red), FLNa (green) and DNA (blue). Co-localization of cyclin B1 and FLNa is indicated by the yellow colour in the merged picture. (Right panels) As a control, HeLa cells were also stained with Myt1 (green), FLNa (red) and DNA (blue). The lack of yellow colour in the merged picture indicates that Myt1 does no co-localize with FLNa in either interphase or mitotic cells. The yellow scale bar is 10 μM.

mpm-2 epitope. Mpm-2 is a phospho-epitope that appears on many proteins during mitosis and is generated by Cdk1, ERK2 and Plk [33,34]. As shown in Fig. 5C, FLNa immunoprecipitated from untreated HeLa cells contains an mpm-2 epitope (upper panel) and mpm-2 reactivity increases following nocodazole treatment (bottom panel). Nocodazole treatment increases cyclin B1 levels (Fig. 2A) and activates intracellular cyclin B1/Cdk1 activity. Nocodazole treatment has no affect on FLNa levels in these cells (Fig. 2A). Taken together, these results indicate that FLNa is an in vivo as well as an in vitro substrate for cyclin B1/Cdk1 activity.

3.6. Cyclin B1/Cdk1 inhibits FLNa-dependent actin gelation

To determine whether cyclin B1/Cdk1 phosphorylation could regulate FLNa function, we next determined whether cyclin B1/Cdk1 could directly regulate FLNa gelation in vitro. Gelation by FLNa involves the solidification of polymerized actin into a cross-linked network [21]. It has been shown previously that Ca^{2+}/calmodulin-dependent protein kinase II (Cam KII) phosphorylation of chicken gizzard FLN caused a decrease in the gelation capacity of FLN [35], and we wanted to determine whether cyclin B1/Cdk1 could similarly affect FLNa's ability to gel actin. As shown in Fig. 6A, FLNa that has been incubated in vitro with cyclin B1/Cdk1 showed a reproducible decrease in its ability to gel actin (Fig. 6A, left panel, lane 4) compared to untreated FLNa (Fig. 6A, lane 3). To confirm the specificity of this inhibition, we tested the ability of the Cdk1 kinase inhibitors roscovitine and olomoucine to rescue gelation from Cdk1 inhibition [36]. As shown in Fig. 6B, both roscovitine and olomoucine (Fig. 6B, lanes 5 and 6) reproducibly prevented cyclin B1/Cdk1 from attenuating the ability of FLNa to gel actin. α-Actinin is a protein used as a positive control for actin gelation (Fig. 6B, lane 7). To confirm these observations, we determined whether phosphorylation of FLNa by cyclin B1/Cdk1 altered the ability to FLNa to increase the apparent viscosity of an actin polymer solution. Solution viscosity is a measure of gelation. As shown in Fig. 6C (left panel), FLNa increases the viscosity of an actin solution in a dose-dependent manner. However, incubation of FLNa with increasing amounts of cyclin B1/Cdk1 inhibits this viscosity increase (right panel). Incubation of FLNa with BSA had no effect.

3.7. Serine 1436 is an in vitro phosphorylation site on FLNa

In order to identify domains of FLNa that were phosphorylated by cyclin B1/Cdk1, we tested the GST-FLNa constructs from Fig. 3 (GST-FLNa 1–4) as substrates for cyclin B1/Cdk1 kinase activity. As shown in Fig. 7A, the FLNa-3 fragment, corresponding to repeats 11–16, showed the highest level of phosphorylation by cyclin B1/Cdk1. We observed phosphorylation in the other FLNa fragments, but at a lower level than was observed in FLNa-3. We then generated smaller FLNa constructs based on the previous results and narrowed the major phospho-acceptor sites in FLNA-3 to repeats 12–15 of FLNa (not shown). Phosphorylation of these repeats by cyclin B1/Cdk1 generates an mpm-2 epitope in vitro (Fig. 7B), consistent with the idea that this sequence contains one or more sites for cyclin B1/Cdk1 activity. There is endogenous mpm2 phosphorylation present in our purified protein and cyclin B1/Cdk1 autophosphorylates itself generating an mpm2 epitope. Cdk1 has a phosphorylation consensus sequence of (K/R)(S/T)P X(K/R) or (S/T)P X(K/R) [37] (where

