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Unconventional actin conformations localize on intermediate filaments in mitosis

Thomas Hubert, Joël Vandekerckhove, Jan Gettemans *

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ABSTRACT

Different structural conformations of actin have been identified in cells and shown to reside in distinct subcellular locations of cells. In this report, we describe the localization of actin on a cage-like structure in metaphase HEK 293T cells. Actin was detected with the anti-actin antibodies 1C7 and 2G2, but not with the anti-actin antibody C4. Actin contained in this structure is independent of microtubules and actin filaments, and colocalizes with vimentin. Taking advantage of intermediate filament collapse into a perinuclear dense mass of cables when microtubules are depolymerized, we were able to relocalize actin to such structures. We hypothesize that phosphorylation of intermediate filaments at mitosis entry triggers the recruitment of different actin conformations to mitotic intermediate filaments. Storage and partition of the nuclear actin and antiparallel "lower dimer" actin conformations between daughter cells possibly contribute to gene transcription and transient actin filament dynamics at G1 entry.

1. Introduction

Cellular division is achieved by cooperation of all cytoskeletal systems: microtubules, actin filaments and intermediate filaments. Although the specialized functions of microtubules and actin filaments in mitosis have been thoroughly explored, less is known about the role of mitotic intermediate filaments. Nonetheless, it is known that all major cytoskeletal filaments are in permanent communication and association with one another [1].

Microtubules play a major role in cell division but actin has also major functions in mitosis. Before nuclear envelope breakdown, cortical actin is required to separate the duplicated centrosomes and position them at the spindle poles [2]. Thereafter, actin filaments surrounding the mitotic spindle and myosin-10 at the spindle poles regulate the length of the mitotic spindle [3]. Once the sister chromatids have been separated and positioned to opposite poles of the cell, the actomyosin ring contracts to physically separate the daughter cells [4].

Intermediate filaments act as mechanical stress absorbers and stabilize cell shape [5]. To provide each cell type with its own structural requirements, there are approximately 73 intermediate filament genes that can be differentially expressed, depending on cell type [5]. The cytoplasmic intermediate filament network reaches from the nuclear envelope to the plasma membrane and adhesion sites, and is used as scaffold and signal transduction device [6–8]. In the interphase cytoplasm, the position of intermediate filaments is controlled by the microtubule network with which they co-align [9–11]. Anterograde transport of intermediate filaments is driven by the plus-end-directed motor kinesin, while the retrograde transport depends on the minus-end motor dynein/dynactin (reviewed in [1]).

In mitosis, each intermediate filament acts differently, depending on cell type. In the course of mitosis, intermediate filaments are progressively phosphorylated by mitotic kinases, including Cdk1, Plk1, AuroraB and Rho-kinase (also named RhoA-binding kinase α) [12–14]. These phosphorylation events are thought to induce dramatic reorganization of intermediate filaments [15]. While some intermediate filaments are disassembled at mitosis entry, others remain filamentous until cytokinesis. But the structural changes of a specific type of intermediate filaments, such as vimentin for example, fully depend on cell type [16]. Mitotic intermediate filament structures are most prominent at metaphase, when they are organized in a typical cage-like structure that surrounds the mitotic spindle [16–21]. The functions of intermediate filaments in mitosis are diverse: tethering of spindle assembly factors that are essential for proper mitotic spindle formation, storage of nuclear matrix proteins and nuclear membrane vesicles to ensure equivalent distribution of these components to daughter cells [22–24]. Furthermore, phosphorylation of intermediate filaments sequesters 14-3-3 proteins [7]. As a consequence, some interaction partners of 14-3-3 proteins are displaced, many of which are involved in cell cycle regulation [8,25].

