Non-visual Arrestins Are Constitutively Associated with the Centrosome and Regulate Centrosome Function

Haripriya Shankar†, Allison Michal‡, Ronald C. Kern‡, Dong Soo Kang‡, Vsevolod V. Gurevich‡, and Jeffrey L. Benovic‡†

From the †Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and the ‡Department of Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37232

In addition to regulating receptor activity, non-visual arrestins function as scaffolds for numerous intracellular signaling cascades and as regulators of gene transcription. Here we report that the two non-visual arrestins, arrestin2 and arrestin3, localize to the centrosome, a key organelle involved in microtubule nucleation and bipolar mitotic spindle assembly. Both arrestins co-localized with the centrosomal marker γ-tubulin during interphase and mitosis and were found in purified centrosome preparations. In vitro binding assays demonstrated that both arrestins directly interact with γ-tubulin. Knockdown of either arrestin by RNA interference resulted in multinucleation, centrosome amplification, and mitotic defects, although only the loss of arrestin2 triggered aberrant microtubule nucleation. Importantly, overexpression of wild type arrestin rescued the multinucleation phenotype and restored normal centrosome number in arrestin siRNA-transfected cells. Moreover, overexpression of arrestin2 or -3 rescued the multinucleation defect observed in MDA-MB-231 breast cancer cells. Taken together, our data reveal that non-visual arrestins are novel centrosomal components and regulate normal centrosome function.

Centrosomes are the major microtubule organizing centers in mammalian cells and organize the astral microtubule arrays during interphase and regulate most microtubule (MT)2-dependent cellular processes such as cell polarity, motility, and structure (1, 2). During mitosis, the centrosomes nucleate the bipolar mitotic spindle, which is essential for ensuring fidelity of chromosome segregation. They also contribute to cell cycle regulation, as components of the anaphase-promoting complex (3, 4) and cell cycle regulatory proteins (5, 6) localize to the centrosome. In addition, centrosomes are important for determining the position of the cleavage furrow and for cytokinesis (1, 7–11).

Understandably, the process of centrosome replication is very tightly regulated to ensure that replication occurs only once during the cell cycle and is coordinated with DNA replication. After replication, the duplicated centrosomes separate and undergo maturation that involves dynamic re-organization of the pericentriolar material (PCM) and increase in size and MT nucleating ability. Several protein kinases, including polo-like kinase 1 (PLK1), aurora kinase (aurora-A), and Nek2, have been implicated in these processes (12, 13).

Centrosomes consist of a core of centrioles surrounded by amorphous PCM that contains a variety of proteins vital for MT nucleation (14) including γ-tubulin (15). γ-Tubulin is a highly conserved centrosomal protein that forms ring-like structures in association with other proteins in the PCM that serves as a template for MT nucleation (16, 17). Pericentrin is another well characterized pericentrosomal protein that forms a 3-MDa complex with γ-tubulin, which can assemble into lattice-like structures in the PCM and provide additional platforms for MT nucleation (18, 19). Pericentrin aids in anchoring the γ-tubulin ring complex to the mitotic spindle during metaphase and is essential for proper assembly of the mitotic spindle (20). Thus, besides MT nucleation, γ-tubulin and pericentrin are also critical for centrosome structure and assembly and correct mitotic spindle formation (18, 21).

Non-visual arrestins (arrestin2 and -3, also known as β-arrestin1 and -2) belong to a family of proteins that was initially identified as regulators of G protein-coupled receptor signaling (22). They are ubiquitously expressed and specifically bind to agonist-activated, phosphorylated G protein-coupled receptors. Arrestin binding terminates G protein signaling by physically uncoupling the receptor from the heterotrimeric G protein (23). Since their discovery, arrestins have been shown to promote receptor internalization (24, 25) and to mediate G protein-independent signaling (26). Additional studies have demonstrated that arrestins can also translocate to the nucleus and regulate gene transcription (27–29). Despite high homology, the two non-visual arrestins differ in their ability to bind various proteins (30) and regulate different classes of receptors (31). Interestingly, their intracellular localization is also considerably different with GFP-tagged arrestin2 being diffuse in both cytoplasmic and nuclear compartments, whereas arrestin3-GFP is strictly cytoplasmic (31, 32). The reason for this differential pattern of distribution is attributed to the presence of a leucine-rich nuclear export signal in the C-terminal region of arrestin3.

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†To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Thomas Jefferson University, 233 South 10th St., Philadelphia, PA 19107. Tel.: 215-503-4607; Fax: 215-503-5393; E-mail: benovic@mail.jci.tju.edu.

‡The abbreviations used are: MT, microtubule; PCM, pericentriolar material; arr2, arrestin2; arr3, arrestin3; GFP, green fluorescent protein; arr2-GFP, arrestin2 with C-terminal GFP tag; arr3-GFP, arrestin3 with C-terminal GFP tag; PBS, phosphate-buffered saline; siRNA, small interfering RNA; GST, glutathione S-transferase; G, inhibitory guanine nucleotide-binding protein; MEF, mouse embryonic fibroblasts; TBS, Tris-buffered saline; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; PIPES, 1,4-piperazineethanesulfonic acid; ERK2, extracellular signal-regulated kinase 2.
It is worth noting that the majority of studies have overexpressed tagged versions of arrestin2 or -3 to visualize their co-localization with other cellular proteins and receptors or relocation to different intracellular compartments (27, 33, 34).