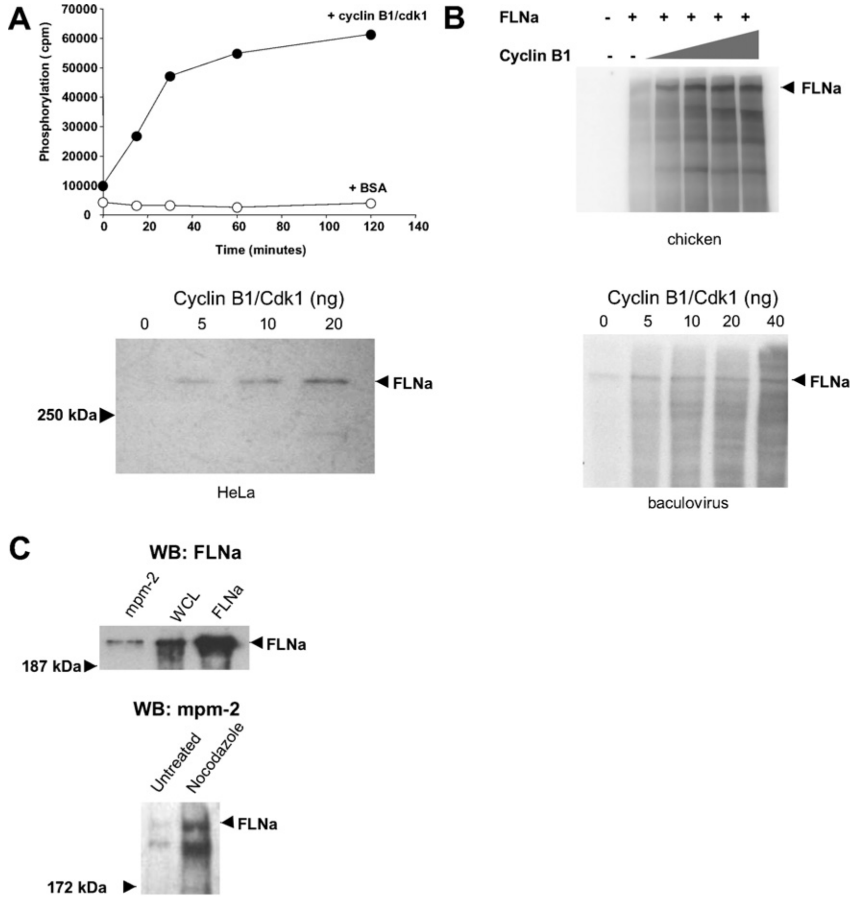

Fig. 5. Cyclin B1/Cdk1 phosphorylates FLNa. (A) Cyclin B1/Cdk1 phosphorylate FLNa in vitro. (Upper panel) Purified recombinant cyclin B1/Cdk1 (20 ng) phosphorylates human FLNa purified from HeLa cells (250 ng) in a time-dependent manner. BSA has no effect on FLNa phosphorylation. (Lower panel) Purified recombinant cyclin B1/Cdk1 phosphorylates human FLNa (250 ng) in a dose-dependent manner. Incubation time was 60 min. Figures are the representative of three independent experiments. (B) Immunopurified cyclin B1/Cdk1 is able to phosphorylate chicken gizzard FLN (top panel). As well, active recombinant cyclin B1/Cdk1 phosphorylates baculovirus purified human FLNa (bottom panel). Phosphorylation in both cases is dose-dependent. Incubation time was 45 min. (C) FLNa in HeLa cells contains an mpm-2 epitope. The upper panel shows a Western blot for FLNa from either a whole cell lysate (WCL) or mpm-2 immunoprecipitation and is blotted with FLNa. The lower panel shows a Western blot for mpm-2 from HeLa cells that have been treated with 100 ng/ml nocodazole. There is increased abundance of the FLNa mpm-2 epitope in the nocodazole treated lane.