The binding between, and co-alignment of intermediate filaments and microtubules are well established. In contrast, the existence of a similar relationship between intermediate filaments and actin filaments has not been fully explored yet. Nonetheless, some studies indicate that both cytoskeletons colocalize [26]. BPAG1n

* Corresponding author at: Department of Medical Protein Research, VIB, Ghent University, Faculty of Medicine and Health Sciences, Albert Baertsoenkaai 3, B-9000 Ghent, Belgium. Fax: +32 9 2649490.
E-mail address: jan.gettemans@vib-ugent.be (J. Gettemans).

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has been identified as a linker protein that connects intermediate filaments with actin filaments in neurons [27]. In the present study, we studied a possible physical connection between actin and intermediate filaments. We provide evidence that non-filamentous actin colocalizes with intermediate filaments in mitotic but not in interphase HEK 293T cells.

2. Materials and methods

2.1. Reagents

Cytochalosin D, nocodazole and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma–Aldrich (C8273; M1404; D8417).

2.2. Antibodies

The following antibodies were used: rabbit anti-γ-tubulin (Sigma–Aldrich; T6557), rabbit anti-detyrosinated-α-tubulin (glu tubulin) (Chemicon; AB3201), rabbit anti-vimentin (Abcam; ab7783), mouse anti-actin clone 1C7 and mouse anti-actin clone 2G2 were a kind gift from Dr. B.M. Jockusch (Technical University of Braunschweig, Germany). Alexa Fluor 488-conjugated goat anti-rabbit antibody and Alexa Fluor 594-conjugated goat anti-mouse antibody were from Molecular Probes.

2.3. Cell culture

HEK 293T and MDA-MB-231 cells were maintained at 37 °C in a humidified 10% CO_2 incubator and grown in DMEM (Gibco) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin. For nocodazole treatment, cells were seeded on collagen-coated coverslips, allowed to recover for 24 h and treated with 33 μM nocodazole for 2 h (Fig. 2B and Fig. S1) or 4 μM nocodazole for 4 h (Fig. 1C) at 37 °C to depolymerize microtubules. For cytochalasin D treatment, cells were seeded on collagen-coated coverslips, allowed to recover for 24 h and treated with 0.1 μM cytochalasin D for 30 min at 37 °C to depolymerize actin filaments.

2.4. Immunostaining and immunofluorescence microscopy

Cells were washed with PBS, fixed with 3% paraformaldehyde for 25 min at room temperature and permeabilized with 0.25% Triton X-100 in PBS for 5 min. Paraformaldehyde was neutralized with 0.75% glycine for 20 min. Cells were then blocked in 1% BSA in PBS for 30 min and incubated with primary antibody for 1 h at 37 °C. Cells were washed in PBS, then incubated with secondary antibody and 4,6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature. Following immunostaining, samples were analyzed using a Carl Zeiss Axiovert 200 M Apotome epifluorescence microscope (63 × 1.4NA oil objective) equipped with an Axiocam cooled CCD camera and processed using Axiovision software (Zeiss).

3. Results and discussion

Several actin-specific antibodies have been developed that are suitable for immunofluorescence microscopy [28–30]. One of them, anti-actin antibody 1C7, has been generated by immunization with cross-linked "lower-dimer" actin to obtain a conformation-specific antibody (epitope corresponds to amino acids 194–203) [30]. Mouse monoclonal anti-actin antibody 1C7 recognizes antiparallel actin dimers ("lower-dimer" actin) and actin monomers but not filamentous actin [30]. We previously identified actin at the centrosome of interphase MDA-MB-231 cells using this antibody [31]. In mitotic MDA-MB-231 cells, actin was absent from centrosomes and localized uniformly in the mitotic cytoplasm (data not shown). We wondered if this localization was cell type-specific. In this perspective, we examined actin 1C7 staining in HEK 293T cells. In methanol-fixed cells, no centrosomal staining was visible in interphase cells (data not shown). In formalin-fixed cells, 1C7 staining revealed a uniform distribution in the cytoplasm and was present in a faint punctuated pattern in the nucleus, as described previously (Fig. 1A, upper panels and Fig. S1, upper panels) [30]. Surprisingly, however, actin was present on cable-like structures in mitotic cells (Fig. 1A, middle and lower panels). Most strikingly, the actin-containing cables formed a cage-like structure around the mitotic spindle in metaphase (Fig. 1A, middle panels). Further on, we will refer to these structures as "actin-containing cables".