Here we dissected the intracellular localization and function of endogenous arrestins. We found that both arrestins co-localize with the centrosome at every stage of the cell cycle, independent of MTs. Both arrestins co-fractionate with γ-tubulin in centrosome-rich fractions and directly interact with purified γ-tubulin. Furthermore, siRNA-mediated knockdown of endogenous arrestins produced multinucleated cells and abnormal centrosome number, size, and function that could be rescued by arrestin overexpression. Our data suggest that loss of arrestin triggers centrosome hypertrophy concomitant with aberrant MT re-growth, resulting in chromosome segregation errors and ultimately producing cells with multiple nuclei. These findings shed new light on arrestins as regulators of normal centrosomal function.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—HeLa and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 20 mM Heps. MDA-MB-231 cells were obtained from the ATCC and cultured in modified Eagle’s medium supplemented with sodium bicarbonate, nonessential amino acids, sodium pyruvate, and 10% fetal bovine serum. HeLa and HEK293 cells were transfected with either GFP vector, arrestin2-GFP, or arrestin3-GFP using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. Transfected cells were processed for immunofluorescence 48 h post-transfection. Lipofectamine 2000 (Invitrogen) was used for transfecting HEK293, HeLa, HeLa-centrin-GFP, and MDA-MB-231 cells with control, arrestin2, or arrestin3 siRNAs, and protein expression was analyzed 72 h post-transfection. Briefly, 20 μl of Lipofectamine 2000 reagent was mixed with 1.5 ml of Opti-MEM (Invitrogen). In another tube, 600 pmol of control, arrestin2, or arrestin3 siRNA was mixed with 1.5 ml of Opti-MEM. After incubating the two tubes at room temperature for 5 min, siRNA-Opti-MEM mix was added to the Lipofectamine-Opti-MEM mix and briefly vortexed. After incubating the reaction for 20 min at room temperature, it was added dropwise to 70–80% confluent plates containing 2 ml of serum- and antibiotic-free media. After 4 h of incubation at 37 °C, 5 ml of fresh medium supplemented with 20% fetal bovine serum was added to the transfected cells. For the rescue experiments, HEK293 cells were transfected simultaneously with siRNAs and 1 μg of GFP vector, arrestin2-GFP, or arrestin3-GFP using Lipofectamine 2000 and processed for immunofluorescence 72 h post-transfection. For overexpressing centrin-GFP concomitant with arrestin knockdown, 0.5 μg of centrin-GFP (kindly provided by Dr. Alexey Khodjakov, Wadsworth Center, Albany, NY) was mixed with control, arrestin2, or arrestin3 siRNAs and transfected using Lipofectamine 2000 as described above. Non-target plus control and on-target plus arrestin2 and arrestin3 siRNAs were purchased from Dharmacon (Thermo Scientific).

**Stable Cell Lines**—pcDNA3, pcDNA3-arrestin2, and pcDNA3-arrestin3 were linearized with PVU-I to facilitate stable integration. MDA-MB-231 cells were transfected with 6 μg of linearized plasmid with Lipofectamine 2000 and selected in 1.2 mg/ml G418. HeLa cells stably overexpressing centrin-GFP were kindly provided by Dr. Tim Yen (Fox Chase Cancer Center, Philadelphia, PA).

**Antibodies**—Monoclonal antibodies used were anti-γ-tubulin (1:1000, Abcam, Cambridge, MA), anti-α-tubulin (1:2000, Sigma), and anti-PLK1 (1:500, Zymed Laboratories Inc., Invitrogen), whereas the polyclonal antibodies used were anti-γ-tubulin (1:1000, Sigma), anti-pericentrin (1:2000, Abcam), and anti-arrestin2 and anti-arrestin3 (1:250) (35, 36). Secondary antibodies used were Alexa Fluor 594-conjugated goat anti-mouse, Alexa Fluor 488-conjugated goat anti-rabbit, and Alexa Fluor 633-conjugated goat anti-rabbit (Molecular Probes).

**Purification of Arrestin Antibodies**—Purified arrestin2 or -3 (50 μg/well) were electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose, and stained with Ponceau S solution (Sigma) to visualize the purified proteins. The region containing the protein was cut into strips, destained with TBS, 1% BSA, and proteins were separated into aliquots, and stored at −20 °C.

**Immunofluorescence Microscopy**—Cells were split on poly-L-lysine-coated coverslips 24 h before fixation. For staining endogenous arrestins and γ-tubulin, cells were washed once with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde/PBS on ice, and permeabilized with −20 °C methanol. In some experiments cells were first pre-extracted with PBS, 0.25% Triton X-100 for 2 min on ice before fixation with 4% paraformaldehyde/PBS. Cells were incubated with quench buffer (PBS, 2.5% nonfat milk, and 150 mM sodium acetate) to reduce paraformaldehyde autofluorescence and then block buffer (PBS, 2.5% nonfat milk) to block nonspecific antibody binding. Primary antibody incubations were done for 1 h at room temperature or overnight at 4 °C for endogenous arrestin2 and -3 staining. Secondary antibody incubations were done for 1 h at room temperature. All washes were done with PBS, 0.1% Tween 20. Cells were then incubated with DAPI (Molecular Probes, Eugene, OR) to stain DNA and mounted using Prolong antifade reagent (Molecular Probes). To stain endogenous pericentrin, cells were directly immersed in −20 °C methanol for 8 min, re-hydrated in PBS for 15 min at room temperature, and washed once with TBS, 1% BSA. Primary and secondary antibody (diluted in TBS, 1% BSA) incubations were done for 1 h at room temperature. All washes were done with TBS, 1% BSA, and cells were mounted as described above. For staining endogenous α-tubulin, cells were either fixed using 4% paraformaldehyde/PBS or by direct immersion in −20 °C methanol.

For confirming the specificity of the arrestin2 and -3 antibodies, the antibodies were incubated with purified GST or GST-arrestin2 or -3 C-terminal tail domain fusion proteins (epitope...
for antibody production) in TBS, 1% BSA for 2 h at room temperature. These were then applied to fixed and permeabilized HEK293 cells and incubated overnight at 4 °C. In addition, incubating cells with Alexa-Fluor-conjugated secondary antibodies in the absence of primary antibodies did not elicit any staining (data not shown).

