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Actin and Arp2/3 localize at the centrosome of interphase cells

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ABSTRACT

Although many actin binding proteins such as cortactin and the Arp2/3 activator WASH localize at the centrosome, the presence and conformation of actin at the centrosome has remained elusive. Here, we report the localization of actin at the centrosome in interphase but not in mitotic MDA-MB-231 cells. Centrosomal actin was detected with the anti-actin antibody 1C7 that recognizes antiparallel (“lower dimer”) actin dimers. In addition, we report the transient presence of the Arp2/3 complex at the pericentriolar matrix but not at the centrioles of interphase HEK 293T cells. Overexpression of an Arp2/3 component resulted in expansion of the pericentriolar matrix and selective accumulation of the Arp2/3 component in the pericentriolar matrix. Altogether, we hypothesize that the centrosome transiently recruits Arp2/3 to perform processes such as centrosome separation prior to mitotic entry, whereas the observed constitutive centrosomal actin staining in interphase cells reinforces the current model of actin-based centrosome reorientation toward the leading edge in migrating cells.

1. Introduction

The centrosome is the microtubule-organizing center of the interphase cell. It has the ability to nucleate, anchor and release microtubules [1]. This organelle is positioned near the nuclear membrane and is oriented towards the cell front when cells are migrating [2]. Structurally, it is composed of two barrel-shaped centrioles, 0.5 μm long, that are embedded in a dense pericentriolar matrix (PCM) that contains the γ-tubulin ring complex (γ-TuRC), which nucleates microtubules [1]. This organelle contains hundreds of proteins, many of which are involved in cell cycle control, since duplication of the centrosome and duplication of the cell as a whole are intimately intertwined [1,3].

As mitosis progresses, the duplicated centrosomes form the core of the spindle poles and nucleate microtubules necessary for assembling and positioning of the mitotic spindle [1]. Meanwhile, mitotic kinases activate centrosomal proteins to support cell division [4]. Quite surprisingly, a significant part of mitosis can proceed in the absence of the centrosome, but, nonetheless, the centrosome is essential for the fidelity of cytokinesis and completion of cell division [5,6].

It is striking that proteome analysis of typical microtubule-based organelles such as the centrosome and the midbody reveals the presence of numerous actin-binding proteins (ABPs) that could sustain actin dynamics [3,7]. Purified centrosomes contain, among others, cofilin, profilin, L-plastin, myosin and beta-filamin [3]. But for what purpose are these ABPs localized at the centrosome? For some ABPs, an answer has already been formulated in the context of cell division. At the G2/M transition, phosphorylation of centrosomal cortactin results in actin filament-mediated separation of duplicated centrosomes and transport of these centrosomes to opposite ends of the nucleus to form the spindle poles [8]. In mitosis, myosin-10 localizes in close proximity to the spindle poles and regulates mitotic spindle length in conjunction with actin filaments [9]. In yeast, ArpC2 interacts with γ-tubulin and regulates microtubules in mitosis, independently of other subunits of the Arp2/3 complex [10]. ArpC1 is also a stand-alone subunit that activates the mitotic kinase Aurora A at the centrosome in order to regulate entry into mitosis [11].

Arp2/3 is an actin-filament nucleating complex consisting of the two actin-related proteins Arp2 and Arp3 and five Arp-complex subunits: ArpC1, ArpC2, ArpC3, ArpC4 and ArpC5, ranked by decreasing molecular mass [12]. The Arp2/3 complex has no significant nucleating activity on its own but is activated by nucleation promoting factors (NPF) such as WASP, N-WASP, SCAR/WAVE, and WASH [12,13]. Activation of Arp2/3 results in branched actin filament networks that contribute to lamellipodial protrusion, substrate adhesion, exocytosis and endocytosis, vesicle and organelle motility [12]. In the nucleus, both the Arp2/3 complex and its activator N-WASP associate with the RNA polymerase II machinery, and their function in actin polymerization is essential for RNA polymerase II-mediated transcription [14,15].