The similarity between the actin-containing cable structures seen in mitotic HEK 293T cells and mitotic intermediate filaments described in literature is remarkable. The cage-like structure around the mitotic spindle in metaphase is typical for intermediate filaments (Fig. 1A, middle panels and Fig. 1B, middle panels) [16–21]. During cytokinesis, the actin cables stretched from one daughter cell to the other, with an interruption in the cleavage furrow (Fig. 1A, lower panels and Fig. 1B, lower panels). Such interruptions at the cleavage furrow are typical for intermediate filament disassembly as a consequence of phosphorylation by Rho-kinase [12,32].

Subsequently, we examined if the "lower dimer" actin-containing cables in mitosis contained or depended on *bona fide* actin filaments. We therefore treated cells with varying amounts of cytochalasin D, which disrupts long actin filaments and induces short aggregates of filamentous actin (Fig. 1B and data not shown) [33,34]. As expected, the morphology of interphase cells changed (Fig. 1B, upper panels). In contrast, 1C7 staining of mitotic cells remained unchanged (Fig. 1B, middle and lower panels). These data indicate that the actin fraction detected on mitotic cables is not part of conventional actin filaments and does not depend on it.

The possibility that stable microtubules attract actin in mitosis was analyzed as well. Microtubules are stabilized by plus-end capping proteins and are characterized by detyrosination of α-tubulin (referred to as Glu tubulin because detyrosination exposes a C-terminal glutamate residue) [35]. Notwithstanding the fact that the majority of mitotic spindle microtubules are dynamic and, consequently, not detyrosinated, a minor fraction is stable and detyrosinated [36,37]. However, we observed that HEK 293T cells did not contain Glu tubulin at or around the mitotic spindle and, therefore, could not be the source of the actin-containing cables (Fig. 1A, middle and lower panels). Glu tubulin staining was found at the centrosome and the midbody, as expected (Fig. 1B, lower panels). Fig. 1B (lower panels) also shows that the actin-containing cables did not colocalize with stable microtubules in the midbody and, in contrast, appear curved, indicative of intermediate filaments.

We took advantage of an exceptional consequence of nocodazole treatment for the intermediate filament network in interphase cells. Microtubule depolymerization results in the collapse of the intermediate filament network into a perinuclear dense mass of intermediate filament cables [38]. GEF-H1 is a microtubule-associated guanine exchange factor that activates RhoA upon nocodazole-induced release from microtubules [39]. RhoA activates RhoA-binding kinase α (Rho kinase) that phosphorylates intermediate filament subunits and results in the collapse of intermediate filament network [32].

When we applied nocodazole to interphase cells, we observed a dramatic relocalization of actin to a perinuclear dense mass of cables, which resulted in depletion of actin from the cytoplasm (Fig. S1, lower panel). When lower concentrations of nocodazole where used during a longer incubation time, some cells displayed a network of actin cables that closely surrounded the nucleus