Images were acquired using a Carl Zeiss LSM 510 META confocal microscope (inverted) with a Plan-Apo 63×/H1.4 or 100× oil immersion lens at room temperature and analyzed using Zeiss AIM 4.2 SP1 software. Images were prepared using Adobe Photoshop and Illustrator applications.

**Microtubule Re-growth Assay**—For MT re-growth experiments, siRNA-transfected HEK293 cells were treated with nocodazole (10 μg/ml) for 1 h at 37 °C to completely depolymerize the MT network. The cells were quickly rinsed 3 times with warm Dulbecco’s modified Eagle’s medium, released in warm medium for 10 min to allow for MT re-growth, and fixed by immersion in −20 °C methanol for 8 min. The cells were rehydrated in PBS for 15 min at room temperature followed by incubation with TBS, 1% BSA for 5 min at room temperature. Primary and secondary antibody incubations were done for 1 h at room temperature. All washes were done with TBS, 1% BSA.

**Quantification of Microtubule Nucleation**—Quantitation of the aster area (μm²) was done using Meta-morph software. MTs nucleated by both single and paired centrosomes in control, arrestin2, and arrestin3 siRNA-treated cells were utilized for the analysis. The focus was adjusted to obtain the best pixel intensity for pericentrin staining at the centrosome. Images were acquired from 3–6 randomly selected frames after adjusting the fluorescence intensity to subsaturation levels using the “color palette” tool in the AIM software. The region of interest was drawn around the periphery of the nucleated MTs, and the corresponding area of the region of interest was measured. Each aster was treated independently for aster area measurements. At least 180 asters from six independent experiments were used for quantification.

**Purification of Centrosomes and Western Blotting**—Centrosomes were purified as previously described (37). Briefly, HEK293 cells were treated with 10 μg/ml nocodazole and 5 μg/ml cytochalasin B (Sigma) for 90 min at 37 °C to depolymerize the MTs and actin cytoskeleton. The cells were scraped and pelleted by centrifugation at 1000 × g. The cell pellet was then washed successively with ice-cold TBS and 0.1× TBS, 8% sucrose solutions. Cells were lysed in buffer containing 8 ml of lysis buffer (10 mM Hepes, pH 7.4, 0.5% Nonidet P-40, 5 mM EDTA, 0.1% β-mercaptoethanol, 1 mM MgCl₂, protease inhibitors) and 2 ml of 0.1× TBS, 8% sucrose. The lysate was centrifuged at 2500 × g for 15 min at 4 °C to pellet the insoluble cell debris. The soluble portion (whole cell

**FIGURE 1.** Localization of endogenous arrestin2 and -3. HEK293 cells were transfected with control, arrestin2, or arrestin3 siRNA as described under “Experimental Procedures.” A, shown is a Western blot (IB) demonstrating knockdown of endogenous arrestin2 and -3 levels. Actin staining demonstrates equal protein loading in each lane. B, shown is quantification of percent knockdown of endogenous arrestin2 and -3 levels after siRNA transfection from six independent experiments. C, siRNA-transfected cells were processed for immunofluorescence microscopy using affinity-purified arrestin2 or -3 antibodies. Note the significant decrease in the intensity of arrestin2 and -3 staining in siRNA-transfected cells. D, control siRNA-treated cells were incubated with purified GST protein or with immunizing GST fusion proteins (Block). Note significant dampening of arrestin2 and -3 staining in the presence of the blocking proteins. Ab, antibody; Con, control.
lysate) was incubated with 1 μg/ml DNase and 9 mM Hepes for 30 min on ice and centrifuged through 3 ml of 50% sucrose in sucrose gradient buffer (10 mM PIPES, pH 6.9, 0.1% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100) at 10,000 rpm for 30 min in a Sorvall RC2-B centrifuge using a SS34 rotor. The supernatant was carefully removed, and the sucrose cushion containing the centrosomes was vortexed thoroughly (crude centrosomes) and subjected to stepwise sucrose gradient (30–70%) centrifugation at 32,000 rpm for 1 h in a Beckman ultracentrifuge (L8-M) using an SW41 rotor. The gradient was made with 1.5 ml of 30% sucrose (w/v), 40% sucrose, 60% sucrose, and 70% sucrose (in sucrose gradient buffer). Fractions were manually collected from the bottom, and equal aliquots of the fractions were mixed with 2× SDS sample buffer and boiled for 5 min. Samples were loaded onto a 10% SDS-PAGE gel and transferred to nitrocellulose. The membranes were blocked in 5% nonfat milk in TBST (TBS, 0.1% Tween 20) and incubated with primary antibodies overnight at 4 °C or room temperature. Blots were washed with TBST and incubated with appropriate secondary antibodies for 1 h at room temperature, washed again, and developed using enhanced chemiluminescence (Pierce).

**Arrestin Purification**—Bovine arrestin2 and -3 were expressed in *Escherichia coli* and purified by sequential heparin-Sepharose and Q-Sepharose chromatography to apparent homogeneity essentially as described (38).