X is any amino acid). There are three perfect matches in repeats 12–15: serines 1436, 1533 and 1630. We next created FLNa mutants where each of these serines is singly mutated to non-phosphorylatable residues. We also generated a FLNa variant where all three residues are mutated. As can be seen in Fig. 7C, the mutation of 1436 dramatically reduces the ability of the fragment to become phosphorylated. Mutants in 1533 and 1630 remain phosphorylated, but to a lesser extent that in the wild type protein. We hypothesize that serines 1436, 1533 and 1630 are all phosphorylated by cyclin B1/Cdk1 in vitro, but phosphorylation of serine 1436 is somehow necessary to direct the phosphorylation of serines 1533 and 1630. Cyclin B1/Cdk1 was unable to phosphorylate the triple mutant. These results suggest that serine 1436 is an in vitro cyclin B1/Cdk1 phosphorylation site.

4. Discussion

Here, we show that the cyclin B1/Cdk1 mitotic regulator binds and phosphorylates FLNa and reduces its ability to gelate actin in vitro. Using a yeast two-hybrid screen, we find that cyclin B1 binds to FLNa residues centred around FLNa repeat 9. The two proteins reciprocally co-immunoprecipitate in human cells and show specific co-localization in mitotic cells. The minimal cyclin B1 residues that interact with FLNa are amino acids 1–40.

Cyclin B1/Cdk1 can phosphorylate FLNa in vitro and this phosphorylation reduces the ability of FLNa to gel actin. Gelation by FLNa involves the solidification of polymerized actin into a cross-linked three-dimensional network, and is believed to reflect the ability of FLNa to create high-order actin structures within cells [14]. Previously, chicken gizzard FLN was identified as a substrate for calcium/calmodulin-dependent protein kinase II (CaM kinase II) [35]. Using an in vitro gelation assay, Ohta and Hartwig [35] demonstrated that the phosphorylation of FLN by CaM kinase II decreased its ability to gel actin. This suggests that modulating FLNa's ability to cross-link actin in cells by phosphorylation is a mechanism to regulate the mechanical properties of cytoplasmic actin networks. We found the incubation of FLNa with cyclin B1/Cdk1, like CaMK II decreased its ability to gel actin. Since we have observed that cyclin B1/Cdk1 can phosphorylate purified FLNa concomitant with a reduction in gelation, we pre-