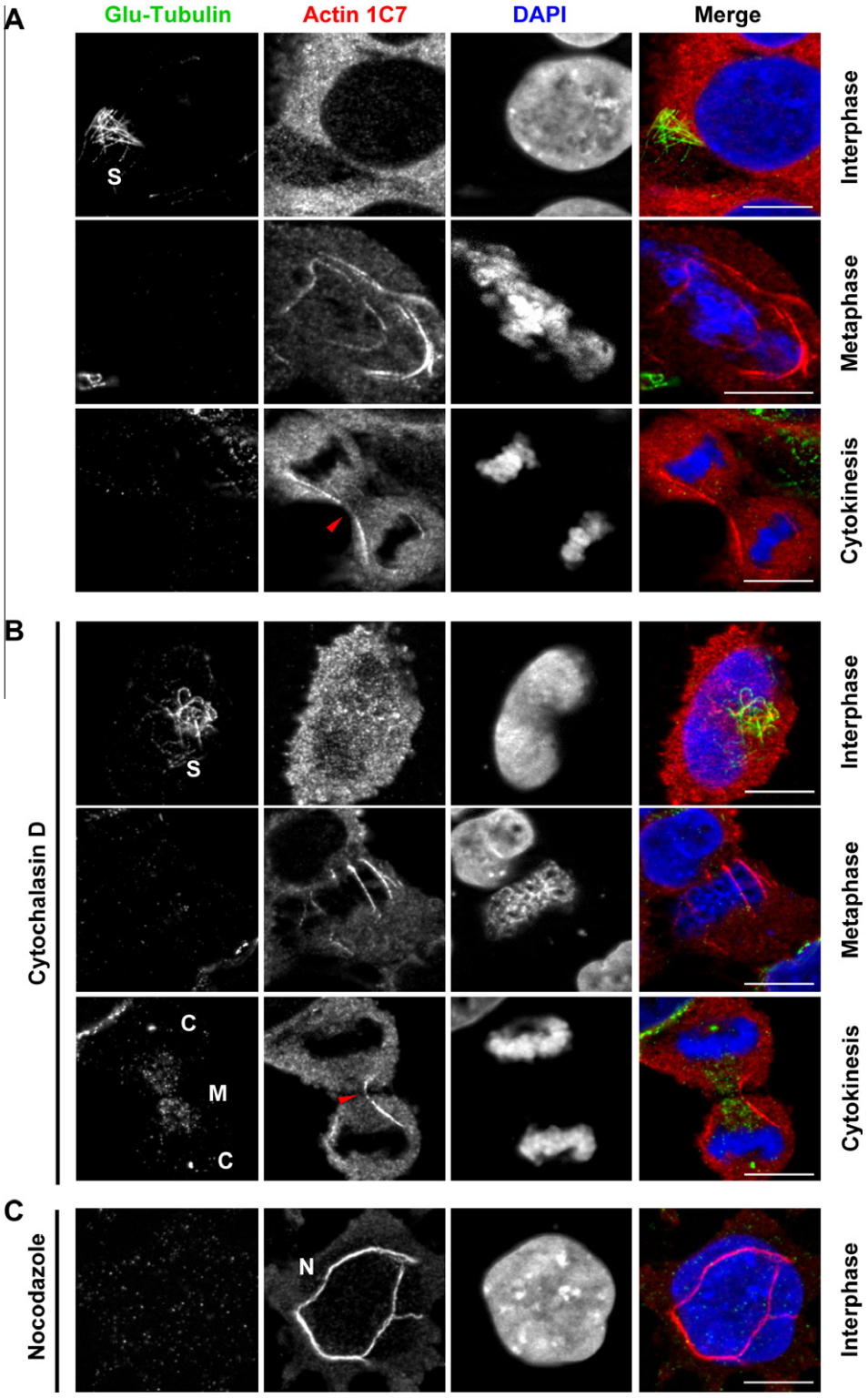

Fig. 1. Actin accumulates in mitotic cables that are distinct from microtubules and actin filaments. (A, B, C) Staining of interphase and mitotic HEK 293T cells with monoclonal anti-actin antibody 1C7, polyclonal antibody to stable microtubules (Glu-tubulin) and DAPI. (B) Cells were treated with cytochalasin D to disrupt actin filaments. (C) Cells were treated with nocodazole to depolymerize microtubules. This cell shows close association of newly formed actin-containing cables with the nuclear envelope. Arrowhead = discontinuation of actin cables at the cleavage furrow. C = centrosome, M = midbody, N = nocodazole-induced actin-containing cables in interphase cells, S = stable microtubules. Scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

(Fig. 1C). This might be the result of interaction between collapsed intermediate filaments with the nuclear envelope [22,40]. Surprisingly, nocodazole depolymerizes both the dynamic and stable microtubules in HEK 293T cells (Fig. 1C). Of note, the collapsed cables did not localize near the centrosome (Fig. S1) and were not altered by microtubule regrowth for a short time (Fig. S2).