**Recombinant His-tagged γ-Tubulin Purification and in Vitro Binding Assays**—pTrcHisB γ-tubulin was generated by PCR using full-length γ-tubulin in pH-13 vector as the template (kindly provided by Drs. Alexey Khodjakov, Wadsworth Center, Albany, NY and Berl Oakley, Ohio State University, Columbus, OH) following ligation into pTrcHisB (Invitrogen) using the XhoI and EcoRI restriction sites. Constructs were transformed into BL21(DE3)pLysS cells and induced with 0.5 mM isopropyl 1-thio-D-galactopyranoside for 4.5 h at 37 °C when the A600 was 0.6. The induced cells were pelleted and lysed in cell lysis buffer (PBS, 0.2% Triton-X-100) containing EDTA-free protease inhibitors (Roche Applied Science). The lysate was sonicated and centrifuged at 14,000 rpm for 30 min to pellet insoluble cell debris. The supernatant containing His-γ-tubulin was incubated with NiTA (Invitrogen) beads for 1 h at 4 °C with rotation. The NiTA beads were pelleted and washed extensively with cell lysis buffer, and His-γ-tubulin was eluted with 750 mM

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**FIGURE 2. Endogenous arrestin2 and -3 co-localize with γ-tubulin.** A, HEK293 cells were fixed and stained with affinity-purified polyclonal arrestin2 (a–h) or arrestin3 (i–p) antibodies and monoclonal γ-tubulin antibody. Representative images of cells in interphase and metaphase are depicted. Arrowheads indicate centrosomes and co-localization (yellow color). Scale bar, 10 μm. b, pre-extracted HEK293 cells were labeled with antibodies to detect endogenous arrestin2 or -3 and γ-tubulin. Arrowheads indicate centrosomes and co-localization. Panels e–h represent images of cells pretreated with nocodazole to de-polymerize the MTs before pre-extraction. Scale bar, 10 μm.
imidazole. Pulldown assays were performed by incubating an equal amount of purified His-\(\gamma\)-tubulin with either control beads (no arrestin) or arrestin2 or arrestin3 beads. Pulldown assays were performed for 1 h at room temperature, and beads were washed extensively with cold cell lysis buffer, eluted with 2× SDS sample buffer, and boiled. One-third of the total sample was loaded onto 10% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with purified arrestin2, arrestin3, or \(\gamma\)-tubulin antibodies.

\(\gamma\)-Tubulin-rich centrosome fractions were pooled, dialyzed overnight against sucrose gradient buffer (without sucrose), and concentrated using a Vivapin concentrator (Sartorius). 200 \(\mu\)l of the concentrated fraction were incubated with control, arrestin2, or arrestin3 beads for 1 h at room temperature. Beads were washed extensively with sucrose gradient buffer, eluted with 2× SDS sample buffer, and boiled. The sample was loaded onto 10% SDS-PAGE gels and immunoblotted with \(\gamma\)-tubulin antibody.

**Statistical Analysis**—Statistical significance was calculated using Student’s \(t\) test.

## RESULTS

### Localization of Endogenous Arrestins by Immunofluorescence

To examine the intracellular localization of endogenous arrestin2 and -3, we initially used HEK293 cells as these cells express readily detectable amounts of both proteins (Fig. 1A). In addition, treatment of these cells with arrestin2 or arrestin3 siRNAs resulted in a significant and specific suppression of arrestin2 (\(~60\%) and arrestin3 (\(~85\%) levels 72 h post-transfection (Fig. 1, A and B). Because there have been very few studies analyzing endogenous arrestins, we next established the specificity and sensitivity of our affinity-purified arrestin2 and -3 antibodies by immunofluorescence in control, arrestin2, and arrestin3 siRNA-transfected cells. Interestingly, both arrestins exhibited significant punctate staining, and siRNA transfection to knock down arrestin expression resulted in a specific reduction in staining as compared with the control (Fig. 1C). Because our antibodies were raised against the C-terminal region of arrestin2 or -3, we also preincubated the antibodies with GST-arrestin2 or GST-arrestin3 (C-tails) or with purified GST as a control. As expected, arrestin2 and -3 staining was significantly attenuated in the presence of the immunizing GST fusion protein containing the arrestin C-tail (Fig. 1D).

As noted above, arrestin2 and -3 staining appeared to be punctate throughout the cell. These results differ from most previously published reports using overexpressed GFP-tagged...
Endogenous Arrestin2 and -3 Co-localize with γ-Tubulin at the Centrosome—We next obtained z-stack images of a number of cells stained for endogenous arrestin2 or -3 to further study the staining pattern. Distinct foci near the nuclear membrane periphery were observed with arrestin2 and -3 antibodies (Fig. 2A). In mitotic cells, we clearly noted intense punctate staining for arrestin2 and -3 at either side of the condensed chromatin periphery were observed with arrestin2 and -3 antibodies (Fig. 2A). In mitotic cells, we clearly noted intense punctate staining for arrestin2 and -3 at either side of the condensed chromatin

arrestins, which show that arrestin2 is localized in the cytosol and nucleus, whereas arrestin3 is strictly cytosolic (31, 32). Interestingly, in certain focal planes, we observed intense perinuclear staining, more predominant for arrestin2 than arrestin3. In some cells the staining was more punctate, reminiscent of the centrosome (Fig. 1C).

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arrestin staining at the centrosome. Similar to our earlier findings, in control cells endogenous arrestin2 (supplemental Fig. S1A) and -3 (supplemental Fig. S1B) staining at the centrosome was clearly observed. However, in cells transfected with arrestin2 or arrestin3 siRNA, there was significant loss of arrestin staining at the centrosome with no difference in γ-tubulin staining (supplemental Fig. S1). This suggests that loss of arrestin2 or -3 does not affect γ-tubulin localization in the centrosome.

Furthermore, we have also studied arrestin2 and -3 co-localization with γ-tubulin in wild type and arrestin2/3 double knock-out mouse embryonic fibroblasts (MEFs) to validate the specificity of our arrestin antibodies and localization

(FIG. 2A, panels e and m). These staining patterns were reminiscent of the centrosome, the microtubule-nucleating center in the cell that resides in the poles of the mitotic spindle. To test whether arrestins reside in the centrosome, HEK293 cells were dually labeled with antibodies to detect endogenous arrestin2 or -3 and γ-tubulin, a well established marker of the centrosome (15). As seen in Fig. 2A, clear co-localization was observed between γ-tubulin and endogenous arrestin2 and -3 in interphasic (panels d and l) and mitotic (panels h and p) cells. In fact, co-localization was observed in more than 90% of the interphasic cells counted for arrestin2 and -3 (n = 200). These results suggest that a fraction of both arrestins is constitutively associated with the centrosome.