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In interphase, the centrosome is attached to the nuclear membrane via a complex of the outer and inner nuclear membrane proteins ZYG-12 and SUN-1 [16]. In migrating cells, the centrosome is positioned between the nucleus and the leading edge, where it is surrounded by organelles such as the Golgi and the recycling endosomes. Although nuclear positioning mainly depends on microtubules, the nuclear movements that are necessary to orient the centrosome toward the leading edge of migrating cells are actin-dependent [17,18]. This process is dependent on the interaction between actin cables and a complex of the outer and inner nuclear membrane proteins nesprin and SUN-2 [18]. Although this model explains how force is generated to reorient the centrosome, the coupling of the actin cables to the centrosome remains unknown.

The increasing number of actin-binding proteins that are detected at the centrosome and the coincident failure to visualize actin itself at the centrosome is intriguing, especially because it is not without precedent [19]. Many actin-binding proteins have been reported in the nucleus and, although evidence indicates that their actin-binding properties are necessary to perform essential functions such as gene transcription, the actual structural form that nuclear actin adopts is still unknown [19]. In this report, we illustrate that centrosomal actin can be visualized by means of an antibody that recognizes antiparallel actin dimers (actin "lower dimer"). In addition, we add Arp2/3 to the growing list of centrosomal actin-binding proteins.

2. Materials and methods

2.1. Plasmids

The ArpC1b cDNA was subcloned into a modified pEGFP-N1 vector (Clontech), in which the EGFP-tag had been substituted by a V5-tag, in order to obtain the expression vector ArpC1-V5.

2.2. Antibodies

The antibodies used were: mouse anti-γ-tubulin (Sigma–Aldrich; T6557), rabbit anti-γ-tubulin (Sigma–Aldrich; T6557), rabbit anti-Arp2 (Abcam; ab47654), rabbit anti-Arp3 was homemade, rabbit anti-ArpC1b (Abcam; ab51243), rabbit anti-ArpC5 (Sigma–Aldrich; HPA004832), mouse anti-Exo70 (Abcam; ab57402), mouse anti-V5 (Invitrogen; R96025), mouse anti-actin clone 1C7 was a kind gift from Dr. B.M. Jockusch (Technical University of Braunschweig, Germany). Alexa Fluor 488 or Alexa Fluor 594-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were from Molecular Probes (Merelbeke, Belgium).

2.3. Cell culture and cell processing

HEK 293T cells 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. HEK 293T cells, seeded on rat tail collagen-coated coverslips, were transfected using the calcium phosphate method.

2.4. Immunostaining and immunofluorescence microscopy

Cells shown in Figs. 1 and 3 were washed with PBS, fixed with 100% ice-cold methanol for 5 min at −20 °C and blocked in 1% BSA in PBS overnight. Cells shown in Fig. 2 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. In order to visualize centrosomal proteins, coverslips were additionally treated with 100% ice-cold methanol for 2 min at −20 °C. Paraformaldehyde was neutralized with 0.75% glycine for 20 min and samples were blocked in 1% BSA in PBS overnight. Coverslips were incubated with primary antibody for 1 h at 37 °C. Cells were extensively washed in PBS containing 1% BSA, then incubated with secondary antibody and 4,6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature. For double staining, this protocol was repeated. Rabbit antibodies were used prior to mouse antibodies. 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

The Arp2/3 complex has been the focus of many studies and, surprisingly, the possible localization of this complex at the centrosome has only very recently been brought to attention [11]. Certain facts account for this lag. First, the centrosome is the major microtubule nucleation center in the cell and there is no direct evidence of actin filament nucleation. Second, no actin or actin-based structures have been reported at the centrosome yet. Third, the vast majority of fixation protocols for cellular immunofluorescence microscopy studies use a 3% paraformaldehyde solution in PBS to cross-link proteins. Under these conditions, most centrosomal proteins are invisible. Visualization of centrosomal proteins requires precipitation of proteins with ice-cold 100% methanol [20].