Staining for the intermediate filament protein vimentin revealed that actin colocalized with filamentous vimentin in mitosis

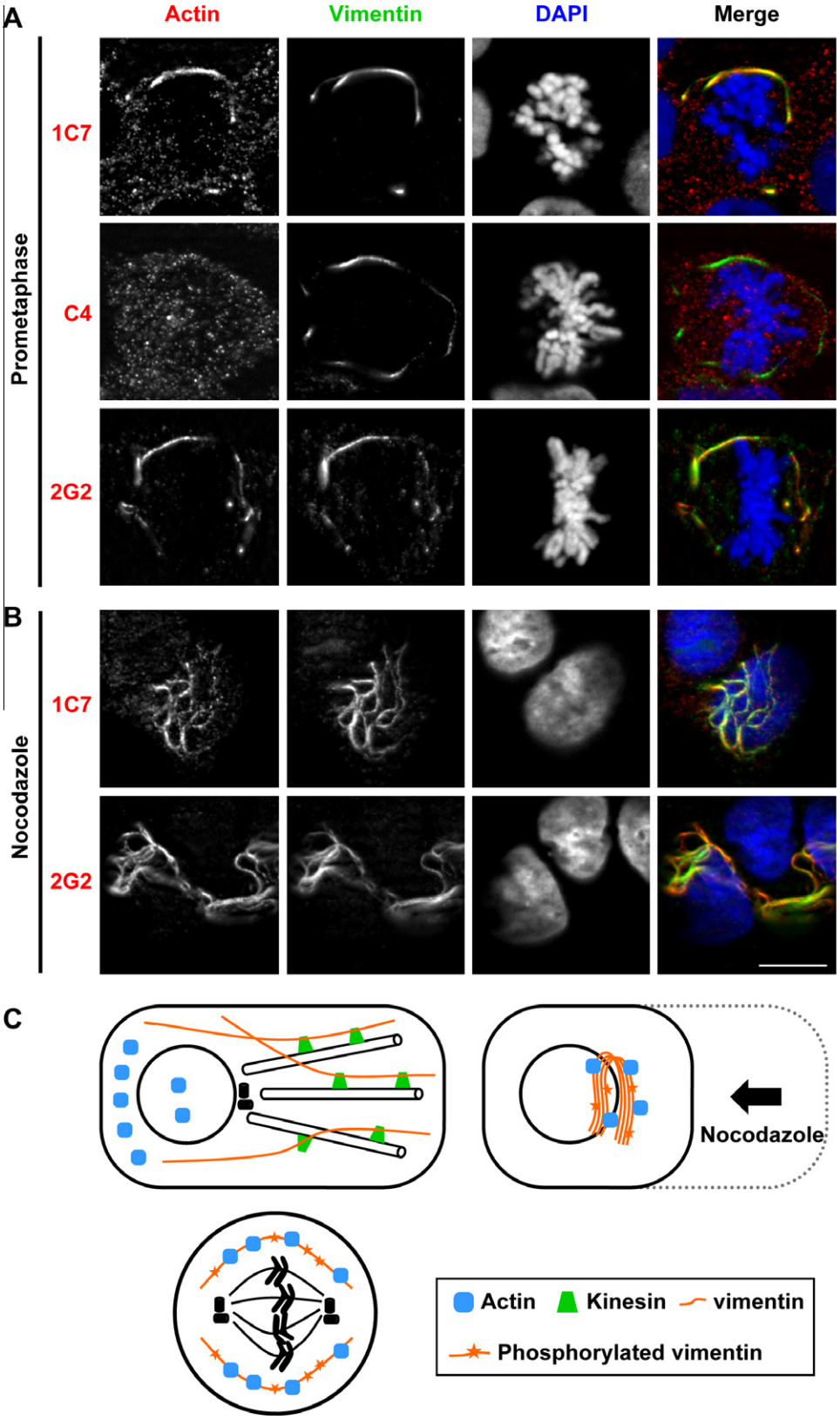

Fig. 2. Conformation dependent localization of actin on mitotic vimentin filaments. (A) Staining of mitotic HEK 293T cells with monoclonal anti-actin antibody 1C7, C4 or 2G2, polyclonal anti-vimentin and DAPI. (B) Identical staining of interphase cells treated with nocodazole to induce dense perinuclear masses of vimentin filament cables. (C) Schematic representation summarizing our observations and model. Atypical actin conformations associate with vimentin intermediate filaments when they become phosphorylated in mitosis (left lower panel). Aberrant phosphorylation events as a consequence of nocodazole treatment could explain the recruitment of actin to the perinuclear bundles of intermediate filament cables (upper right panel). Cylinders represent microtubules. Scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

(Fig. 2A, upper panels). It was also confirmed that actin and vimentin colocalize in the nocodazole-induced perinuclear dense mass of cables (Fig. 2B, upper panels). Noteworthy, vimentin clearly localized in the nucleolus, as predicted by a proteomic study [41] (Fig. S3).

The type of actin conformation colocalizing with metaphase vimentin filaments was investigated using two additional anti-actin antibodies: anti-actin antibody C4 that binds to monomeric actin [29], and anti-actin antibody 2G2 that recognizes specifically a nuclear conformation of actin (nonsequential epitope corresponds to amino acids 131–139, 155–169 and 176–187) [28]. Surprisingly, antibody 2G2, but not antibody C4, stained mitotic vimentin filaments (Fig. 2A, middle and lower panels). In addition, antibody 2G2 stained nocodazole-induced perinuclear cables (Fig. 2B, lower panels), confirming the data obtained with untreated cells.