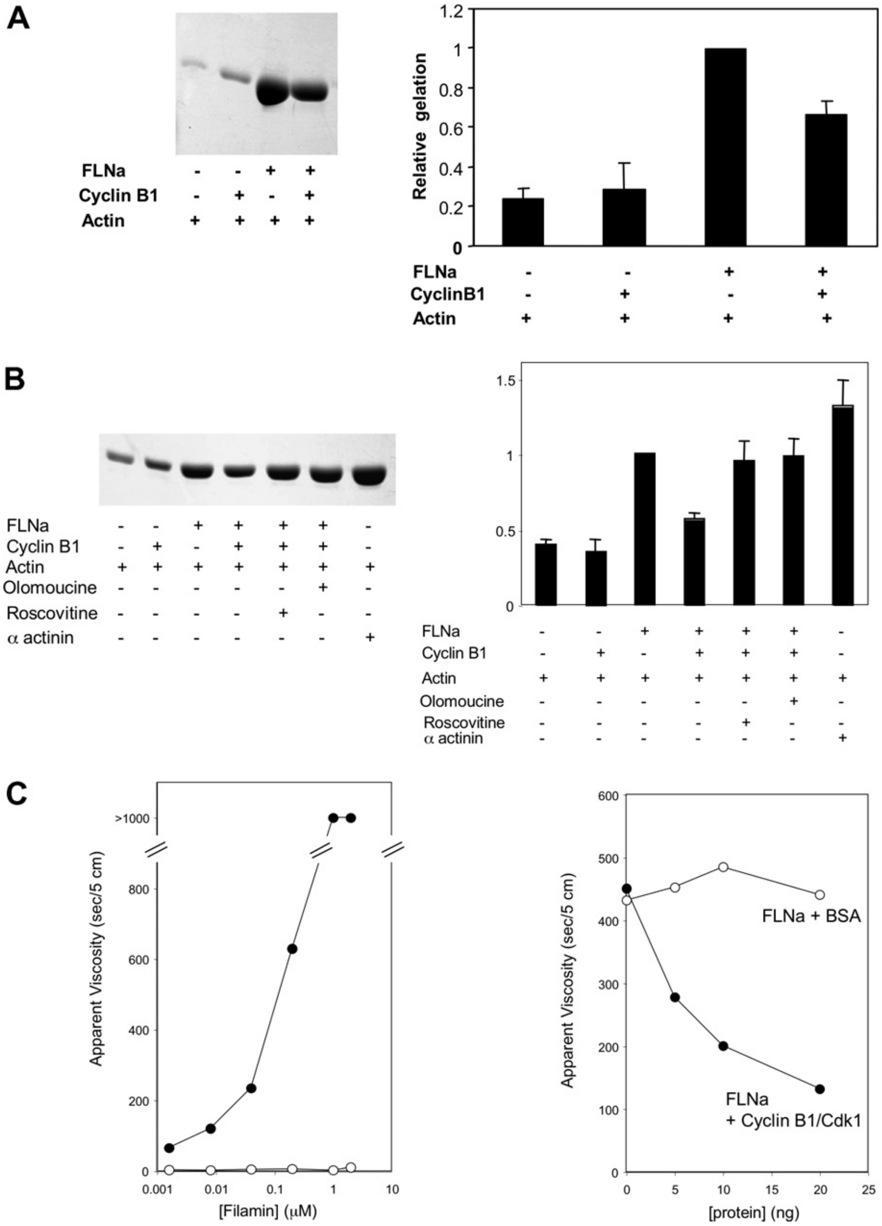

Fig. 6. Cyclin B1/Cdk1 are able to decrease FLNa gelation. (A) Cyclin B1/Cdk1 phosphorylation inhibits the ability of FLNa to gel actin. The left panel shows that phosphorylation of FLNa by immunopurified cyclin B1/Cdk1 decreases its ability to gel actin. There is reduced actin content in lane 4 relative to lane 3. The histogram shows a densitinometric quanitation of gelled actin in three independent experiments. The amount of gelled actin is normalized to the FLNa control. (B) Cdk1 kinase inhibitors rescue inhibition of FLNa gelation. Treatment of immunopurified cyclin B1/Cdk1 with either roscovitine or olomoucine causes FLNa induced actin gelation to return to the same level as FLNa alone, indicating that the inhibition of gelation caused by cyclin B1/Cdk1 is dependent on Cdk1 kinase activity. α-actinin acts as a positive control for actin gelation. The histogram shows a densitinometric quanitation of gelled actin in three independent experiments. The amount of gelled actin is normalized to the FLNa control. (C) (Left panel) Human FLNa (closed circles) increases the apparent viscosity of a solution of actin polymers (23 μM) in a dose-dependent fashion (left panel). Open circles are FLNa without actin. (Right panel) Incubation of FLNa (200 nM) and actin with increasing concentrations of either active cyclin B1/Cdk1 (closed circles) or BSA (open circles) are shown.. The treatment of FLNa with increasing concentrations of cyclin B1/Cdk1 reduces the ability of FLNa to increase the apparent viscosity of a 23 μM actin solution (closed circles). Treatment of FLNa with BSA had no effect on FLNa-dependent viscosity changes (open circles). Figures are the representative of three independent experiments with triplicate viscosity measurements in each.

dict that FLNa phosphorylation affects its ability to gel actin. The mechanism by which phosphorylation of FLNa affects its ability to gel actin is, however, unknown; phosphorylation may reduce FLNa's ability to bind actin, to dimerize, or both.