Untreated HEK 293T cells were fixed with methanol and co-stained for the centrosome marker γ-tubulin and four components of the Arp2/3 complex (Fig. 1A). The perinuclear γ-tubulin-positive centrosome was very conspicuous, particularly because the visibility of the cytoplasmic fraction of γ-tubulin was greatly reduced by methanol fixation (Fig. 1A) [20]. In a subpopulation of interphase HEK 293T cells (~10%), we observed clear colocalization of Arp2, Arp3, ArpC1 and ArpC5 with γ-tubulin, suggesting that the full Arp2/3 complex colocalizes at the centrosome (Fig. 1A). However, the overlap was not complete (Fig. 1A, magnification). The γ-tubulin staining could be subdivided into a bright signal representing the centrioles (orange arrow) and a less intense signal that represents an expanded form of the pericentriolar matrix (green bracket, Fig. 1A, magnification). The Arp2/3 components consistently colocalized with γ-tubulin at the pericentriolar matrix but not at the centrioles (Fig. 1A). These data indicate that the cells in which centrosomal Arp2/3 was found had expanded their pericentriolar matrix, possibly in preparation for cell division when centrosomes have to be separated, repositioned and prepared to augment their microtubule nucleating activity. In contrast, we never detected Arp2/3 subunits at centrosomes in mitosis (data not shown).

Clearly, the Arp2/3 complex must be targeted and anchored in some way to localize at the pericentriolar matrix. At the lamellipodium, the Exo70 subunit of the exocyst complex is responsible for recruitment of the full Arp2/3 complex through direct binding with the ArpC1 subunit [21]. Interestingly, Exo70 localizes at the centrosome and has an effect on microtubule polymerization [22,23]. We hypothesized that Exo70 could target Arp2/3 to the centrosome in the same way as Exo70 recruits Arp2/3 at the lamellipodium [21]. In this scenario, Exo70 and ArpC1 would colocalize on the same part of the centrosome. Therefore, we stained the centrosome for γ-tubulin and Exo70 and uncovered that Exo70 was, similarly to the Arp2/3 subunits, localizing on the expanded pericentriolar matrix, but not on the centrioles (Fig. 1B). Exo70 and its direct interaction partner ArpC1 colocalized perfectly (Fig. 1B). Similar to ArpC1 and the other Arp2/3 subunits, we did not detect Exo70 on the centrosomes of interphase cells that had not expanded their pericentriolar matrix, and on mitotic centrosomes (data not shown). The colocalization of Exo70 and Arp2/3 in space and time