To further improve the visualization of endogenous arrestins at the centrosome, cells were pre-extracted with PBS-Triton before fixation. Pre-extraction eliminated most of the cytosolic arrestin staining and produced concentrated centrosomal staining (Fig. 2B, panels a and c). Under these conditions, co-localization between arrestin and the centrosome was observed in every cell. Importantly, both arrestins remained associated with the centrosome even in the presence of the microtubule-depolymerizing drug, nocodazole, demonstrating that the centrosomal localization is independent of MTs (Fig. 2B, panels e and g). In addition, we pre-extracted the control, arrestin2, and arrestin3 siRNA-transfected cells to compare the loss of arrestin staining at the centrosome. Similar to our earlier findings, in control cells endogenous arrestin2 (supplemental Fig. S1A) and -3 (supplemental Fig. S1B) staining at the centrosome was clearly observed. However, in cells transfected with arrestin2 or arrestin3 siRNA, there was significant loss of arrestin staining at the centrosome with no difference in γ-tubulin staining (supplemental Fig. S1). This suggests that loss of arrestin2 or -3 does not affect γ-tubulin localization in the centrosome.

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of arrestins. There was obvious co-localization of endogenous arrestin2 and -3 with γ-tubulin in wild type MEFs that was not observed in arrestin2/3 double knock-out MEFs (supplemental Fig. S2, panels A and B). These results support our earlier observations that both non-visual arrestins are enriched at the centrosome.

We also tested whether endogenous arrestin2 and -3 co-localize with the centriole marker centrin. HEK293 cells expressing centrin-GFP were stained with arrestin and γ-tubulin antibodies. Both arrestins were found to co-localize with centrin upon pre-extraction (supplemental Fig. S3), suggesting that they associate with the centriole and PCM. In addition, we also observed co-localization of both arrestins with pericentrin, another centrosomal marker (data not shown).

**Figure 3A**

Centrosome-containing fractions were purified from HEK293 cells using established procedures (37) and analyzed for the presence of arrestin2, arrestin3, and the centrosomal proteins γ-tubulin, α-tubulin, PLK1, and Hsp90 (39) and the non-centrosomal protein Lamin B. As shown in Fig. 3A, arrestin2 and -3 were enriched in fractions 4–7 similar to γ-tubulin and other centrosomal proteins, suggesting that they co-fractionate with γ-tubulin in purified centrosome preparations. Because both arrestins co-localized and co-fractionated with γ-tubulin, we next studied whether they could interact with γ-tubulin. Fractions 4–7 were pooled, dialyzed, concentrated, and utilized for in vitro binding assays using control beads or purified arrestin2- or arrestin3-coupled beads. As shown in Fig. 3B, γ-tubulin selectively bound to arrestin2 and -3. Interestingly, a slower migrating γ-tubulin band was also observed in arrestin2 pull-down assays, possibly reflecting a modified form of γ-tubulin (40). To determine whether arrestins could directly interact with γ-tubulin, we performed in vitro binding assays by
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incubating control or arrestin2- or arrestin3-coupled beads with purified recombinant His-tagged γ-tubulin. As shown in Fig. 3C, γ-tubulin bound specifically to arrestin2 and -3, similar to our results with the purified centrosome preparation. Our results reveal that arrestin2 and -3 can directly interact with γ-tubulin and suggest that this may be the mechanism that mediates arrestin localization in the centrosome. Although arrestins have previously been shown to associate with MTs, which are composed of α- and β-tubulin polymers (41), we now demonstrate that both non-visual arrestins can also directly interact with another member of the tubulin family, γ-tubulin.

Loss of Arrestin Expression Results in Accumulation of Multiple Nuclei and Centrosome Abnormalities—Ablation or overexpression of many centrosomal proteins including pericentrin, PLK1, and aurora-A triggers multinucleation, centrosome amplification, and/or other defects of centrosome function (42, 43). Since we found that arrestin2 and -3 are resident centrosomal components, we used RNA interference to study the functional role of arrestins in centrosome function. First, we analyzed control, arrestin2, and arrestin3 siRNA-transfected HEK293 cells for multiple nuclei by co-staining cells for pericentrin and counting nuclei by DAPI staining. Any cell with multiple, micro-, or fragmented nuclei were included in the >1 nuclei category. Loss of either arrestin2 or -3 triggered an ∼2-fold increase in the percentage of cells with >1 nuclei and a 2–3-fold increase in the percentage of cells with 3 or more nuclei (Fig. 4, A and B). A significant increase in the percentage of cells with micronuclei was also observed with arrestin knockdown (Fig. 4B). However, the double knockdown of arrestin2 and -3 did not have an additive effect over single knockdowns and, therefore, was not included in additional functional experiments (data not shown).

We next assessed the effect of knocking down arrestin2 or -3 on centrosome structural and functional defects (44). Centrosome number and size were measured by using pericentrin as the marker and any centrosome bigger than 2 μm in diameter was categorized as enlarged. Centrosome amplification was defined as an increase in the number of centrosomes per cell. Cells usually have 1–2 centrosomes, and any cell with 3 or more centrosomes was quantified as amplified (45). We observed a significant increase (∼2-fold) in the percentage of cells with 3 or more centrosomes after depletion of arrestin2 or -3 (Fig. 4C). There was also a 3–4-fold increase in cells with abnormally large centrosomes in arrestin2 and -3 siRNA-transfected cells; however, statistical significance was achieved only for arrestin2 knockdown (Fig. 4C).