While we observed the reduction of FLNa-dependent gelation by cyclin B1/Cdk1, we do not know whether this has physiological consequences in a living cell. We have transfected active cyclin B1 alleles into FLNa-deficient M2 cells and their FLNa containing A7 counterpart. M2 cells have defects in cell locomotion and show high amounts of membrane blebbing [14]. These defects have been rescued in the A7 cell line [38]. We reasoned that cyclin B1 should alter the actin cytoskeleton in M2, but not A7. However, we were unable to confidently identify FLNa-dependent changes in actin structure upon

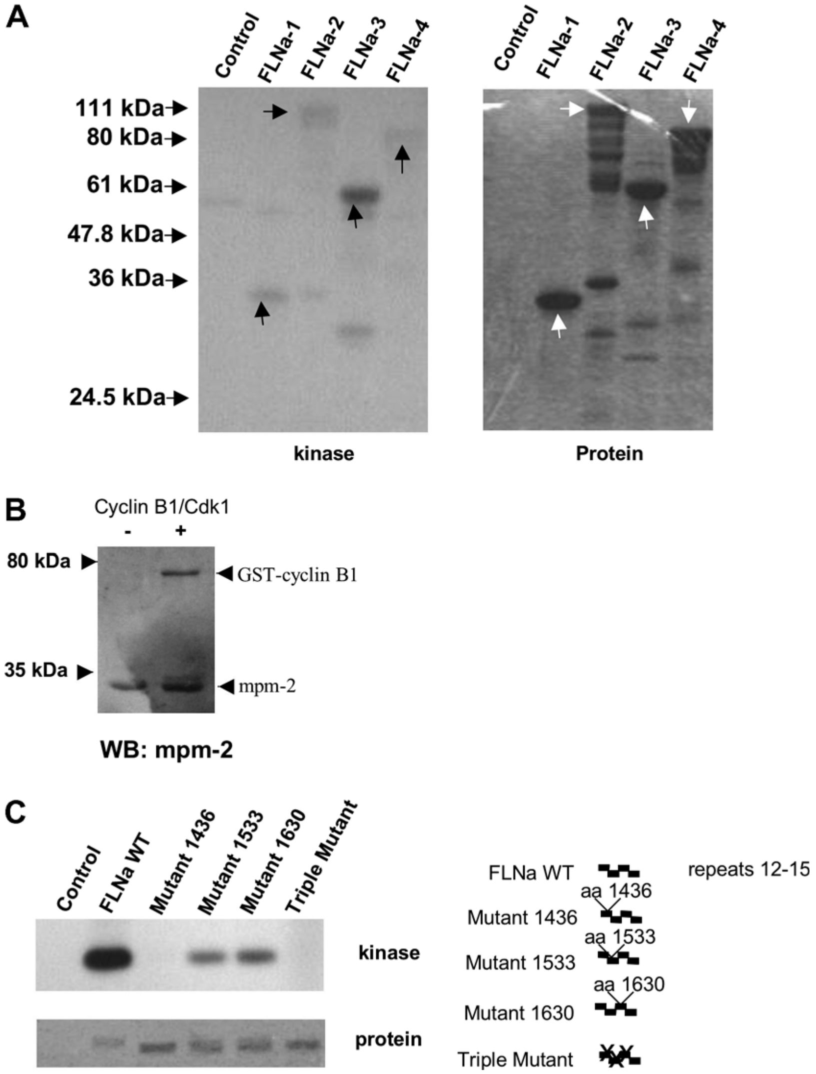

Fig. 7. Cyclin B1/Cdk1 phosphorylates FLNa at serine 1436. (A) Cyclin B1/Cdk1 preferentially phosphorylated a FLNa fragment containing repeats 11–16 (FLNa-3) (left panel). The positions of the FLNa fragments are seen in the Coomassie stained gel (right panel). All FLNa fragments were produced as GST fusion proteins, which were treated with thrombin to release the protein fragment. (B) Generation of an in vitro mpm-2 site in repeats 12–15. A recombinant GST protein containing repeats 12–15 was incubated with or without active cyclin B1/Cdk1 followed by Western blotting for mpm2. (C) Mutation of serine 1436 greatly decreases the ability of cyclin B1/Cdk1 to phosphorylate FLNa repeats 12–15 (FLNA WT). The triple mutation also shows greatly reduced phosphorylation (top panel). A schematic diagram for the partial FLNa constructs is shown (right panel). The Coomassie stained gel appears in the bottom panel.

cyclin B1 transfection (data not shown). Thus, a cyclin B1 and FLNa-dependent pathway of actin remodelling is an idea that requires further investigation. However, we have found that FLNa contains an mpm-2 epitope in HeLa cells, consistent with the idea that cyclin B1/Cdk1 could phosphorylate FLNa in vivo and that FLNa might be a regulator of mitotic actin remodelling.

We have identified serine 1436 as a major in vitro phospho-acceptor site. Three FLNa serines (1436, 1533 and 1630) in repeats 12–15 match the Cdk1 consensus phosphorylation sequence (K/R) (S/T)P X(K/R) or (S/T)P X(K/R) [37]. Mutation of serine 1436 to non-phosphorylatable glycine dramatically reduced in vitro phosphorylation. However, mutations of serines 1533 and 1630 also reduced phosphorylation. We favour a model where serines 1436, 1533 and 1630 are all phosphorylated by cyclin B1/Cdk1 in vitro, but phosphorylation of serine 1436 is somehow necessary to direct the phosphorylation of serines 1533 and 1630. Alternatively, 1436 may be the only residue phosphorylated but the interaction between cyclin B1/Cdk1 and serines 1533 and 1630 are necessary for maximal 1436 phosphorylation. These three phospho-acceptor sites are physically distinct from the cyclin B1 interaction site on FLNa. Cyclin B1 binds to repeat 9 while serines 1436, 1533 and 1630 are in FLNa repeats 12, 13, and 14, respectively.