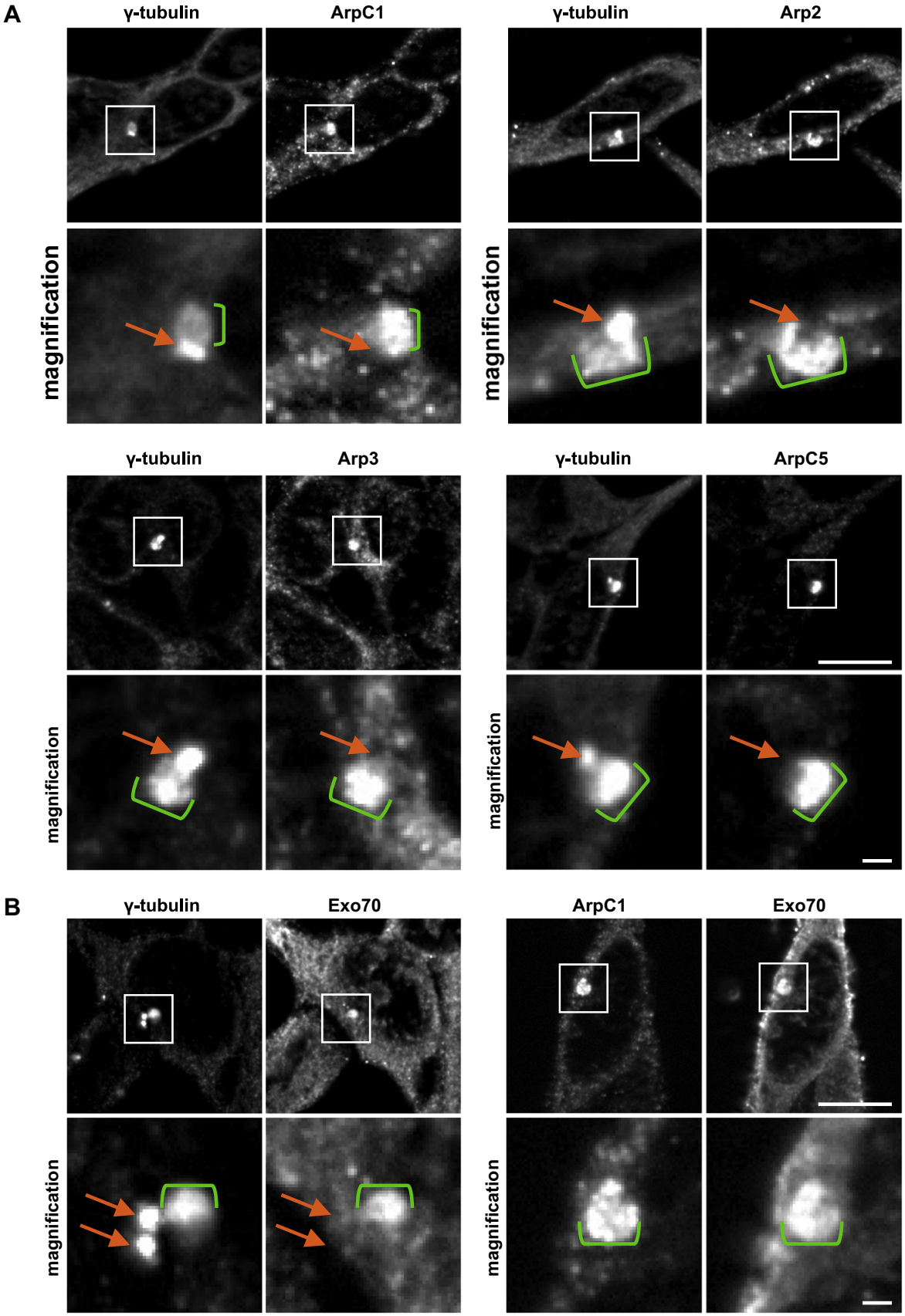

Fig. 1. The Arp2/3 complex localizes to the expanded pericentriolar matrix of the centrosome. (A) Methanol-fixed interphase HEK 293T cells were stained for γ-tubulin and Arp2/3 complex components ArpC1b, Arp2, Arp3, ArpC5. (B) Methanol-fixed interphase HEK 293T cells were stained for γ-tubulin, ArpC1b and Exo70. Orange arrows point to centrioles. Green brackets indicate expanded pericentriolar matrix. Scale bar = 10 μm (upper pannels) and 1 μm (magnifications). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