It is known that pericentrin can associate with certain structures called acentriolar bodies, which lack centrioles but can still nucleate microtubules. To ascertain whether the amplification of centrosomes observed in pericentrin-stained cells after arrestin knockdown is due to centrosome fragmentation or de facto centriole amplification, we compared centrin (marker of centriole) and pericentrin (marker of PCM) staining in control, arrestin2, and arrestin3 siRNA-treated cells. Centrin-GFP was expressed in HEK293 cells concurrent with siRNA transfection to visualize the effect of arrestin knock-
down on the number of centrioles/cell. It has been shown that overexpression of centrin-GFP does not produce defects in cell cycle progression or centrosome function (46). As shown in Fig. 5, arrestin knockdown triggered a significant increase in cells with $>4$ centrin spots, supporting our earlier findings. In addition, when we stained arrestin-knockdown centrin-GFP expressing cells for pericentrin, we observed several phenotypes including concomitant amplification of centrosomes and centrioles, with each centriole surrounded by a cloud of PCM (Fig. 5A). In some cells centriol spots colocalized with pericentrin, although there were a few that did not have any associated PCM. We also noticed that although some centrosomes in the arrestin siRNA-treated cells contained a normal number of centrioles, they were associated with excessive PCM or PCM fragments lacking centrioles (Fig. 5A). Importantly, similar results were observed in HeLa cells stably expressing centrin-GFP (Fig. 6, A and C). In addition, we also noted a 2-fold increase in the occurrence of multinucleated cells upon arrestin2 or -3 depletion in centrin-GFP-expressing HeLa cells (Fig. 6B). Interestingly, in both HEK293 and HeLa cells, arrestin knockdown-induced centriole amplification was observed not only in binucleate/multinucleate cells but also in mononucleate cells. These results suggest that centriole amplification is not an outcome of failed cytokinesis but is more likely due to centriole over-duplication.

**Arrestin2 Is Important for Regulating Microtubule Nucleation**—Microtubule nucleation is the most critical function of the centrosome, and indeed ablation of certain centrosomal proteins results in either nucleation of larger asters (47) or small/fewer asters (48). In addition, breast cancer cell lines that have multiple centrosomes such as MDA-MB-231 and Hs578T also nucleate larger and longer asters (49). Consequently, we studied the effect of reducing arrestin expression on the MT-nucleating capacity of the centrosome. HEK293 cells were treated with nocodazole to completely depolymerize the MTs, washed, and then released into warm media to allow MT regrowth. Aster area was measured for the newly nucleated MT asters in control, arrestin2, and arrestin3 siRNA-treated cells. Interestingly, a significant increase in aster area was observed only in arrestin2 siRNA-transfected cells (supplemental Fig. S4, panels A and B). These results provide further evidence for the critical role of arrestins in regulation of distinct aspects of centrosome function and suggest that loss of arrestin2 induces centrosome hyperactivity, similar to that observed with the loss of BRCA1 (47).

**Overexpression of Wild Type Arrestin2 or -3 Rescues the Multinucleate and Centrosome Amplification Phenotypes**—Loss of arrestin2 or -3 led to a significant increase in the number of cells with multiple nuclei and centrosomes. Accordingly, we

**FIGURE 7.** Overexpression of arrestin2 and -3 rescues the multinucleate and centrosome amplification defects. Control, arrestin2, and arrestin3 siRNA-treated HEK293 cells were transfected with GFP vector, arrestin2-GFP, or arrestin3-GFP. Only interphasic cells expressing low, medium, or high levels of GFP were analyzed for the rescue of the multinucleate phenotype and centrosome amplification. Pericentrin was used to visualize centrosomes, and nuclei were stained with DAPI. A, for quantification of percent rescue of the multinucleate phenotype, more than 140 cells were analyzed from 4 independent experiments. B, for quantification of percent rescue of centrosome amplification, more than 100 cells were analyzed from 4 independent experiments. The single asterisk (*) indicates $p < 0.05$, and the double asterisk (**) indicates $p < 0.005$. Data are represented as the average ± S.E. C, shown is quantification of the rescue of the multinucleate phenotype ($\geq 3$ nuclei). HEK293 cells were transfected with control siRNA plus GFP, arrestin2 siRNA plus GFP, arrestin2 siRNA plus arrestin2-GFP, arrestin3 siRNA plus GFP, or arrestin3 siRNA plus arrestin3-GFP. At least 140 cells were counted from 3 independent experiments and were analyzed for $\geq 3$ nuclei per cell. Data are represented as the average ± S.E.
checked whether restoring arrestin levels would rescue these defects. HEK293 cells were simultaneously treated with arrestin2 siRNA plus GFP vector, arrestin2 siRNA plus bovine arrestin2-GFP, arrestin3 siRNA plus GFP vector, or arrestin3 siRNA plus bovine arrestin3-GFP. Control cells were treated with control siRNA and GFP vector. This allowed us to identify cells expressing GFP and selectively examine them for multinucleate phenotype and centrosome amplification defects. Overexpression of arrestin2-GFP or arrestin3-GFP in arrestin2- or arrestin3-depleted cells, respectively, resulted in a significant rescue of both multinucleation and centrosome amplification defects induced by the loss of arrestin (Fig. 7, A and B). In addition, restoring arrestin2 or -3 levels produced a significant reduction in the percentage of cells with three or more nuclei (Fig. 7C). These findings demonstrate that the observed centrosomal defects are due to reduction of arrestin2 or -3 expression and verify that arrestins play an important role in regulating centrosome number and function.