While we have identified 1436, 1533 and 1630 as in vitro cyclin B1 substrates, we have yet to identify a mechanism by which these residues could affect in vivo or in vitro gelation. This issue is complicated because we have not yet been able to confidently establish an experimental system where the ectopic expression of cyclin B1 modulates some facet of FLNa-dependent actin remodelling in living cells (see above). In addition, FLNa is a large 280 kDa protein and cyclin B1/cdk1 can phosphorylate FLNa fragments that do not contain serines 1436, 1533 and 1630 (see Fig. 7A), suggesting that there are additional in vitro phospho-acceptor sites. Moreover, we do not yet know whether 1436, 1533, and 1630 are authentic in vivo FLNa phosphorylation sites, and the mechanism by which cyclin B1 alters in vitro FLNa/dependent gelation is currently unknown. As such, we have not yet determined whether 1436, 1533 and 1630 have an obligate role in mediating actin gelation in vivo or in vitro. Other kinases known to phosphorylate FLNa include p21-activated kinase 1 [31], protein kinase Cα [32] and ribosomal S6 kinase [39] and these may co-operate with Cdk1 to regulate FLNa activity. The comprehensive identification of FLNa residues phosphorylated by cyclin B1/Cdk1 in vitro and in vivo is an important issue for continued study.

Cyclin B1 is one of the three proteins identified to date that binds between the actin-binding domain and the first hinge region of FLNa [14]. Protein kinase C α (PKCα), a member of a family of phospholipid-dependent serine/threonine kinases was found to bind FLNa between repeats 1–4 and repeats 22–24 [32]. The significance of the binding however, between repeats 1–4 has not yet been fully determined. The other protein is furin, a membrane associated endoprotease [40]. Interaction of furin with FLNa results in its tethering to the cell surface, allowing for the sorting of furin into its proper cellular compartments [40]. Other identified FLNa-interacting proteins including TRAF2, SEK-1 and granzyme B, bind to repeats in the carboxy-terminal portion between the hinges and the dimerization domain [17,41,42]. While we find that phosphorylation by cyclin B1 reduces the ability of FLNa to gel actin, it is also possible that FLNa phosphorylation could alter the binding of other proteins to FLNa. However, this idea remains to be explored.