Altogether, our results point toward association of atypical actin conformations with vimentin intermediate filaments when they become phosphorylated in mitosis. Aberrant phosphorylation events as a consequence of nocodazole treatment (see above) could explain the recruitment of actin to the perinuclear bundles [12,32,38,39]. Fig. 2C summarizes our data and model. Our results suggest that nuclear actin and antiparallel "lower dimer" actin, but not regular monomeric actin are recruited to mitotic vimentin filaments. Interestingly, mitotic intermediate filaments act as temporary storage compartments for nuclear matrix proteins and nuclear membrane vesicles [22,23]. Possibly, intermediate filaments also stabilize nuclear actin complexes and antiparallel actin dimer-containing complexes that are subsequently activated at G1 entry to start gene transcription and transient actin nucleation, respectively [42,43].

Nuclear actin conformation might be preserved by interacting proteins. Candidate interactors are members of chromatin remodeling complexes and RNA polymerase complexes [44]. Maintenance of subcomplexes of these large machineries could be advantageous for the reconstruction of the original complexes at G1 entry. Antiparallel actin dimers are a first step in actin filament polymerization [42]. They bind to gelsolin [45] that can nucleate actin filaments *in vitro* [46]. In addition, we previously reported that antiparallel actin dimers also localize at the centrosome where they could be involved in actin filament-based centrosome reorientation toward the leading edge in migrating cells [31,47]. Thus, it might be beneficial for cells to dispose of antiparallel actin dimer-complexes at G1 entry.

In sum, mitotic, phosphorylated intermediate filaments might inactivate, store and redistribute different actin complexes to daughter cells. At the end of mitosis, they may act as transport adaptors as well. In this respect, there would be no need to accurately store and protect "regular" polymerization competent G-actin that can easily be distributed through passive diffusion. Clearly, cells make efforts to conserve unconventional actin conformations throughout the cell cycle, emphasizing their physiological importance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.002.

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