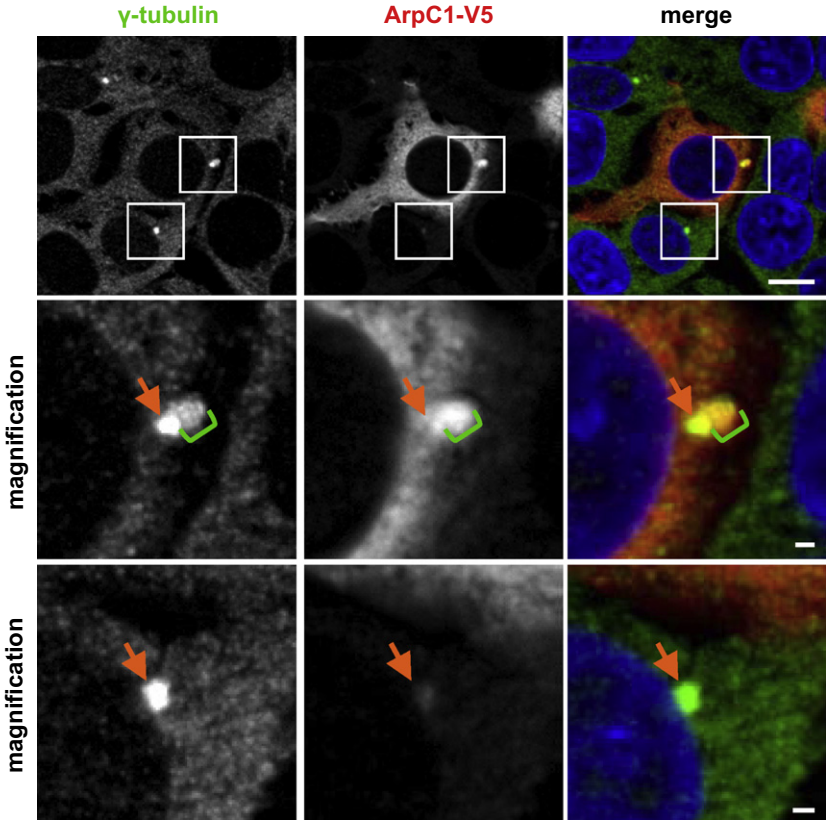

Fig. 2. Overexpressed ArpC1 expands and specifically accumulates at the pericentriolar matrix of the centrosome. Transfected HEK 293T cells expressing ArpC1b-V5 were stained for γ-tubulin (green) and ArpC1b (anti-V5 antibody; in red). The centrosomes of a transfected cell and a neighboring, untransfected cell were magnified and displayed in the lower panels. In the magnifications, orange arrows point to centrioles and green brackets indicate expanded pericentriolar matrix. Scale bar = 10 μm (upper pannels) and 1 μm (magnifications). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at the centrosome is remarkable. Collectively, these data imply that Exo70 could recruit the full Arp2/3 complex to the perinucleolar matrix of the centrosome.

To confirm the localization of endogenous Arp2/3 specifically at the expanded pericentriolar matrix of the centrosome, we expressed V5 epitope-tagged ArpC1 and observed that ArpC1 was able to expand the pericentriolar matrix and accumulate exclusively in this part of the centrosome (Fig. 2, green bracket and orange arrow in the first magnification). This experiment perfectly reproduced the results obtained from the endogenous stainings.

The most prominent component of the pericentriolar matrix is γ-tubulin. To examine the localization of Arp2/3 at the centrosome further, we expressed ArpC1 in combination with (1) γ-tubulin, (2) GCP2, a component of the γ-tubulin small complex (γ-TuSC) 3) GCP-WD, a component of the γ-tubulin ring complex (γ-TuRC) (Supplementary Fig. S1). All components of the γ-tubulin machinery convincingly recruited ArpC1 to the centrosome, reinforcing our previous data (Supplementary Fig. S1).

In order to assess if the functionality of centrosomal Arp2/3 was related to actin, we performed immunofluorescence microscopy of several cell lines with several anti-actin antibodies [24–26]. Interestingly, actin was consistently detected at the centrosome of interphase but not mitotic MDA-MB-231 cells, using the monoclonal anti-actin antibody 1C7 (Fig. 3 and Supplementary Fig. S2). This antibody recognizes antiparallel ("lower dimer") actin [26]. The monoclonal anti-actin antibodies C4 and 2G2 that recognize monomeric actin and the nuclear conformation of actin, respectively, did not stain the centrosome (data not shown), suggesting that centrosomal actin has a specific conformation [24,25]. Exo70 localization was similar to actin in MDA-MB-231 cells (Supplementary Fig. S3). Thus both actin and Exo70 localized at the centrosome of all interphase cells. In contrast, no Arp2/3 staining was observed at the centrosome of MDA-MB-231 cells (data not shown). Conversely, no actin staining was observed at the centrosome of HEK 293T cells (data not shown).