Arrestin Expression Rescues the Multinucleate Defect in MDA-MB-231 Breast Cancer Cells—MDA-MB-231 cells are breast cancer cells that exhibit centrosome amplification, enhanced microtubule nucleation, and defects in cytokinesis (49). As a logical extension of our findings, we examined whether stable overexpression of arrestin2 or -3 could rescue some of the defects observed in these cells. Fig. 8 B demonstrates the level of overexpression of arrestin2 and -3 as compared with endogenous levels. We also analyzed the co-localization of endogenous and overexpressed arrestin2 and -3 with the centrosomes in these cells. Clear co-localization between arrestin2 and -3 with α/β-tubulin was observed in cells transfected with vector (panels d–h) or arrestin3 (panels i–p) antibodies. Arrowheads denote centrosomes and co-localization. Scale bar, 10 μm. B, shown is a Western blot (IB) demonstrating the levels of arrestin2 or -3 in stably overexpressing cells as compared with pcDNA3-transfected cells. The ERK2 blot indicates equal amounts of protein loading in each lane. C, for the quantification of percent multinucleated cells after overexpression of arrestin2 or -3, at least 430 cells were counted from 6 independent experiments. D, shown is a Western blot demonstrating significant reduction in levels of endogenous arrestin2 and -3 after siRNA transfection. The ERK2 blot indicates equal amounts of protein loading in each lane. E, wild type MDA-MB-231 cells were transfected with control, arrestin2, or arrestin3 siRNA and examined for a multinucleate defect. For quantification of percent multinucleate cells, at least 150 cells were analyzed from 3 independent experiments. The single asterisk (*) indicates p < 0.05. Data are represented as the average ± S.E.
was very effective for both arrestins (Fig. 8D), and consistent with our previous findings, we noted a significant increase in the percentage of cells with multiple nuclei after reduction in arrestin expression (Fig. 8E). Interestingly, arrestin2 knockdown resulted in a stronger defect compared with arrestin3 knockdown. It is plausible that these differences could either be
Reducing Arrestin2 or -3 Expression Produces Mitotic Defects—Centrosome amplification is usually associated with multipolar spindles during mitosis, which is in part responsible for improper chromosome segregation and accumulation of multiple nuclei. Thus, we also evaluated the effect of reducing arrestin2 or -3 expression on mitotic bipolar spindle formation. Most of the cells selected for quantifying the mitotic defects were in metaphase with a few in pro-metaphase. HEK293 cells with condensed chromatin and pericentrin-decorated spots at the poles of the spindle were identified as normal mitotic cells (Fig. 9A, panels a–c). There was a significant decrease in mitotic cells with 1–2 centrosomes and an increase in cells with 3 or more centrosomes in arrestin-depleted cells (Fig. 9D). There was also a concomitant decrease in the percentage of bipolar mitotic spindles and enhanced multipolar mitotic spindles (three or more) in arrestin2 and -3 siRNA-transfected cells as compared with control cells (Fig. 9, A and B). These findings are in accordance with our data showing a pronounced increase in cells with supernumerary centrosomes during interphase after depletion of arrestin2 or -3. However, it is also interesting to note that in some cells multiple centrosomes clustered at the poles but still nucleated a normal bipolar spindle (Fig. 9A, panels j–l and s–u). Thus, these results demonstrate that these cells can form a bipolar spindle even in the presence of centrosome amplification. Studies have suggested that this could also potentially introduce defects in chromosome segregation (50).

In addition to multipolar spindles, we also observed significant anomalies in spindle morphology and position in arrestin2 and arrestin3 siRNA-transfected cells (Fig. 9C). The spindles were considered normal if they had the typical fusiform appearance and were located on either side of the condensed chromatin (Fig. 9A, panels a–c). In some cases both spindle poles were aberrantly located on one side or the center of the condensed chromatin (Fig. 9A, panels d–f and m–o) or were abnormally small (Fig. 9A, panels m–o). In other cases the spindle was bipolar but was not focused correctly (data not shown). Thus, a range of spindle defects were noted after depletion of arrestin2 or -3 that could contribute to inaccurate chromosome segregation.

**DISCUSSION**

This study was undertaken to examine the intracellular localization and function of endogenous arrestins. We provide evidence for the localization and functional role for arrestin2 in the centrosome. Using confocal microscopy, we have shown co-localization of endogenous arrestins with different centrosomal markers (γ-tubulin, pericentrin, and centrin) in multiple cell lines. In addition, we have shown that arrestins are enriched in purified centrosome fractions and can directly interact with γ-tubulin in each and every phase of the cell cycle. Therefore, we conclude that arrestin2 and -3 are constitutively associated with the centrosome.

We have also analyzed the functional role of the arrestins by suppressing the expression of arrestin2 or -3 using specific siRNAs. Loss of arrestin2 or -3 produced a significant increase in the number of cells with multiple nuclei, centrioles, and centrosomes. Importantly, expression of wild type arrestin2 or -3 in arrestin-depleted cells rescued the multinucleate and centrosome amplification defects, verifying their role in normal centrosome function. It is worth noting that greater rescue was observed with arrestin2 overexpression as compared with arrestin3 in HEK293 and MDA-MB-231 cells. Loss of arrestin2 also produced a significant increase in abnormally large centrosomes. We envision that in arrestin2 knockdown cells, some of the amplified centrosomes either undergo fusion and form unusually large centrosomes or there is excess PCM associated with each centrosome. Reduction in arrestin2 expression also generated hyperactive centrosomes as arrestin2-siRNA-transfected cells nucleated larger MT asters. Therefore, our results support a more critical role for arrestin2 in centrosome function, although arrestin3 is also important.

In a recently published study, it was reported that arrestin3 but not arrestin2 co-localizes with the centrosome in HeLa cells (56). In addition, this previous study did not note any increase in multinucleated cells or any defect in MT re-growth after release from the nocodazole block in arrestin3-deficient MEFs. Thus, the authors concluded that arrestin3 does not contribute to any of the classical centrosome functions. We believe that in the Molla-Herman et al. study (56), the high background in their arrestin2-GFP-expressing cells could have made it difficult to discern the presence of arrestin2 in the centrosome. In our experiments we observed that both non-visual arrestins are core components of the centrosome in multiple cell lines including MEFs. Interestingly, another study demonstrated that both arrestin2 and -3 co-localize with the cilia, where they regulate hedgehog signaling (57). Specifically, it was reported that endogenous non-visual arrestins co-localize with pericentrin in the basal body of the cilia, in agreement with our observations that both arrestins co-localize with pericentrin in the centrosome.