To date, filamins have been found to interact with over 30 cellular proteins of great functional diversity [13]. Many filamin interacting proteins are involved in cell signalling and it is believed that filamins physically connect several signalling complexes to the actin cytoskeleton [13]. For example, FLNa is known to act as a scaffold for β-arrestins (β-arr), multifunctional adaptor proteins [43]. β-arr and FLNa act cooperatively to activate the MAPK extracellular signal-regulated kinase (ERK) because FLNa facilitates the formation of the β-arr–ERK2 complex [43]. FLNa could act as a molecular scaffold to recruit active cyclin B1/Cdk1 into a position where it can phosphorylate other cytoskeletal regulatory proteins. The knowledge that FLNa acts a scaffold for other proteins suggests the possibility that it could recruit active cyclin B1/Cdk1 and other proteins to the same position in a cell. For example, the cyclin B1/Cdk1 complex has previously been reported to phosphorylate actopaxin, caldesmon and paxillin, focal adhesion proteins that associate with actin [28–30]. This mitosis-dependent phosphorylation may play a role in co-ordinating the disassembly of focal adhesions at the onset of mitosis. It is also observed that as cells progress out of mitosis, actopaxin and paxillin become dephosphorylated, independent of cell adhesion, suggesting that dephosphorylation of both proteins precedes the establishment of post-mitotic cell-extracellular matrix (ECM) adhesion [28]. It has been reported that an early event in mitosis is the accumulation of active cyclin B1/Cdk1 in the cytoplasm [44]. FLNa could potentially recruit cyclin B1/Cdk1 to actopaxin, paxillin and caldesmon and other cytoskeletal regulatory proteins [28–30]. However, the intracellular localization of endogenous or transfected cyclin B1 was largely similar between the M2 and A7 cell lines (not shown), suggesting that FLNa does not have an obligate role in controlling cyclin B1 localization. It is probable that the large number of actin cytoskeleton binding, cross-linking and regulating proteins necessitates multiple pathways for actin rearrangement, some dependent on cyclin B1/Cdk1 and others not.

In summary, we find that the cyclin B1/Cdk1 mitotic regulator binds the actin cross-linking protein FLNa as well as co-localizing with it. We also show that cyclin B1/Cdk1 phosphorylates FLNa and reduces its ability to gel actin. We have identified serine 1436 as one site of cyclin B1/Cdk1 phosphorylation in FLNa. Our observations are consistent with the idea that cyclin B1/Cdk1's phosphorylation of FLNa could play an important role in regulating actin dynamics. This is interesting considering the possibility that FLNa could be acting as a scaffold allowing cyclin B1/Cdk1 to phosphorylate other actin associated proteins such as caldesmon and paxillin at mitosis. We believe that our observations provide evidence that cyclin B1/Cdk1 are involved in the regulation of actin rearrangement at the onset of mitosis and that the cyclin B1 binding and cyclin B1/Cdk1 phosphorylation of FLNa may represent only one of many possible pathways leading to the control of actin cross-linking at mitosis.

Acknowledgements: This work is supported by a grant from the National Cancer Institute of Canada, with funds from the Canadian Cancer Society (J.M.L.). We thank Steven Innocente, Nisha Anand, Tahir Feroz and Sujeeve Jeganathan for their insight into this project. We are grateful to Rick Austin, Tom Stossel, Fumihiko Nakamura, and Peter Whyte for many helpful discussions. We thank Stanley Hollenberg for reagents used in the yeast two hybrid system, Fumihiko Nakamura for FLNa cDNA, Tom Stossel for M2 and A7 cell lines and Anja Hagting for the cyclin B1 GFP constructs.

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