Many actin-binding proteins that could support actin dynamics are present at the centrosome [3,8,27]. Recent reports have complemented the inventory of centrosomal actin-binding proteins with, for example, the Arp2/3-activator WASH and, in this study, the Arp2/3 complex [13]. However, the presence and conformation of actin at the centrosome has remained undetermined. The antibody used to detect centrosomal actin in this study is indicative of an actin conformation that shows similarity to, or equals, antiparallel actin dimers. Antiparallel actin dimers are presumed to be important in the initial phase of actin polymerization and alter the function of actin-binding proteins such as gelsolin [28,29]. The failure to detect centrosomal actin with the anti-actin antibodies C4 and 2G2, or with phalloidin further emphasizes the specific nature of centrosomal actin. In conclusion, we hypothesize that the transient presence of Arp2/3 at the centrosome of HEK 293T cells is a cell cycle event [8]. The constitutive presence of actin at the centrosome of interphase MDA-MB-231 cells more likely points toward a function in centrosome orientation in migrating cells [18].

Throughout the last decade, the emerging research field of nuclear actin has revealed many surprises [19]. The fascinating developments of actin binding proteins at the centrosome promise to be quite interesting as well.

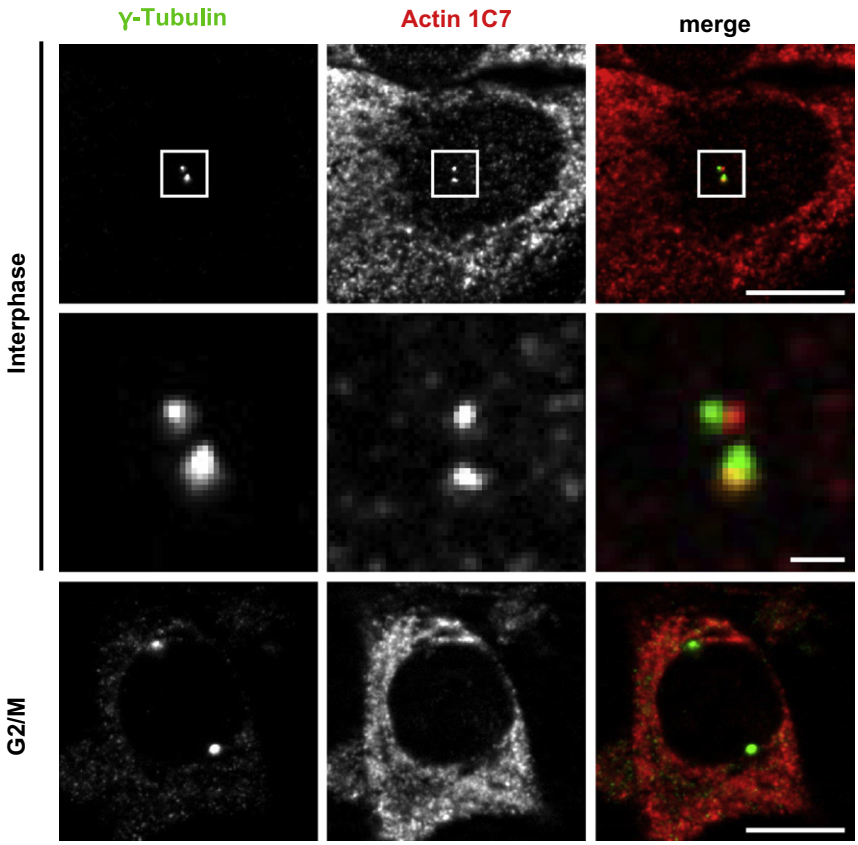

Fig. 3. Antiparallel actin dimers ("lower dimer" actin) localize at the centrosome of interphase but not mitotic MDA-MB-231 cells. Staining for γ-tubulin and actin, using the monoclonal anti-actin antibody 1C7. Actin was always present at both centrioles. Scale bar = 10 μm and 1 μm (magnifications).

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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.2010.11.084.

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