**FIGURE 9.** Loss of arrestin2 or -3 promotes multipolar mitoses and spindle defects. A, representative images are shown of metaphasic HEK293 cells transfected with control (panels a–c), arrestin2 (panels d–i), or arrestin3 (panels m–u) siRNA. Pericentrin was used to visualize centrosomes, α-tubulin was used to stain the mitotic spindle, and condensed chromatin was stained with DAPI. Note two control cells in the depicted frame with normal pericentrin staining at the spindle pole and a normal bipolar spindle. Panels d–i and m–o represent arrestin2 and arrestin3 siRNA-treated cells with abnormal spindles; g–i and p–r represent arrestin2 and -3 siRNA-treated cells with multipolar spindles; j–l and s–u depict arrestin2 and -3 siRNA-treated cells with centrosome clustering at the poles. Scale bar, 10 μm. B, for quantification of percent multipolar mitoses, 250 mitotic cells were counted from an asynchronous population. C, for quantification of spindle defects, at least 90 cells were analyzed from four independent experiments. D, shown is quantification of centrosome amplification (≥3) in mitotic cells. The single asterisk (*) indicates p < 0.05, the double asterisk (**) indicates p < 0.005, and the triple asterisk (***)) indicates p < 0.001. Data are represented as the average ± S.E.
Arrestins Regulate Centrosome Function

Because arrestins have been shown to associate with MTs (41), we wanted to determine whether endogenous arrestins required MTs for centrosome localization and function. Because pretreatment of cells with nocodazole to disrupt MTs did not alter arrestin localization in the centrosome, this localization appears to be independent of MTs. Although we believe that arrestin association with MTs does not contribute to the centrosomal defects observed in cells with reduced arrestin expression, a possible role for this interaction in centrosomal function cannot be excluded.

There are several possible mechanisms by which non-visual arrestins could modulate their effect on centrosome number and function. First, this could be mediated by the scaffolding functions of arrestins. Indeed, many studies have demonstrated that arrestins can form complexes with multiple cytoplasmic and nuclear proteins, which is essential for their ability to regulate different cellular responses. We have demonstrated that arrestin2 and -3 can directly interact with γ-tubulin, and we believe that this represents a mechanism contributing to arrestin localization in the centrosome. Binding to γ-tubulin might enable arrestins to associate with other centrosomal proteins. Two other centrosomal proteins, BRCA1 and PLK1, have also been shown to associate with γ-, α-, and β-tubulins (40, 58, 59).

Interestingly, knockdown of arrestin expression displays many of the functional centrosome defects noted after loss of BRCA1 or overexpression of PLK1, including multinucleation, centrosome and centrosome amplification (42, 60–63), and enhanced microtubule nucleation (47). These observations suggest that arrestins, similar to BRCA1, might be involved in the negative regulation of centrosome activity and help to prevent inappropriate centrosome duplication. Therefore, γ-tubulin, BRCA1, PLK1, arrestin2, and arrestin3 could exist as a complex in the centrosome, and altering the expression of any of these proteins may lead to disruption of the complex, resulting in deleterious consequences. In addition, arrestin2 and -3 can also form hetero-oligomers (64), providing a possible explanation for the lack of compensation by arrestin2 or -3 when the expression of either protein is selectively diminished. Conceptually, this might suggest that multiple proteins need to act in concert to ensure proper functioning of this organelle.

Our findings and other published studies show that in addition to non-visual arrestins, other regulators of G protein-coupled receptors are also localized in the centrosome. For example, the sphingosine 1-phosphate receptor (65), Gβγ (66), Gβγ, and RGS14 (55) have been shown to co-localize with γ-tubulin and/or pericentrin. Moreover, fluorescence resonance energy transfer experiments reveal that Gαi1 directly interacts with RGS14 in the centrosome, and its normal expression and function is essential for proper cytokinesis (66). Indeed, silencing Gαi or RGS14 expression produced cells with multiple nuclei (55), mirroring the defects observed after loss of arrestin2 or -3 expression.

Previous studies revealed that reduced arrestin2 expression led to an increase in the number of cells in G1 phase, whereas overexpression of arrestin3 led to a G2/M arrest (34, 54), suggesting a role in cell cycle progression. Moreover, although a reduction in arrestin3 levels triggered uncontrolled proliferation by positively regulating ciliogenesis (58), loss of arrestin3 in PC12 cells impaired nerve growth factor-induced growth arrest. Similarly, inhibition or loss of many centrosome-associated proteins or the removal of the centrosome induces arrest at G1-S (11, 51–53). These findings led to the speculation that the centrosome is required for the G1-S transition, and in its absence the checkpoint system is activated. Thus, it is likely that arrestins could regulate the cell cycle via their localization in the centrosome, and altered cell cycle progression could be responsible for the multinucleate phenotype observed in arrestin knockdown cells. In summary, we provide evidence that arrestin2 and -3 are constitutively associated with the centrosome and positively regulate centrosomal function. These observations suggest that arrestins regulate different aspects of cell cycle progression, thus implicating a novel role for the arrestins as possible suppressors of tumorigenesis.

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Arrestins Regulate Centrosome Function

Non-visual Arrestins Are Constitutively Associated with the Centrosome and Regulate Centrosome Function
Haripriya Shankar, Allison Michal, Ronald C. Kern, Dong Soo Kang, Vsevolod V. Gurevich and Jeffrey L. Benovic

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